Advances in diagnosis and treatment of malignant mesothelioma

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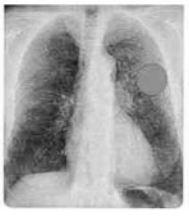
ISBN: 90-8559-229-1



STICHTING ASBESTKANKER

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The work presented in this thesis was performed at the Department of Pulmonary Medicine at Erasmus MC, Rotterdam, The Netherlands. This work was supported by Stichting Asbestkanker Rotterdam (IKR-stimuleringsfonds, among others), and the Mesothelioma Applied Research Foundation (MARF).
Printing of this thesis was financially supported by: IKR-stimuleringsfonds, Dr. Ir. van de Laar Stichting, J.E. Jurriaanse Stichting, Eli Lilly Nederland, tebu-bio, BD biosciences, Harlan Nederland, and others.

Printing: Optima grafische communinatie, Rotterdam, The Netherlands.

Advances in diagnosis and treatment of malignant mesothelioma

Verbetering van diagnostiek en behandeling van kwaadaardig mesothelioom

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 6 december 2006
om 15.45 uur

door

Josephus Petrus Johannes Jacobus Hegmans
geboren te Heesch

We are branches of the same old tree
Shakira

Preface

In late 1960's, physician Dr. J. Stumphius identified twenty-five cases of a rare aggressive tumor known as mesothelioma among shipyard "Royal Schelde" workers due to asbestos exposure (1,2). Further observations showed an increase of mesothelioma cases among these workers. In 1974, the number of cases totaled 42; in 1978, the number rose to 57. The commercial use of asbestos in the Netherlands peaked in the 1970's with the import of 50,000 tons of this 'magic' mineral and 150,000 tons of products annually (3). Five million tons of asbestos containing material still remains incorporated within our national infrastructure. Eternit, an asbestos cement factory in Goor processed asbestos until a ban on its use was introduced in 1993. For years, the factory dumped its asbestos-containing waste by simply putting the waste at the disposal of anyone who wanted to pave a road or a farmyard. So right now, there are miles of road paved around Goor with asbestos, exposing the local people to asbestos dust each time they use these roads. In The Netherlands, the public is getting increasingly familiar with the word asbestos and asbestos related disease, including mesothelioma. There is media interest during renovation, demolition or fire in buildings containing asbestos and the threat that the dust then poses to the public when carried by wind to surrounding areas. Also persons wearing respiratory and other personal protection equipment while removing asbestos containing materials can easily be encountered. Almost everyone who lives in industrialized areas of the western world has asbestos fibers in their lungs, and many can remember being exposed to asbestos incidentally. In the United States, the gray cloud that covered New York on September 11th, 2001, brought about much more awareness of the public to the word mesothelioma (Figure 1).

The collapse of the World Trade Center by terrorist attacks involves not only

Figure 1: Impression of the 9/11 World Trade Center collapse. Dust clouds containing a variety of substances (e.g. asbestos fibers) advances (left). Unprotected tower workers, local residents and first responders exposed to the dust (right). Photos: Aris Economopoulos.

the death of 2749 individuals directly by the assault, but experts fear collateral deaths and injuries over the next two to three decades due to the release of toxic compounds and pulverized carcinogenic fibers into the air. The Twin Towers were insulated with at least 400 tons of asbestos fibers as fire retardant*. A serious asbestos exposure of approximately 114.000 residents, office workers, and first responders took place at the scene and most of them were poorly or not protected against such an exposure**. In 2003, measurements in a building across the street from the WTC that was left untouched after the collapse detected concentrations of asbestos fibers that were 61.000 times the maximum appropriate level***. Therefore, it is more than likely that a public health crisis will unfold over the next two to three decades unless a cure for mesothelioma is found. Mesothelioma is often caused by occupational exposure to asbestos and this results in complex medical–legal aspects of the disease. From January 2003 onwards, the Compensation Asbestos Regulation provides compensation in advance of around € 16,000 to each asbestos victim in The Netherlands. If it can be shown that the employer was negligent in its duty of care, the compensation will be around € 50.000 in total. The number of asbestos-related court cases and requests for asbestos settlements is growing. A simple Google search of 'lawyers' and 'mesothelioma' identified nearly 7 million webpage results. Asbestos is predicted to cost the economy of the western world around $ 300.000.000.000 in compensation in the coming decades (Figure 2)(4). This all leads to more media interest in mesothelioma that does not exist for most other sporadic cancers of comparable incidence. Although some advances are being made, a cure for mesothelioma is still lacking, while mesothelioma cases continue to increase annually. I thank the Department of Pulmonary Medicine Rotterdam, Stichting Asbestkanker, and MARF for supporting us in our aim to gain more insight into this horrible disease and hope that we, together with other researchers worldwide, may once prove that mesothelioma is no longer a life-ending disease.

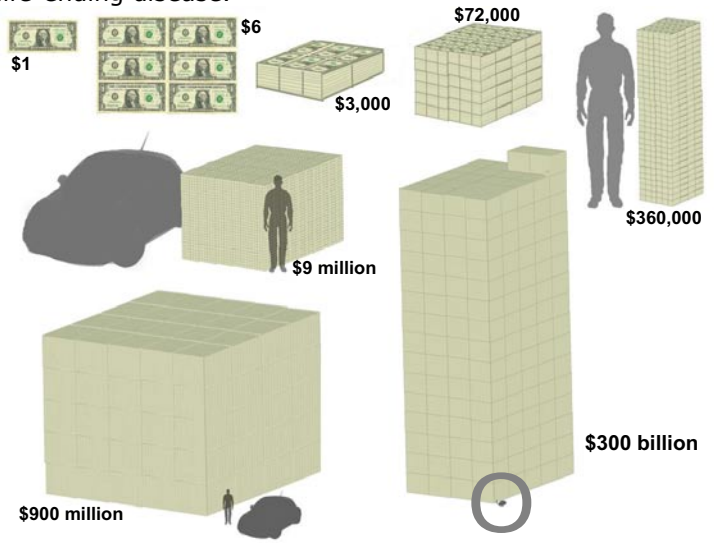

Figure 2: Visualizing the $ 300 billion that are the predicted costs in the western world for asbestos settlements in the coming decades, in addition to healthcare costs associated with the disease.

* Nordgrén, Megan *et al*., The Environmental Impacts of the World Trade Center Attacks A Preliminary Assessment, National Resources Defense Council, Feb 2002

** Brauch, "The 9-11 World Trade Center Collapse Asbestos Risk Assessment", November 2004

*** RJ LeeGroup, WTC Damage Assessment 130 Liberty Street, CR-07, December 2003

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Chapter 1

Malignant mesothelioma

Chapter 1 Malignant mesothelioma

1.1 *Anatomy*

The thoracic cage in humans is constructed like a vertical, cone-shaped bellow with the diaphragm as the moving part in the lowermost and widest end. Within the protected but limited space of the thoracic cage, the lung has to move and change volume constantly to breathe. In order to decrease the friction generated between the lung and the thoracic wall at respiration, the inner surface of the thoracic cage and the outer surface of the lung are covered by a serous, elastic membrane with a smooth and lubricating surface - the parietal layer and visceral layer pleurae, respectively (Figure 1A). The pleura consist of a monolayer of polygonal mesothelial cells (mesothelium) supported by submesothelial connective tissue. These mesothelial cells also line other serosal cavities (pericardial cavity, peritoneal cavity, tunica vaginalis testis) and the organs contained within these cavities. In the pleural cavity, which is the space between these two layers, a small amount of fluid is present under normal physiological conditions consisting of a few milliliters (approximately 0.3 ml per kg body mass). The forces operating on the pleura with respect to movement of liquid are first, the oncotic pressures exerted by the blood in the pleural capillaries and by the liquid in the pleural space and second, the hydrostatic pressures within pleural capillaries and in the pleural space. The fluid is produced continuously and reabsorbed mainly through the lymphatic system (5,6). Under abnormal conditions, the pleural space can be filled with air, blood, plasma, serum, lymph, or pus. This expansion of the pleural space can compress the underlying tissue and causes partial collapse of the lung (Figure 1B).

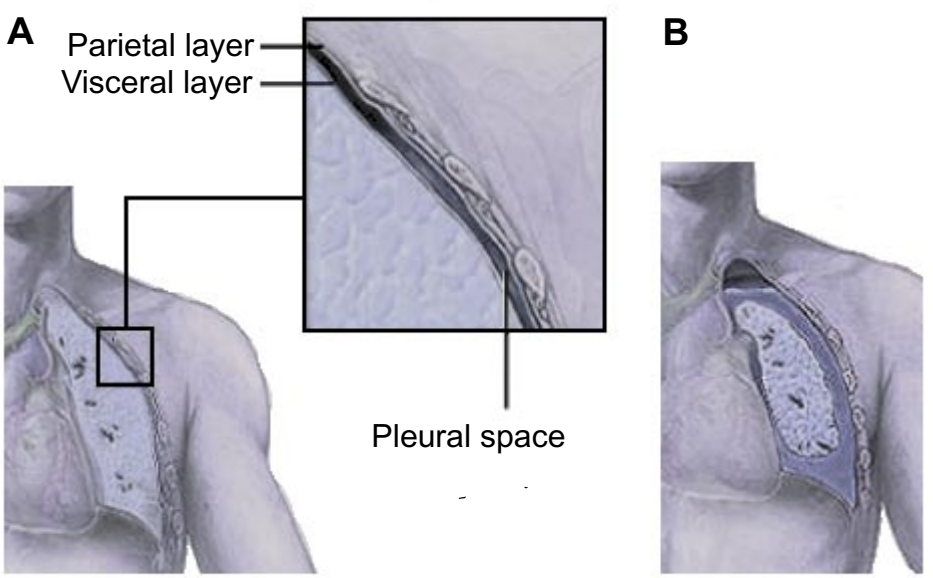

Figure 1: The inner surface of the thoracic cage (parietal layer) and the outer surface of the lung (viceral layer) are covered by a serous, elastic membrane, which enables breathing without any friction. In the pleural cavity, which is the space between the two layers, a small amount of fluid is present under normal physiological conditions consisting of a few milliliters (A). Under abnormal conditions, like malignancies, the pleural space can be filled with up to several liters of fluid (B).

1.2 *Etiology*

In 1960, Wagner and coworkers (7) described the occurrence of a highly aggressive tumor among workers in South-African's Cape Province asbestos mines, followed by a large numbers of reports confirming the association between exposure to airborne asbestos particles and cancer. This cancer, termed malignant mesothelioma (MM), is caused by neoplastic transformation of mesothelial cells and occurs predominantly in the pleura and less frequently in the peritoneum, pericard or tunica vaginalis testis. Asbestos is the major causative agent. Asbestos is a term used for a number of naturally occurring minerals that have crystallized to form long thin fibers and fiber bundles. The Greek word 'asbestos' in its original sense stands for 'indestructible'. Most common is the serpentine group, which includes chrysotile (white asbestos) and which has been the most frequently mined. A second asbestos group known as the amphiboles includes crocidolite (blue asbestos) and amosite (brown asbestos). The fibers have extraordinary tensile strength, conduct heat poorly and are relatively resistant to chemical attack - properties that made asbestos extremely useful. Since the latter part of the nineteenth century asbestos has been used extensively throughout the world and has been incorporated into numerous materials in the construction industry, buildings, ship and aircraft industry and in a diversity of war industries. Besides mesothelioma, inhalation of asbestos fibers can also cause pleural plaque and thickening, slowly progressive, non-cancerous fibrous hardening and scarring of the lungs (asbestosis), and lung cancer.
A few other generally recognized causes of mesothelioma exists: endemic erionite exposure in Turkey (8), ionizing radiation, especially in patients for whom thorotrast was used as a radiographic contrast material in the 1950s (9); and chest injuries (10). Also the potent oncogenic polyoma virus, simian virus (SV)-40, has been implicated as a cofactor in the causation of malignant mesothelioma (11-17) although convincing evidence connecting SV-40 infection and the occurrence of mesothelioma has not been forthcoming (18-21). SV-40 was disseminated widely throughout the world in the Salk polio vaccine in the 1950s and 1960s (22-24). Pedigree analyses in the Cappadocian region of Turkey revealed that the malignant mesothelioma epidemic (~ 50% of deaths in the villages Turkoy, Karain, and Sarihidir) is caused by the interaction between erionite exposure and genetically predisposed individuals (25).

1.3 *Pathogenesis*

Mesothelial cells form a monolayer of specialized pavement-like cells (mesothelium) that line the body's serous cavities (pleural, pericardial and peritoneal) and the internal organs. This single layer of mesothelial cells provide a slippery, non-adhesive and protective surface that aid to the free movement of the surfaces (26). Mesothelioma arises from these mesothelial cells. The paths that mesothelial cells take on their way to become malignant is unknown and probably highly variable dependent of several host factors, including environmental factors, polymorphisms and mutations in susceptibility genes, age and immunity. Several plausible explanations have been proposed as to how asbestos causes malignant changes in these cells. First, the shape and length-to-wide ratio of the asbestos fiber determines how deeply into the lung the fibers are inhaled and whether they then have the capacity to penetrate the lung epithelium and enter or irritate the pleural space (27-29). Secondly, asbestos fibers have the

capacity to interfere with the mitotic process by dividing or piercing the mitotic spindle, disrupting mitosis, which has the potential to lead to chromosome damage, often with extensive aneuploidy and structural rearrangements that characterize mesothelioma (30). The most common abnormalities seen are loss of P16INK4A, P14ARF, and NF2, which implies that a particular pattern of tumor-suppressor gene loss is necessary for mesothelioma development (31-33). Asbestos-induced cell damage can be mediated to some extent by toxic oxygen radicals, which induce DNA damage is a third explanation (34-37). And fourth, asbestos fibers induce phosphorylation of the mitogen-activated protein kinases and extracellular signal-regulated kinases 1 and 2 and elevate expression of early response proto-oncogenes (FOS or JUN or activator protein 1 family members) in mesothelial cells (38,39). When mesothelioma becomes clinically visible 20 to 40 years after asbestos exposure, tumor cells and their products have already been interacting with and affecting host cells for a considerable time to ensure the survival of the tumor. At that time, mesothelioma tumors manifest the six essential alterations in cell physiology that dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion ("hallmarks of cancer"(40)). Tumor development also depends on factors in the microenvironment. Interactions between malignant cells, stromal cells, extracellular-matrix components, various inflammatory cells and a range of soluble mediators contribute to tumor development and progression. There is accumulating evidence that cancer cells can recruit and subvert normal cell types to serve as active collaborators in their neoplastic program (41). Mesothelioma is found to consist of at least three histologic subtypes, epithelial, biphasic (mixed) type, or sarcomatatoid (desmoplastic) type, the latter having the worst prognosis for survival.

1.4 *Epidemiology*

Based on World Health Organization reports, mesothelioma incidence rates from different countries show large differences, rising from the sporadic background rate of around 1 per million to over 35 cases per million per year in some countries (42). Industrialized countries have much higher rates than non-industrialized countries, reflecting the past production and use of asbestos in industry and continued with secondary manufacture, installation, usage, and disposal. At the national level, Scotland, England and The Netherlands have the highest incidence rates (74–88 per million in men) in the western European countries (43, 44). The geographical distribution over The Netherlands shows a pattern with a clear concentration of deaths from mesothelioma in the neighborhood of harbors, shipyards, and heavy industry near the river mouths and along the North Sea coast (45). In Rotterdam, with large shipbuilding plants, busy docks and industrial plants, it is not surprising that the risk is highest at the national level. Due to the past exposure to asbestos, mainly occupational, approximately 90% of the mesothelioma patients are men. In Rotterdam area, malignant mesothelioma is responsible for 1% of overall mortality in men (46). Iceland (1983), Norway (1984), Denmark (1986), and the United States (1989) were the first to introduce bans on some or all asbestos products, while usage of asbestos is forbidden since July 1993 in The Netherlands, followed by France (1996), Poland (1997), Belgium (1998), UK (1999), Latvia (2001), and Slovak

Republic (2002). A ban on asbestos use in world's second-largest economy Japan will not go into effect until 2008. The latency period between the first exposure to asbestos and the onset of disease ranges from 15 to over 60 years (47). Therefore, the incidence of mesothelioma in developed countries is still increasing and the peak of the epidemic is expected around the year 2017. For the Netherlands, the most plausible scenario predicts an increase in pleural mesothelioma mortality up to 500 cases per year in men, with a total death toll close to 12.400 cases during 2000-2028. However, using different assumptions this death toll could rise to nearly 950 cases per year in men (Figure 2). Mortality among women remains low, with a total death toll of about 800 cases (48). Women developing mesothelioma are often living with asbestos workers and therefore exposed to asbestos dust brought home on the clothing and hair of these workers. In pleural mesothelioma, the tumor tends to appear in one lung, with a right/left preference of 60% to 40%. Pleural mesothelioma occurs about ten times more frequently than peritoneal mesothelioma. Both sexes are equally represented with peritoneal mesothelioma. Primary mesothelioma that arises in the tunica vaginalis testis or primary pericardial mesothelioma are rare. Median survival from the first signs of illness is 9 – 12 months (49). Only 13% of mesothelioma patients are still alive 5 years after diagnosis.

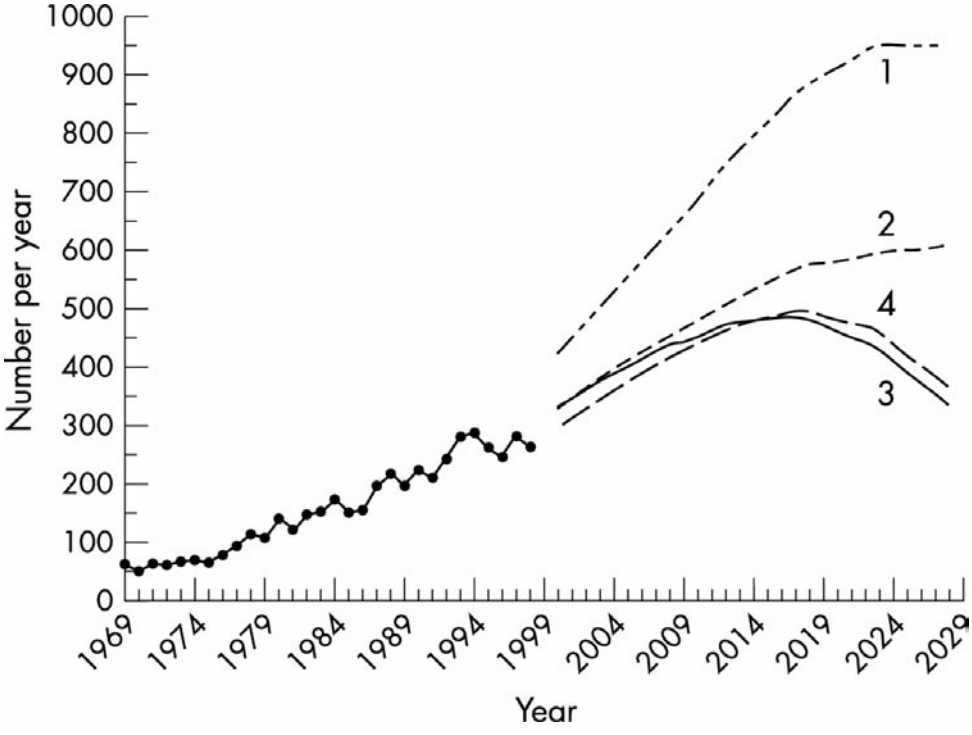

Figure 2: Observed mortality from pleural mesothelioma among men in the Netherlands from 1969 to 1998 and predicted number of deaths from pleural mesothelioma among men for 2000 to 2028. The numbers represent different prediction models of the number of deaths based on differences in available data, statistical modelling, and assigned birth cohort risks (48). The observed mortality in 2006 assumes prediction model 3 as the most plausible scenario.

1.5 *Clinical presentation*

The majority of patients with malignant pleural mesothelioma present initially with dyspnea (breathlessness) and chest pain. These symptoms are caused by an accumulation of fluid between the lung lining and chest cavity, known as pleural effusion, that constricts the underlying lung parenchyma (Figure 1B). Many of the patients have a significant history of asbestos exposure 20-40 years prior to their clinical presentation. Constitutional symptoms such as weight loss and fatigue can be present, but these generally appear later in the course of disease. As the disease progresses, the effusion becomes loculated and the pleural space is replaced by tumor. A large retrospective study on 322 Canadian patients diagnosed with diffuse pleural malignant mesothelioma revealed the following clinical features (50).

Dyspnea and/or chest pain	90%
Pleural Effusion	84%
Weight loss	29%
Cough, weakness, fever, loss of appetite	3%
Asymptomatic features	3%
Hemoptysis, hoarseness, dysphagia, Horner's syndrome	<1%

Patients with peritoneal mesothelioma tend to present with nausea, vomiting, weight loss, stomach pains, and occasionally bowel obstruction. Other symptoms of peritoneal mesothelioma may include blood clotting abnormalities, anemia, and fever. Mesothelioma of the pericardium or tunica vaginalis are rare, but tends to present by blood or fluid accumulation in the space between the myocardium (the muscle of the heart) and the pericardium (the outer covering sac of the heart), or a pocket of (blood-stained) watery liquid that has built up around the testicles, respectively.

Although metastatic deposits of mesothelioma are fairly common at post mortem, these deposits rarely manifest clinically (51). The most common sites of spread are the pleural surface on the other side, the hilar, mediastinal, internal mammary, and supraclavicular / axillary lymph nodes. Distant metastasis may occur to major organs, such as bone, lung and to the peritoneum (52). In the advanced stages of disease, patients will gradually become weaker and having more physical problems as the tumor bulk increases. Eventually, they will develop continuous chest pain secondary to the invasion of the chest wall and intercostal nerves. Dyspnea will worsen due to restriction of lung and chest expansion. Management of mesothelioma depends largely on the staging of the tumor. Early diagnosis and medical intervention may lengthen life expectancy.

1.6 *Diagnosis*

The diagnosis of mesothelioma is not always easy because many of the symptoms in mesothelioma patients are common in a variety of other diseases (e.g. pleural metastases [notably carcinomas] or benign reactive mesothelial proliferations). The diagnosis of mesothelioma will entail scans and biopsies arranged by a physician. Imaging techniques (chest radiography [X-ray], computed tomography [CT], magnetic resonance imaging [MRI], or positron emission tomography [PET] scan) is often used to detect if there is a positive

indication of the presence of a tumor, and the possible presence of an effusion. In certain circumstances a more invasive operation is needed in order to remove tissue sample (thoracoscopy, laparoscopy, or by needle biopsy). This is known as open pleural biopsy and is considered to be the most effective and conclusive ways of diagnosing mesothelioma. Once the tissue samples have been collected, a detailed examination of the cells is performed to check for malignant cells in the tissue. Table 1 shows immunohistochemical stains that are currently used to distinguish epithelial mesothelioma from primary pulmonary adenocarcinoma (53-58). In spite of intensive research efforts, no single immunostain exists that is entirely conclusive for either mesothelioma or serosal metastatic tumor, and for most commercially available antibodies recorded in the literature both the diagnostic value of each one and their various combinations in immunohistochemical panels are still under debate (56,59,60). Other main problem areas in the pathologic assessment of tissue specimens submitted with a clinical suspicion of mesothelioma are the distinction between sarcomatoid mesothelioma and primary or metastatic sarcoma or sarcomatoid carcinoma, and malignant and benign mesothelium.

Table 1: Immunohistochemical markers for differentiating epithelial malignant mesothelioma (M) from pulmonary adenocarcinoma metastatic to the pleura (C), this being the most frequent and impelling diagnostic dilemma. As no single marker can reliably separate all cases, a panel of immunostains that includes members of both groups is recommended (61,62).

Immunostain	preferentially stains *	sensitivity	specificity
B72.3	C	80%	93%
Ber-EP4	C	80%	90%
BG8 (Lewis^y)	C	93%	93%
Calretinin	M	82%	85%
Carcinoembronic antigen (CEA)	C	83%	95%
Cytokeratin (CK) 5/6	M	83%	85%
D2-40 **	M	86%	100%
E-cadherin	C	86%	82%
Human mesothelial cell-1 (HBME-1)	M	85%	43%
Leu-M1 (CD15)	C	72%	93%
Mesothelin	M	97%	53%
MOC-31	C	93%	93%
N-cadherin	M	78%	84%
Podoplanin **	M	86%	100%
Thrombomodulin	M	61%	80%
Thyroid transcription factor-1 (TTF-1)	C	72%	100%
Vimentin	M	62%	75%
Wilms' tumor 1 (WT1) protein	M	77%	96%

* C: preferentially stains pulmonary adenocarcinoma, M: preferentially stains epithelial mesothelioma. ** D2-40 and podoplanin are the most recent additions to this list (62) but have not yet been fully tested in routine diagnostic work.

Detecting characteristic ultrastructural features by electron microscopy can facilitate the diagnosis of mesothelioma. Although a detailed discussion of the ultrastructure of mesothelioma is outside the scope of this thesis, the appearance of the microvilli helps to distinguish epithelial mesothelioma from adenocarcinoma. Mesothelioma is characterized by the presence of abundant long, slender microvilli, while those of adenocarcinomas are short and thick (Figure 3). A length to diameter ratio (LDR) value > 15 is characteristic of mesothelioma; a LDR ratio < 10 is typical of adenocarcinoma (63,64). Also tonofilaments in the cytoplasm and giant desmosomes (more than 1 μm), when present, favour the diagnosis of epithelial mesothelioma.

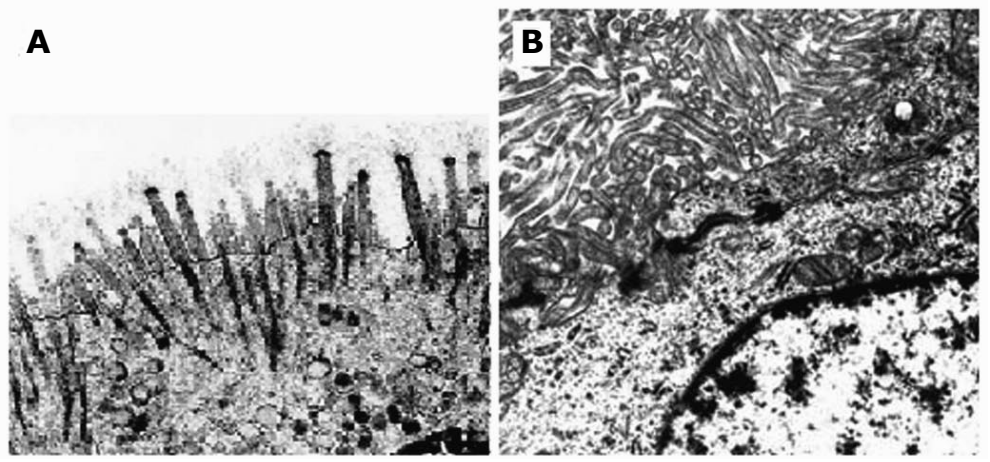

Figure 3: Ultrastructural features by electron microscopy also help to distinguish carcinoma from mesothelioma. Adenocarcinoma (A) typically has few, relatively short blunt microvilli, whereas mesothelioma is characterized by the presence of long and slender ("spaghetti") microvilli (B).

Cytokeratin-derived markers, such as tissue polypeptide antigen (TPA), tissue polypeptide-specific antigen (TPS) and cytokeratin-19-fragments (CYFRA 21-1) in serum are used as tumor markers for lung cancers (65) and although the sensitivity for diagnosis of mesothelioma is low, the concentration changes in proportion to disease activity (66,67).
Recently, osteopontin (68), mesothelin (69), and soluble mesothelin-related protein (SMRP)(70) have been reported as novel potential serum markers for mesothelioma. Analysis of serum osteopontin levels comparing subjects without cancer who were exposed to asbestos with that of patients with pleural mesothelioma had a sensitivity of 78% and a specificity of 86% at a cutoff value of 48.3 ng/ml of osteopontin (68). SMRP is the circulating product of mesothelin, a surface protein thought to be important in mesothelial cell adhesion and possibly signaling. Initial SMRP serum measures reported by Robinson and coworkers (70) had a sensitivity of 84% and specificity approaching 100% compared with pleural metastasis of other tumors but later reports could not confirm this (sensitivity 58.3%, specificity 73.3%)(71). SMRP concentrations parallel disease progression and regression in epithelioid mesothelioma rather than for the mixed subtype and sarcomatoid subtype because only epithelioid mesothelioma cells are positive for mesothelin staining (72). SMRP levels can be measured either in serum or pleural fluid, but pleural fluid SMRPs can better

differentiate mesothelioma from pleural metastatic carcinomas (71). Although promising, the test is only recently available (Fujirebio Diagnostics, Inc.) and therefore not extensively studied. The diagnosis of mesothelioma, if definite, leads to a discouraging prognosis for the patient but without it treatment cannot be planned, and the patient's legal position in terms of compensation remains unclear.

1.7 *Staging and prognostic factors*

Once a diagnosis has been made, further imaging studies (such as CT, MRI, PET, or thoracoscopy) may be required to learn the stage of the disease. Staging is the process of evaluating the progress of cancer in a patient and is determined by the advancement, extent, and spread to other parts of the body. The oldest staging system is the Butchart system which is based mainly on the extent of primary tumor mass and divides mesothelioma into four stages (73). Another staging system has been developed by the International Mesothelioma Interest Group and adopted by the American Joint Committee on Cancer (52). This is a TNM system, similar to staging systems used for most other cancers. T stands for tumor (its size and how far it has spread to nearby organs), N stands for spread to lymph nodes and M is for metastasis (spread to distant organs). The Brigham system is the latest system and stages mesothelioma according to resectability (the ability to surgically remove the tumor) and lymph node involvement (74). Final staging requires surgery (75). Response evaluation criteria in solid tumors (RECIST) are inadequat for objective clinical response evaluation in patients with malignant pleural mesothelioma (76). At the time most mesotheliomas are diagnosed, they are composed of multiple small nodules ranging from 1 mm to occasionally 1 cm, that over a period of time coalesce to form a rind of solid tumor tissue. Determining the stage of the cancer is essential for the treatment and expectations for patients with mesothelioma. Prognose of patients with malignant mesothelioma depends on several factors that can be separated in host-related prognostic factors, tumor-related factors and environment-related factors (see chapter 3). In short, the prognosis is worse in male patients and in patients with extensive disease, poor performance status, weight loss, elevated white-blood cell counts, anemia, thrombocytosis, sarcomatoid histologic findings, or high standardized uptake value ratios on PET (two year survival rates of 0% and a median survival time of only 5.5 months). Patients with epithelial subtype mesothelioma without lymph node involvement, and who are younger than 50, have the best chance for long-term survival.

1.8 *Treatment*

As of this time, there are no medical procedures that are curative. Treatment for mesothelioma is consequently considered purely palliative and has the goals to improve quality of life and to decrease disease progression. Relieving the pleural fluid pressure, with the use of suction when required, followed by talc application provides only short-term symptomatic relief. The treatment being used on mesothelioma depends upon a variety of factors, including the location and histological subtype of the tumor, the extent of the disease, the age, performance status, and medical history of the patient.

Sugarbaker et al. (77) reported that surgical cytoreduction produce long-term survivors although systematic review of the literature provided very limited evidence for the role of surgery in treating this disease (78). In all successful surgery cases, the pleural lining is removed with the lung, including all or part of the affected diaphragm, as well as the outside cover of the heart (extrapleural pneunonectomy). It is extremely difficult, painful and not without serious complications (79). A large percentage of mesothelioma patients are found to be ineligible for surgery. To prevent local recurrence after surgery, patients are subsequently treated with some form of local irradiation and/or systemic therapy. Radiotherapy involves the use of radiation sources to kill off the cancerous cells in the affected area, and systemic therapy uses drugs to kill off the cancerous cells. These multimodality treatments where several treatment approaches are combined, i.e. surgery followed by broad-spectrum cytotoxic chemotherapy, are rapidly becoming the recommended treatment paths for early stage mesothelioma (80).
Currently the combination of pemetrexed (ALIMTA, registered trademark of Eli Lilly and Company) and cisplatin is considered standard of care for patients with mesothelioma, as an increase in median survival of 3 months was noticed (81-85). This combination chemotherapy is the only treatment with activity proven in phase III trials and is approved by the US Food and Drug Administration. The downside of these drugs is the high toxicity level and can therefore cause systemic side effects as nausea, vomiting, hair loss, weight loss and physical fatigue. Dietary supplementation with low-dose folic acid and vitamin B12 limits the toxicities while maintaining clinical activity (86). Because of the limited success of current treatments for extended mesothelioma, novel therapeutic regimens are urgently needed. With new cancer treatments developed through medical research, studies such as gene therapy, immunotherapy, photodynamic therapy, and administration of anti-angiogenic agents, it is hoped that an effective treatment will soon be discovered.

1.9 *Cancer immunosurveillance and dendritic cells*

The concept that the body's immune system can identify and destroy nascent transformed cells was originally embodied in the host-immunity-mediated resistance hypothesis (often referred to as cancer immunosurveillance) of Sir Macfarlane Burnet and Lewis Thomas. In 1957, Burnet stated: "It is by no means inconceivable that small accumulations of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence"(87). There has been an ongoing debate if immune responses and effector mechanisms play a significant role in the spontaneous control of cancer mainly caused by the absence of strong experimental evidence (88,89). However, reports of occasional spontaneous tumor regression, increased cancer incidence in immunocompromised individuals, and immune infiltrates within tumors are now interpreted in terms of immunologic recognition and response against neoplastic lesions. An example of a visible active immunosurveillance event against neoplasia in humans might be the regression of pigmented melanocytic naevi (common moles) in young individuals (90). Most of these neoplastic lesions are clinically benign, although they can express antigens associated with malignant melanoma. Frequently, the growth of a naevus can

stop and the mole can subsequently flatten, depigment, and regress completely. The depigmentation often begins in a region surrounding the initial naevus and is characterised by a depigmented halo ring around the lesion. Histologically, the depigmentation in the halo is caused by death of melanocytes. The degree of depigmentation and melanocytic necrosis is associated with the number of leucocytes that infiltrate the halo and the infiltrating leucocytes are mainly CD8-positive lymphocytes and monocytes (91,92).
Lymphocytes of the innate (NK, NKT cells) and adaptive immune sytem (T-cells) are required to prevent the development of tumors by the ability to produce interferon (IFN)-γ and their ability to kill. Dendritic cells (DCs) are widely acknowledged as the central surveillance cell type and play an important role in the activation and/or modulation of all these lymphocyte subsets to control and/or eliminate human tumors (Figure 4). DCs originate from bone marrow precursors and have the unique capacity to migrate to peripheral tissues, where they differentiate into immature DCs. Upon encountering tumor cells or tumor-associated antigens, DCs engulf this material and begin migrating via lymphatic vessels to regional lymphoid organs. Chemokines and their receptors, matrix molecules and adhesion molecules on the DCs, as well as on the surrounding tissues coordinate this migration. They mature en route; activating their ability to convert antigens to 10- to 15-mer peptides bound to major histocompatability

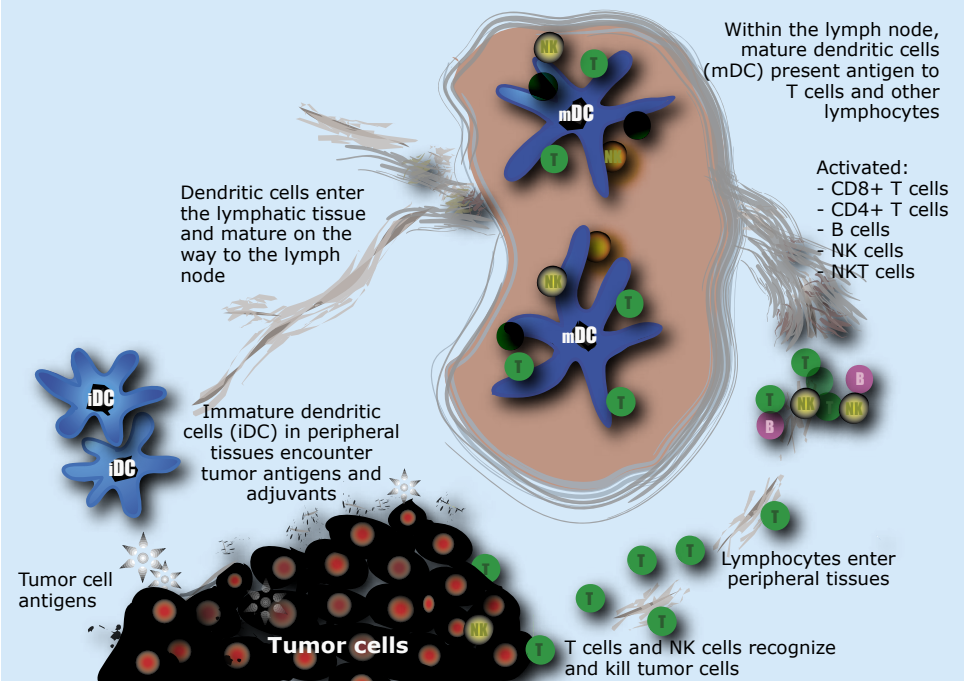

Figure 4: Induction of T cell responses by dendritic cells to tumors.
T cell responses to tumors may be induced by cross-priming, in which the tumor cells or tumor antigens are taken up, processed and presented to CD8+ T cells by dendritic cells (DCs). A second signal besides antigen recognition is required for naïve CD8+ T cells to differentiate into effector cytotoxic T lymphocytes (CTLs), which may be provided by (B7) costimulators on DCs, or by cytokines produced by CD4+ helper T cells. Differentiated CTLs then can kill tumor cells.

complex (MHC) class I and class II molecules. Mature DCs also upregulate production of surface costimulatory molecules (e.g., CD80, CD86) and cytokines needed to stimulate the antigen-specific cytotoxic T lymphocytes (CTL) they encounter in the T-cell areas of the draining lymph nodes. Mature DCs also activate and mobilize B cells, T-cells, natural killer (NK) cells and NKT cells essential for tumor killing (93). However, tumors frequently interfere with the development and function of immune responses and can actively down-regulate anti-tumor immunity. They escape detection and/or elimination by the immune system by down-regulation of MHC expression, low or absent production of specific tumor antigens, or crypticity of epitopes, leaving the tumor free to expand in an uncontrolled manner. Furthermore, tumor cells produce an offensive repertoire of cytokines that can either suppress the incoming effector cells or because of surface expression of molecules such as Fas ligand, which induce apoptosis of incoming T-cells. It has become evident that while protecting the host against cancer development, the immune cells also promotes the emergence of tumors with reduced immunogenicity leading to a complex interplay of tumor growth and tumor regression mechanisms (94).

1.10 *Exosomes*

Dendritic cells do not only trigger T-cell responses through direct cell-cell contact or cytokine production, but also through secretion of exosomes (95,96). Exosomes are small membrane vesicles (60 – 100 nm in diameter) of endosomal origin, which are secreted upon fusion of multivesicular bodies with the plasma membrane (97,98). Exosomes display a discrete set of proteins involved in antigen presentation, such as MHC-I and MHC-II (99). Peptide-pulsed DC-derived exosomes activate CTLs and elicited potent anti-tumor immune responses in tumor-bearing mice (100). Tumor cell types have also been shown to secrete exosomes (101,102). These exosomes are morphologically analogous to exosomes produced by DCs. However, the production of exosomes by tumor cells appears to be lower than that of DCs. The tumor-derived exosomes are capable of transferring MHC-I-peptide complexes to DCs, inducing a $CD8^+$ T-cell-dependent cross-immunization in tumor-bearing mice (102). Exosomes are capable of doing so since they display, amongst others, proteins containing native tumor antigens. Even exosomes derived from poorly immunogenic cancers were therapeutically effective, while the tumor lysate was not capable of inducing anti-tumor responses (100). More surprisingly, tumor-derived exosomes, from mesothelioma, colon, mammary and other carcinomas, loaded on DCs triggered T-cell-mediated anti-tumor immune responses leading to a strong inter-tumor cross-protection (101). This suggests that the exosomes probably contain shared tumor-rejection antigens. Despite numerous questions pertaining to their biological relevance, exosomes are having the potential to be an attractive powerful immunotherapeutic tool combining the anti-tumor activity of DCs with the advantages of a cell-free vehicle (103-105).

1.11 *Immunotherapy*

Cancer immunotherapy attempts to harness the exquisite power and specificity of the immune system to recognize and destroy tumor cells or to prevent tumor

recurrence. The fact that some patients with mesothelioma have tumors that regress spontaneously (106-108) or respond to immunotherapy suggests the immune system can generate anti-tumor reactivity under some circumstances (109). Clinical immunotherapy trials in mesothelioma patients have included intrapleural administration of IL-2, IL-12 or IFN-γ, systemic IFN-α alone or with chemotherapy, intrapleural administration of lymphokine activated killer (LAK) cells with IL-2, intratumoral administration of GM-CSF and subcutaneous administration of GM-CSF in combination with autologous tumor cell lysate (110-114). Administration of GM-CSF activates DCs, which are the most powerful antigen presenting cells of the immune system, and vital in inducing activation and proliferation of $CD8^+$ cytotoxic T-lymphocytes and $CD4^+$ helper T-lymphocytes (see section 1.9). However, DC function is suppressed in cancer patients through release of tumor derived soluble factors that inhibit the differentiation, maturation and therefore immunostimulatory function of DCs, leading to a defective induction of CTL responses (115-123). A major advantage nowadays is that DCs can be generated in large amounts *ex vivo*, in the absence of this suppressing environment, and subsequently loaded with a preparation of antigens, and then infused back into the patient to induce CTL responses. This has prompted their recent application to therapeutic cancer vaccines (124,125). Ebstein et al. have recently shown that human DCs pulsed with dead mesothelioma cells were able to induce a CTL respons *in vitro* directed against the tumor, particularly when DC were loaded with apoptotic tumor material, illustrating that malignant mesothelioma cells contain unknown tumor-associated antigens that can lead to an anti-tumoral immune response (126). Although this strategy was shown to be efficacious *in vitro*, it has not been shown that tumor antigen-pulsed DCs would have an anti-tumoral effect against malignant mesothelioma *in vivo*. Several studies in other cancers in humans, e.g. renal cell carcinoma, melanoma, glioma and lung cancer, have shown that DC-based immunotherapy is still sub-optimal but can induce tumor-specific CTL responses that lead to shrinkage of the tumor and sometimes prolonged survival (127). With more knowledge relevant to basic aspects of tumor immunology, immunotherapy might hold the key to the clinical realization of effective therapeutic treatment for mesothelioma in the future.

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Chapter 2

Aims and outline of the thesis

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Erasmus Medical Centre Rotterdam is situated in an area of high asbestos exposure due to shipyard building industries from 1970 to 1990. As a result the incidence of malignant mesothelioma (MM) in this region is one of the highest in the world. It occurs in about 400 - 500 patients in The Netherlands and in about 10,000 patients world-wide annually. The incidence is still rising. Our clinic is an important referral centre for experimental therapies for patients with MM. The Department of Pulmonary Medicine Rotterdam has a longstanding research interest in this refractory disease. As MM is difficult to diagnose and unresponsive to most therapies and typically recurs even after the most aggressive attempts of surgical resection and multi-modality approaches, new diagnostic and therapeutic measures are necessary.

The aim of the work addressed in this thesis was to improve the diagnosis and treatment of malignant mesothelioma.

The following reagents were at our disposal to perform this research: ten human MM cell lines established from pleural effusions and tissue specimens in our laboratory, a murine MM cell line (AB1), and pleural fluids from MM and other malignancies metastatic to the pleura. The cell lines of human origin were characterized by means of immunocytochemistry and cytogenetic analysis and confirmed to be of MM origin. We have obtained the AB1 cell line through the collaboration with Prof. Dr. B. Robinson, Queen Elizabeth II Medical Centre, Perth, WA, Australia. This murine cell line provides a relevant model for the human disease because it has been induced by crocidolite asbestos and parallels the human MM in several aspects. As our institute is a referral centre for patients with MM, a continuous supply of pleural fluid was guaranteed. The next section will give a brief overview of the experimental work that was performed.

Chapter 3 provides a literature survey of the prognostic factors in MM that may help to predict the future course and outcome.
In ***chapter 4***, the use of phage antibody display technology to generate human antibodies with new specificities is investigated. The methodology uses the functional expression of human antibody fragments on the surface of filamentous bacteriophages, selection of phage antibodies with antigens, followed by amplification of the selected phages in a bacterial host. To overcome problems involving selections on intact cells, we have developed an experimental model system that allows to improve the experimental conditions and to compare the various selection strategies. The optimization of key steps in the selection procedure of specific phage antibodies from a synthetic library on intact MM cells is described here.
Proteomics is the systematic analysis of the protein expression of healthy and diseased tissues and may lead to understanding of how cells actually work and how disease processes operate. In chapter 5 and chapter 6, novel proteomic technologies are utilized to analyze pleural effusions in order to discover changes in expression of pleural proteins that result from MM. ***Chapter 5*** describes the use of 2-dimensional differential gel electrophoresis (2D DIGE) to identify unique proteins by observing concentration changes and modifications on a single protein level. Therefore, serum and pleural effusion of the same patient is labeled with spectrally different fluorescent tags prior to the electrophoretic separation. In

chapter 6, protein profiles of pleural effusions are determined to differentiate MM from other malignancies giving effusions using Surface Enhanced Laser Desorption/Ionization (SELDI) time-of-flight (TOF) technology.
Exosomes are small membrane vesicles of endosomal origin. Their function is largely unknown but literature suggests that tumor-derived exosomes carry shared tumor-rejection antigens on their surface and are able to induce anti-tumor responses in different tumors. Therefore, the protein composition of MM-derived exosomes is studied in more detail. In ***chapter 7***, exosomes are isolated from pleural effusions and matrix-assisted laser desorption ionization (MALDI) TOF mass spectrometry is used to characterize the protein composition. Isolated fractions are subjected to electron microscopy to assure the presence of exosomes. ***Chapter 8*** describes the analysis of exosomes isolated from culture supernatants of MM cells *in vitro*. In ***chapter 9*** the expression profiles of 80 different cytokines in supernatant of MM cell lines and corresponding patient's mesotheliomatous pleural effusions are analyzed. The cellular infiltrate in human MM biopsies is determined using immunohistochemistry. We further investigate the presence of regulatory T-cells in MM tissue and their function in a murine MM model.
In ***chapter 10***, we evaluate the hypothesis that autologous dendritic cells (DCs) presenting tumor antigens might induce a protective immune response in MM. A murine model allows us to study the impact of antigen source, DC maturation status, and timing of administration on outcome. Preliminary results of our phase I clinical study with a combination of chemotherapy and autologous DC vaccinations for a patient with MM are described in ***chapter 11***.
In ***chapter 12***, the main points emerging from the experimental studies are discussed. Also the contribution of these studies to the current therapeutic application of DC-based immunotherapy in MM is described as well as proposals for follow-up investigations.

Chapter 3

3

Clinicopathological prognostic factors and scoring systems in malignant pleural mesothelioma

Oxford University Press 2006
"Malignant Pleural Mesothelioma" Editors K. O'Byrne and V. Rusch

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Clinicopathological prognostic factors and scoring systems in malignant pleural mesothelioma

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Introduction

Prediction of the future, whether it concerns economic welfare, the weather forecast, or health issues, has always fascinated mankind. In ancient Greece and Rome, major events in life were forecast by priests from the position of chicken bones that had been thrown in the air or the appearance of the liver of a goose that had been sacrificed, methods that we nowadays consider a 'wild guess'. In our modern era the wild guess is replaced by a 'calculated risk', introduced by statisticians. Focusing on statistical studies on cancer, we can identify prognostic factors which provide a forecast or statement of probability on a certain clinical outcome [1]. This statement predominantly applies to a group of patients, but can also be used in daily practice to make predictions and clinical decisions on individual patients.

Malignant pleural mesothelioma (MPM) is a disease with a heterogeneous clinical outcome. Although the prognosis is extremely poor in general, individual patients might live for an unexpectedly long time [2]. Similarly, although mesothelioma is known to be resistant to most tumour-directed therapies, individual patients might benefit from chemotherapy, radioradiotherapy, photodynamic therapy, immunotherapy, radical surgery, or multimodal treatment.

In this chapter some key issues on the science of prognosis will be highlighted before the individual prognostic factors that have been identified in MPM are described.

Prognostic factors

Prognostic factors are variables that can account for some of the heterogeneity that is associated with the expected course and outcome of a disease. They guide clinicians in clinical decision-making. The use of prognostic factors is hampered by the fact that the clinical outcome for the individual patient remains hard to predict, even when valid prognostic factors are available. Only groups of patients with mainly good or poor prognostic factors can be discriminated, and the median survival of a given group will be better or worse, respectively.

Not all prognostic factors have a clinically relevant impact on the prognosis. Since prognostic factors are not studied in a structured fashion, their relation and impact on prognosis is not evident in every situation [3].

A prognostic factor should be a significant and independent variable and should predict clinically important issues. Significant is meant to be statistically significant, and many, mostly univariate, tests are used for this purpose. An independent factor adds new and additional prognostic value to the already known prognostic factors. To show the additional value a multivariate analysis

is required, which should preferentially be performed on prospectively collected data with adequate power to detect a small but significant prognostic value [1]. The number of events (e.g. death) should be at least 10 times the number of potential prognostic variables that are included in the model [4]. Therefore the ideal study of prognostic factors has a prospective design with a large study population in which the prognostic factor is evaluated by multivariate analysis.

Although the incidence of MPM is rising worldwide, it remains a relatively rare disease, and prospective evaluation of a novel prognostic factor would take a long period of time. Therefore the majority of studies on prognostic factors in MPM are retrospective analyses of clinical or pathological databases. A major flaw of these retrospective analyses is that data on 'confirmed' or already known prognostic factors are not always available and cannot be incorporated in the multivariate analysis to prove the 'additional' prognostic value of a new factor.

Most studies have an 'exploratory' nature, focusing on the prognostic potential of a factor, rather than 'confirmatory', applying known factors to other patient populations and thereby confirming their prognostic value [4]. Before being applied in clinical practice, a new prognostic factor should be validated in at least one confirmatory study.

Furthermore, prognostic factors not only predict issues relevant to daily clinical practice, but can also contribute to clinical and basic science. Currently, prognostic factors in malignant mesothelioma are most frequently used to select a particular patient population for a particular therapy or study protocol. Patients who participate in trials can be stratified according to their prognostic factors and, conversely, a description of the prognostic factors helps to identify the patients to whom the results can be applied. Proper description of the prognostic variables of all study groups that participate in randomized or other trials adds to the quality of the study reports [5, 6].

Progress in molecular and cellular biology might provide us with new prognostic markers. These markers have an additional benefit, since they reflect a specific feature of the biology of the tumour cell and lead to improved understanding of molecular pathogenesis [7, 8]. In the future, they might guide scientists in the development of new therapeutics, predict the sensitivity of the tumour for a particular treatment, and lead clinicians in their choice of the appropriate therapy.

Prognostic factor studies in malignant mesothelioma

More than 70 papers have been published on prognostic factors in MPM, allof which focus on survival. These papers describe the prognostic value of more than 50 different factors. The majority of the studies are retrospective analyses of a particular patient population from a clinic or registry, or of patients who have participated in clinical trials, or concern reports on data obtained from pathology archives. Four studies which prospectively evaluate data from consecutive patients entering a hospital or receiving a particular treatment have been published [9–12].

One confirmatory study on prognostic factors in MPM has been published. Edwards *et al.* [13] applied the prognostic factors that had been identified by evaluation of the data from mesothelioma studies by the Cancer and Leukemia Group B (CALGB) [14] and the European Organization for Research and Treatment of Cancer (EORTC) [15] to their own population. By retrospective evaluation they showed that prognostic factors that had been determined in MPM patients involved in clinical trials also had a predictive value in a general hospital population [13].

Another landmark study on prognostic factors in mesothelioma is the study on the database of the Surveillance, Epidemiology and End Results (SEER) Program from 1988 [16]. It is the largest study of its kind, involving 1475 histologically confirmed cases of mesothelioma, and shows that age, sex, tumour stage, treatment, and geographical area of residence are important predictors of patient survival.

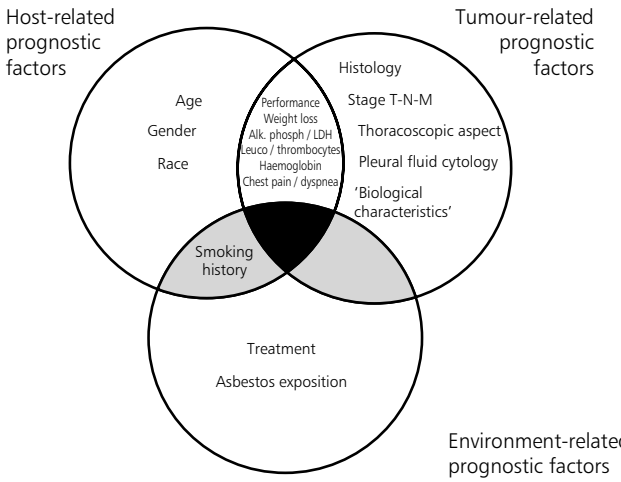


Figure 5.1 Prognostic factors may be incorporated in more than one category, as illustrated in this diagram.

As MPM is generally seen as a disease with a rapid fatal outcome, quality of life is one of the most important issues for a patient. Although the quality of life might be reflected by the performance status, no data are available on the correlation between prognostic factors and quality of life, or on the role of quality of life as a prognostic factor. In this regard the clinical needs do not parallel the academic search for prognostic factors. All prognostic factors in mesothelioma studies, apart from one, focus on survival in an attempt to discriminate patient populations with a relatively good or relatively poor prognosis. Only one study focuses on recurrence-free survival [17], but no factors have been described that predict other clinical issues.

Prognostic factors can be separated into host-related prognostic factors, tumour-related factors, and environment-related factors [18]. The host-related factors typify the patient, the tumour-related factors characterize the disease, and the environment-related factors are external factors not directly related to either the disease or the patient. The prognostic factors that have been described for mesothelioma studies according to this classification are categorized in Figure 5.1. As illustrated, the prognostic factors may be included in more than one group. For instance, performance status is influenced by both comorbidity and tumour load, and therefore might be considered as both a host-related and a tumour-related factor. A remarkably large number of different factors have been evaluated for their prognostic significance, with a large number of biological parameters appearing to have an impact on prognosis. We will focus on the prognostic factors and describe their value as predictors of survival.

Host-related prognostic factors

Host-related prognostic factors include parameters that are unrelated or only indirectly related to the tumour, but have a major impact on the outcome of the disease. Factors of this type have been studied extensively in MPM. Although almost all studies concern retrospective analyses of hospital or study databases, they agree on the most important factors. These studies have been listed in a number of publications [7, 13–15, 19, 20].

Performance status

All MPM studies that included performance status data in their analysis showed a significant effect on the survival, with a better performance correlating with a better survival [10, 11, 13–15,

19, 21–25]. Only one study, which included only patients with WHO-performance status 0 and 1, did not confirm this result [26]. Since, until recently, the natural course of the disease could only be influenced minimally by treatment, the large impact of performance on survival was to be expected [27].

Since the first description of performance status by Karnofsky [28], its use has been widely accepted in clinical oncology [29]. It is considered to be a major parameter of the fitness of a patient and an important prognostic factor for many malignancies. The major drawback of performance status is the fact that it is not systematically documented in the records of all patients and therefore cannot be taken into account in all retrospective analyses [27, 30]. Moreover, oncologists tend to give the healthiest assessment, nurses an intermediate assessment, and patients themselves the poorest assessment of their performance status [31]. Despite these disadvantages, the performance of a patient remains one of the most important prognostic factors, and therefore a systematic and accurate documentation of the performance score should be in the charts of all MPM patients.

Weight loss

Weight loss is a poor prognostic factor in MPM [12–14, 22, 26, 32]. Patients without weight loss appeared to derive most symptomatic benefit from palliative surgery [26]. MPM patients with weight loss undergoing chemotherapy have fewer symptomatic and radiological responses and a shorter overall survival [33]. However, in all these studies weight loss lost its statistical significance as a prognostic factor when evaluated in a multivariate analysis.

Although weight loss has no independent prognostic significance in MPM, it is generally seen as a sign of a poor condition for patients with a malignancy. Clinical trials studying chemotherapeutic agents tend to focus on patients with the best prognosis and often exclude patients with a considerable weight loss. It is easy and reproducible to determine and is a helpful criterion for the selection of patients for chemotherapy or other treatments [34].

Leucocytes, platelets, and haemoglobin

Recently attention has been paid to the prognostic significance of these parameters by evaluation of the CALBG and EORTC databases [14, 15]. Appraisal of the EORTC database revealed that a high leucocyte count ($>8.3 \times 10^9$/litre) had a significant poor prognostic value [15], whereas a leucocyte count $>8.7 \times 10^9$/litre was predictive of a poor prognosis in univariate analysis of the CALBG data [14]. The confirmatory study by Edwards *et al.* [13] found an additive poor prognostic effect of a high leucocyte count in their population. Further but weaker support for this observation is provided by two other studies [19, 22]. One study did not detect a significant contribution of the leucocyte count to the survival [26].

Data concerning the prognostic significance of high platelet counts are variable. Three studies confirm a poor prognostic impact of a platelet count either >400 000/ml [14, 32] or >314 000/ml [17]. One study describes a univariate effect [13], and five studies do not verify any prognostic value of the platelet count [9, 15, 19, 25, 26]. Nevertheless, platelet count remains an interesting parameter theoretically. Platelets may affect the tumour by promoting proliferation of tumour cells by the secretion of agents such as vascular endothelial growth factor (VEGF) [35]. The effect of platelets on angiogenesis and the tumour vasculature via VEGF and other pro- and anti-angiogenic factors has recently been recognized [36]. Additionally, platelets act as a source of several inflammatory mediators [35]. The tumour may affect the number and activation state of platelets. Mesothelioma cells from a patient with a thrombocytosis produced large amounts of interleukin 6 (IL-6), an important promoter of thrombocytosis [37]. Thrombocytosis occurs

rather frequently in mesothelioma patients, although thromboembolic complications such as severe thromboses and concurrent pulmonary emboli are only infrequently reported [11, 32, 38].

The haemoglobin level was one of the most significant prognostic factors in the study by Edwards *et al.* [13]. In two other studies the haemoglobin level had a significant prognostic value in univariate, but not multivariate, analyses [14, 15]. These studies concerned patients who had participated in chemotherapy trials and therefore had to have adequate cell counts. It is not known whether any or how many patients had received a blood transfusion before being entered in the trials, which might have biased the haemoglobin analysis. Two papers concentrating on haemoglobin as a prognostic factor did not confirm these data [19, 26]. Both studies used rather low median haemoglobin values for their patient population with cut-off levels of 12.6 g/dl and 13.2 g/dl, respectively. More data are needed to appreciate better the value of blood cell counts as prognostic indices in MPM.

Gender

Females with MPM tend to have a better prognosis than men in multivariate analysis [9, 10, 13, 15–17, 39]. The female gender is a good prognostic factor in univariate analysis in three other studies [23, 24, 40]. Although 12 studies could not confirm the good prognostic value of the female sex, they did not show the converse.

MPM preferentially affects males, probably because men are more likely to have an occupational exposure [32]. Therefore most studies only involve a small number of female patients, limiting the power to detect the prognostic importance of gender. Nevertheless, most data seem to point to a better prognosis for female patients with MPM. So far, the explanation for this observation remains to be elucidated.

Age

Most studies agree that older age is a poor prognostic factor, although this statement is not undisputed. Some studies state that an age higher than 50 or 60 years is associated with a worse survival [23, 41]. Other studies mention a worse survival at an age above 65 or 75 years [11, 14, 19, 25, 32, 42]. Only one study on 100 patients identifies older age as a good prognostic factor, when analysing age as a continuous variable in a multivariate analysis, but it does not provide an explanation for this contradictory result [30]. Eleven papers have been published that did not detect any significance for age as a prognostic factor. Older patients are likely to have more comorbidity, which again may have an impact on survival, especially since co-morbidity is usually poorly documented. This might upset the interpretation of data on the prognostic significance of age in MPM.

Chest pain and dyspnoea

Chest pain and dyspnoea are common symptoms at presentation of MPM. Chest pain might be a non-specific symptom but can also be caused by infiltration of the structures of the thoracic wall by tumour, and as such can be correlated with locally more advanced tumours. Therefore it moight even be considered as a tumour-related prognostic factor. Several studies have shown an additional poor prognostic impact of the presence of chest pain [14, 32], whereas others showed this prognostic effect by univariate analysis only [13, 22]. However, the majority of studies did not detect any impact of the presence of chest pain on the survival of MPM patients [10, 19, 24, 25, 27, 40, 43].

A similar number of studies have analysed the prognostic effect of dyspnoea, but these unanimously conclude that dyspnoea does not have any impact on survival [10, 19, 23–25, 27, 40, 43].

Apparently, the presence and amount of pleural fluid, which is the major cause of dyspnoea in MPM, is not correlated with the extent of the tumour nor does it predict its natural course.

Race and ethnicity

One study including 19 per cent black and 81 per cent white patients demonstrated a significant survival advantage for white patients [24]. This effect was independent of other prognostic factors such as performance status, therapy, and tumour stage. No explanation was given for this phenomenon. A similar ethnic distribution was described in the SEER study but a better outcome for white MPM patients was not detected [16]. Race and ethnicity are only scantily reported in treatment and prevention trials in solid tumours including MPM [44] . Although the limited size of the patient population in most MPM studies does not allow subgroup analyses, information on the diversity of the participants substantially adds to the validity and applicability of the results [44].

Tumour-related prognostic factors

Tumour-related prognostic factors involve the anatomical tumour stage and histological and biological characteristics of the tumour.

Anatomical staging

Anatomical staging is a major prognostic factor in MPM. Almost all prognostic factor studies that include staging in their analysis reveal a significant impact on survival and show that this effect adds to the other prognostic factors. Twelve studies support this statement whilst only two cannot confirm it, as is summarized in Table 5.1. Despite this, staging is not generally accepted as a good prognostic factor for several reasons. Firstl, several different staging systems are in use, which makes direct comparison of the papers focusing on this subject difficult [40, 45–47]. Indeed, the radical multimodality therapy series of Sugarbaker *et al.* [40] describes a highly significant impact of one staging system on survival, while two other staging systems do not seem to have a predictive value in the same patient population. Secondly, proper staging of MPM requires a surgical procedure, which is a major disadvantage. Thorough staging is currently only indicated in patients eligible for major surgical procedures, and most patients tend to be inoperable at presentation. This implies that surgical staging is not routinely performed in all patients, and data on tumour stage are not available for every patient [27, 41]. Thirdly, the tools used for non-surgical staging differ considerably even within the populations from single hospitals [27, 30].

Some studies highlight specific aspects of the staging of MPM. In early stage MPM in particular, invasion of the visceral pleura was found to be a negative prognostic factor [12], and this feature was subsequently incorporated in staging systems [46]. In fact, a normal or purely inflammatory macroscopic appearance of the visceral pleura was associated with better survival than pleura with small nodules, which again had a better prognosis than completely involved visceral pleura (24months, 10.5 month, and. 6.9 months, respectively) [12]. A single observation points to the potential significance of the absence of neoplastic cells in the pleural fluid [25]. Negative microscopic resection margins positively influenced the survival of patients who had extrapleural pneumonectomy as part of trimodality treatment [40]. The statement 'negative resection margins' requires histological examination of at least 20 sections of the extrapleural pneumonectomy specimen, and therefore is difficult to reproduce since it is only valid for the minority of the patients who were candidates for this extensive therapy.

Although radiological techniques like CT and MRI scanning have their limitations in predicting local tumour spread and presence of mediastinal lymph node metastases, most centres rely

Table 5.1 Tumour stage as a prognostic factor in malignant pleural mesothelioma: different studies focusing on tumour stage as a prognostic factor in malignant mesothelioma

Reference	n	Patient selection	Staging system	Significance[a]	Staging method
Rusch and Venkatraman [9]	231	Prospective registry	TNM [46]	+	Thoracotomy
			T1and 2 vs. T3 and 4	+	
			N0 vs. N2	+	
Tammilehto [10]	98	Prospective database	Mattson [111] stage I and IIA vs. higher stages	+	Clinical and surgical
Boutin *et al*. [12]	125	Prospective series	Butchart *et al*. [45]	+	Thoracoscopy
			Stage Ia vs. Ib	+	
Ohta *et al*. [39]	54	Random samples	Stage III and IV vs. I and II	+	Clinical
Metintas *et al*. [19]	100	Consecutive patients	Stage I vs. higher stage	+	CT
Van Gelder *et al*. [42]	167	'New cases'	Butchart	+	Clinical
Spirtas *et al*. [16]	1475	SEER Registry	Localized vs. regional vs. distant vs. unknown	+	
Ruffie *et al*. [32]	332	Pathological diagnosis	Butchart	+	From charts
Alberts *et al*. [24]	262	Histologically confirmed	Butchart	+	CT and thoracotomy
De Pangher Manzini *et al*. [25]	80	Consecutive patients	Butchart I and II vs. III and IV	Univariate[b]	Clinical
Sugarbaker *et al*. [40]	183	EPP and chemoradiotherapy	Sugarbaker *et al*. [47]	+	Extrapleural pneumonectomy
			Revised system [40]	+	
			TNM	–	
			Butchart	–	
Tammilehto *et al*. [21]	88	'With adequate CT'	TNM	+	CT
			T	+	
			N	–	
			M	–	

Direct comparison between the studies is severely hampered by the major differences in patient selection, staging systems, and methods used to stage the patients.

a+ The stage was a significant variable in a multivariate analysis of this series; – the stage was not significant.

[b]This study was not significant in multivariate analysis, possibly because 20 patients with an unknown stage and only four patients with stage II or IV were included.

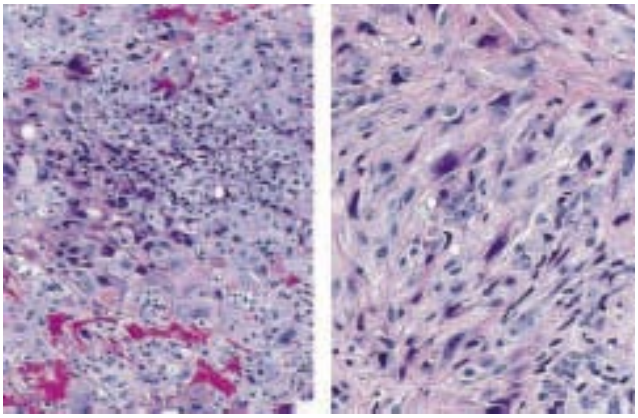
on these tools for staging MPM. Estimation of the TNM stage from 88 preoperative CT scans revealed significant differences in prognosis correlated with the T categories and the TNM stages, but not with the N or M categories [21], although some centres feel that retrospective TNM staging is not sufficiently accurate [13]. Another CT parameter, tumour volume estimated by three-dimensional CT scan reconstruction, did predict the survival of patients who underwent resection of MPM [17]. The median survival for patients with a preoperative tumour volume less than 100 cm^3 was 22 months compared with. 11 months if the volume was more than 100 cm^3.

MPM with a high metabolic activity, as assessed by fluorodeoxyglucose positron emission tomography (FDG PET), may have a worse prognosis. [48] A study of 17 patients with predominantly epithelioid and biphasic MPM showed a significantly worse survival for patients with a standardized uptake value (SUV) greater than 4 compared with the group with lower uptake of deoxyglucose and a lower metabolic activity. No information was available on other prognostic factors, which implies that this interesting finding needs to be confirmed by further studies.

Histology

The histological subtype is the best studied and most important prognostic factor of malignant mesothelioma (Fig. 5.2). Patients with an epithelioid type MPM have a significantly better prognosis than those with a sarcomatoid subtype [9, 40, 43, 49]. Differences in median survival may be as great as 200 days [27, 50]. Some patients have a mixed histology, with both epithelioid and sarcomatoid features. When larger pleura samples are taken for diagnosis, the mixed subtype of MPM is diagnosed more frequently [51, 52]. In survival analyses, mixed and sarcomatoid histology are often combined in one group since their incidence compared with the epithelioid subtype is rather low [9].

Almost all prognostic factor studies reveal data on the histological subtyping of the tumour. More than 50 per cent of the studies, including all prospective analyses, confirm its prognostic significance. Only six studies do not identify histological subtype as a significant factor [17, 19,



(a) **(b)**

Figure 5.2 The most important prognostic factor in malignant pleural mesothelioma. (a) A mesothelioma with predominantly epitheloid cells in solid sheets; (b) a mesothelioma characterized by spindled cells with a fascicular pattern. The former epitheliod tumour has a far better prognosis than the latter sarcomatoid mesothelioma. In this particular situation both micrographs were obtained from the same patient, who consequently has an intermediate prognosis. (Courtesy of Dr M. den Bakker, Erasmus MC, Rotterdam, The Netherlands.)

24, 39, 41], among which is the largest study on prognostic factors in mesothelioma [16], but this paper listed the histological subtype for only 291 of the 1475 cases described. Other negative studies were also hampered by missing data [19], inter-observer error, leading to a lack of clarity regarding the histological subtype [24], or a long period of time over which the cases were gathered [27].

One study suggests that patients with a definite diagnosis of MPM survive longer than those with a possible or probable diagnosis [15]. Explanations given for this feature were a shorter time to obtain the diagnosis in the 'definite' group and/or the presence of more undifferentiated tumours in the 'probable or possible' group. The only other paper including this factor in its analysis could not confirm the prognostic significance of the certainty of histological diagnosis [13].

Origin

The influence of the site of origin on the survival of malignant mesothelioma is not clear. A malignant mesothelioma can originate from many serosal tissues, as is illustrated by the large SEER database which included pleural mesothelioma (81 per cent), peritoneal mesothelioma (15 per cent), and mesothelioma from the ovary, tunica vaginalis, heart, and lung [16]. Mesothelioma from the heart probably reflects pericardial mesothelioma, and that from the lung most likely reflects MPM. A survival difference between the different primary tissues was not described.

One series, including 136 pleural and 37 peritoneal mesotheliomas, revealed a poorer survival of patients with a pleural origin [23]. Otherwise, analysis of data from 57 patients with the pleura and 12 patients with the peritoneum as initial site of the tumour revealed a better survival for patients with pleural disease [11]. A further study with relatively few patients with peritoneal mesothelioma did not detect any survival difference [10]. Differentiation between peritoneal mesothelioma and female genital cancer can be difficult, and it is not clear to what extent the misdiagnosis of peritoneal mesothelioma confounds the results described earlier [10, 16].

Tumours from the testicular serosa are generally detected at a relatively early stage with a small tumour load and this might be reflected in the good survival observed. This probably also accounts for two patients with a mesothelioma originating from this site who had better survival than patients with primary pleural disease, despite the fact that their disease was metastasized [23].

Left- or right-sided pleural mesothelioma

Only one suggestion has been published so far on the favourable effect of left-sided tumours [41]. This study describes the prognosis of 167 MPM patients who were selected from the files of a pathology laboratory. No explanation is given for this observation, but it does not seem to be secondary to a higher complication or mortality rate for patients who had surgery for right-sided tumours. Other studies report similar survival rates for patients with right- and left-sided tumours [19, 25, 40].

Tumour markers

Recently, new markers have been explored by molecular and cellular biological techniques. Some markers seem to predict a better outcome of the disease in selected MPM patients, but confirmation by larger independent series is still lacking. More importantly, these markers give insight into the (extra)cellular processes associated with the development of MPM and shift the approach to MPM from anatomical, focusing on surgical staging and local (radiotherapeutic) treatment, towards systemic, focusing on the selection of patients for systemic therapy.

In general, tumour markers might be used for screening purposes, as a diagnostic, staging, or monitoring tool, or as a tool to predict the response to treatment or to foresee tumour recurrences [53]. Guidelines for the use of tumour markers in the management of various solid tumours such as breast, colorectal and prostate cancer have been published [53, 54]. Our knowledge of tumour markers in MPM is still too scanty to allow the implementation of the use of the markers in daily clinical practice, but they might be of help in the development of new diagnostic or therapeutic strategies. The tumour markers that have been tested for their prognostic value are described in Chapter 3.

Chromosomal changes

Chromosomal abnormalities associated with MPM are complex, involving both numerical and structural changes. Controversial results have been reported regarding the prognostic significance of, for example, DNA ploidy and lower S-phase fractions [55–58]. The number of copies of the short arm of chromosome 7 and hyperdiploid mean chromosomal number seem to correlate with shorter survival rates [59]. The proliferation index determined by flow cytometry and the expression of the cell-cycle-related proteins $p27^{kip1}$, PCNA and MIB1 (Ki-67 nuclear antigen) has been described as an independent prognostic factor [55, 60–63]. A low mitotic count and a low apoptotic index defined by *in situ* end-labelling is associated with a significant survival advantage [64]. Low expression of the p27 antigen in tissue sections of MPM is associated with a significantly worse prognosis [65].

Recent research has demonstrated that mice that developed pleural tumours induced by conditional knockout of different individual genes differed dramatically in overall survival time [66]. Whether this has its human counterpart in the expression-ratio-based microarray analysis that was able to predict treatment-related outcomes in selected MPM samples is not yet clear [67].

Tumour suppressor genes

Analogous to the frequent chromosomal aberrations, numerous mutations and deletions of tumour suppressor genes have been described in malignant mesothelioma [68–70]. An analysis of the significance of the expression of the tumour suppressor genes p53, Kristen ras (K-*ras*) and *rhoA* did not reveal a prognostic value of these markers [71, 72].

Aberrant methylation of the tumour suppressor gene RASSF1A has been associated with a poor prognosis in lung cancer patients [73]. Although a significant relationship with prognosis was not demonstrated, aberrant methylation was notably absent in four cases with survival that exceeded 36 months [74]. Expression of the cell cycle inhibitor $p21^{WAF1/CIP1}$ (p21), a downstream target of p53, bears a prognostic significance in patients in whom the SV40 sequence is found in the tumour tissue [75].

Simian virus 40

One study has shown a trend for increased survival in SV40-negative MPM patients [76]. Simian virus 40 (SV40), a DNA virus with potential transforming and carcinogenic effects, has been mainly studied as a possible aetiological, rather than prognostic, factor of mesothelioma [77]. DNA encoding SV40 Tag or SV40 Tag protein expression is primarily found in biphasic and sarcomatoid mesotheliomas [76], but it is still not clear whether SV40-postitive MPM tumours behave more aggressively than SV40-negative tumours.

Angiogenesis

Angiogenesis, the process of generating new blood vessels, is essential for tumour growth beyond a few millimetres in diameter [78]. Increased microvascular density present mainly at the periph-

ery of the tumour correlates with a shorter survival [22, 79]. Also, the spatial arrangement of the vessels, in addition to vessel density, correlated with survival, a feature that also applies to patients with cervical and colorectal cancers [80]. A number of factors are involved in the regulation of tumour angiogenesis. Although high levels of vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (FGF-1), and transforming growth factor β (TGF-β) can be detected in serum and effusions, only FGF-2 and its binder syndecan-1 is significantly correlated with tumour aggressiveness and the prognosis of MPM [81, 82]. This study also showed that syndecan-1 is modulated by the Wilms tumour 1 transcriptional suppressor gene (WT1) product, which correlates with the histological type but did not correlate with prognosis.

The role of thrombospondin 1 (TSP-1) is still unclear. It was originally reported as an inhibitor of angiogenesis [83], but studies now suggest that it also may function as stimulator of angiogenesis [84]. The utility of TSP-1 overexpression as a prognostic factor in MPM had little value [85].

Cyclo-oxygenase-2 (COX-2) is overexpressed in MPM [86] and correlates with a worse survival in both univariate and multivariate analyses [87, 88]. COX-2 catalyses the initial rate-limiting steps of prostaglandin E_2 (PGE_2) synthesis from arachidonic acid in cell membranes. PGE_2 activates specific epithelioid receptors that increase cyclic AMP production, which in turn stimulates synthesis of VEGF, a trigger of angiogenesis.

Serum and pleural fluid markers

Hyaluronan is an extracellular polysaccharide present in pleural exudate from MPM patients. A retrospective analysis of 100 MPM patients by Thylen *et al.* [30] showed the prognostic significance of hyaluronan levels in the pleural fluid, with elevated levels indicating a longer survival.

High serum lactate dehydrogenase (LDH) levels (>500 IU/l) are associated with worse prognosis, and since LDH is commonly measured in daily clinical practice it might be an useful marker for patient selection for specific treatments [14, 19]. More recently, Robinson *et al.* [89] assayed serum concentrations of soluble mesothelin-related proteins (SMRP) using a double-determinant ELISA and found that it correlated with the size of the tumour and increased during tumour progression. Although not tested widely, SMRP in serum could be a useful marker for monitoring MPM progression [89].

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) and their endogenous inhibitors (TIMPs) may have prognostic significance in MPM [90]. These proteins are involved in extracellular degradation of matrix proteins, such as collagen, laminin, and fibronectin, during normal tissue remodelling processes and implicated in the pathogenesis of diverse invasive processes including local cancer spread and metastasis. MMP-1 is overexpressed in MPM, but there is no significant variation in MMP and TIMP expression in patients with a better or worse prognosis [90]. Also, MMP-2 is abundantly present in MPM specimens [91]. High MMP-2 expression and activity seemed to be correlated with a poorer survival outcome.

Tissue polypeptide antigen and Cyfra 21–1

Tissue polypeptide antigen (TPA) assay measures a specific epitope structure of human cytokeratin 8, 18, and 19 fragments. The Cyfra 21–1 assay is specific for the cytokeratin 19 fragment. In contrast with cytokeratins themselves, cytokeratin fragments are soluble and thus detectable in serum and pleural effusions of MPM patients. In a retrospective study in 52 patients Schouwink *et al.* [92] showed that high serum TPS and Cyfra 21–1 levels were predictive for poorer survival in multivariate analysis. TPA and Cyfra 21–1 could not discriminate MPM from other malignant pleural diseases.

Antioxidant enzymes

Catalase is a hydrogen peroxidase scavenging enzyme which decomposes hydrogen peroxide to water and oxygen. Together with manganese superoxide dismutase, a superoxide scavenging enzyme, these antioxidant enzymes are more highly expressed in MPM than in healthy mesothelium or metastatic adenocarcinoma of the pleura [93–95]. High catalase, particularly high coordinated expression of catalase and manganese superoxide dismutase, in mesothelioma is associated with a better prognosis [96]. High prevalence of other detoxification enzymes such as glutathione S-transferases (GSTs) are in good agreement with the low responsiveness of mesothelioma to chemotherapy but their prognostic value is debatable [97, 98].

Proteomics and genomics

New powerful cellular, molecular, and proteomic-based technologies will undoubtedly lead to the identification of novel tumour markers. The application of new or improved techniques for immunohistochemistry [99], tissue immunoblotting, differential display, laser capture microdissection, phage antibody display technology [100], and flow cytometry [55] together with proteomic-based approaches, such as two-dimensional gel electrophoresis, mass spectrometry (MALDI–TOF/QTOF/SELDI–TOF) and protein (chip-based) expression array technology will enable this process to develop over the coming years and will undoubtedly affect clinical management of MPM in the future.

The advent of microarray gene expression profiling represents one such area. Early studies using microarray technology, which have been able to stratify mesothelioma according to outcome, have now been completed. The first prognostic set of genes, consisting of eight discriminatory genes, was identified by Bueno and colleagues, and a six-gene model basede on these could significantly predict patient outcome after treatment [101]. Building on this, Bueno's group carried out a study to identify markers which had prognostic value for patients with widely divergent survival times. From this analysis 46 putative markers were identified. The authors identified four genes [*KIAA097*, GDP-dissociation inhibitor 1 (*GDIA1*), cytosolic thyroid hormone binding protein (*CTHBP*), and an EST similar to the L6 tumour antigen] which could correctly classify (100 per cent) a training sample. These genes were subsequently tested on 29 samples not previously subjected to microarray analysis, and were found to significantly predict outcome in these samples [102]. A later study compared two different microarray analysis methods to identify a common subset of 27 genes which could be used to predict both survival and progression of malignant pleural mesothelioma [103].

In summary, many molecular markers have been studied in MPM. The detection of these markers has led to the evaluation of a range of new targeted therapeutic agents, including inhibitors of angiogenesis and cell signalling, and survival pathways and immunomodulatory agents in the management of this disease [104]. In the future the powerful new technologies of proteomics and genomics will allow us not only to predict outcome for the individual patient and identify novel targets for therapy but also to individualize patient treatments based on likely outcomes from the various therapeutic options available.

Environment-related prognostic factors

Environment-related factors are external to the patient and his or her disease. They include treatment, exposure to asbestos, the availability of clinical care and social support, and the level of education and socio-economic status of the patient. Papers focusing on environment-related prognostic factors generally include patients with different malignancies. It is likely that the poor prognostic factors from these surveys, such as lower socio-economic status, lower educational

level, and greater distance from medical centres and experience of medical professionals, also apply to mesothelioma patients and may account for regional survival differences [105–108]. Therefore a description of the clinical setting in which a study has been performed is potentially important when reporting on clinical trials. In this regard multicentre and multinational trials should consider stratification of the patients by centre of referral when analysing outcomes.

Exposure to asbestos

One paper describes a negative association between asbestos exposure and survival in 332 patients from the area around Ontario and Quebec [32]. Other studies, which investigated the correlation between asbestos exposure and survival, could not confirm this result. A history of exposure to asbestos *per se* is not an ideal prognostic factor since it is rather poorly reproducible. Many patients do not recall whether they have been exposed to asbestos, and when exposure to asbestos has occurred it is difficult to quantify. A more objective measure might be the amount of asbestos fibres in pleural biopsies or resection specimens after a surgical procedure. Correlation of asbestos fibre count with survival in 28 patients revealed that a lung tissue fibre count below 1×10^6 fibres/g tissue seemed to predict a relatively good prognosis, but its prognostic value was no longer apparent in a multivariate analysis [10].

The analysis of the SEER data for 1475 patients revealed poorer survival of patients from registries in areas that had shipbuilding as major industry [16]. A possible explanation for this phenomenon was higher asbestos exposure in these areas, but since individual data on exposure to asbestos were not available, the evidence for this statement was rather thin.

Smoking

The absence or presence of a smoking history does not have any prognostic significance for patients with MPM [10, 19, 23, 24, 32, 40].

Treatment

The data from the first phase III randomized trial in MPM have recently become available, and for the first time a particular chemotherapy schedule was proved to offer a survival benefit [109]. Until then only less conclusive data were available, including that from the studies focusing on therapy as a prognostic factor (Table 5.2).

Several papers show a beneficial effect on survival of tumour-directed therapy. However, three papers describing prospectively obtained data showed only a statistically weak or no treatment effect [9, 10, 12]. Three other papers which used a multivariate analysis and included the performance status as a prognostic factor did show a treatment effect. Pleuropneumonectomy and chemotherapy [23], any therapy with doxorubicin and radiotherapy as a major component [24], and chemotherapy and more extensive surgery [11] were all predictors of a better survival of the MPM patients. Nevertheless, the retrospective nature of the studies made it impossible to relate the difference in prognosis solely to therapy [41].

As described earlier, performance status is a valuable prognostic factor that can be used to select patients for a particular therapy. Therefore analysis of the factor 'treatment' on survival may be biased by the performance of the patients [24]. For instance, a better outcome for patients receiving chemotherapy might well be due to selection of patients with a better performance status [12]. Patients with better performance status might show better responses to chemotherapy [11]. In some studies the significance of radiotherapy or chemotherapy [10], or radiotherapy and surgery [21], in univariate analysis is lost when performance status is taken into account in the analysis.

Table 5.2 Treatment as a prognostic factor in malignant pleural mesothelioma: summary of papers comparing survival data for different treatment modalities.

Reference	n	Patient selection criteria	Therapies compared	Statistical significance[a]	Prognostic significance of the performance score
Tammilehto [10]	98	Prospective analysis	Debulking surgery vs. none Hemithorax RT vs. none Chemotherapy vs. none	Univariate[b] Univariate Univariate	+
Rusch and Venkatraman [9]	231	Prospective data	EPP vs. pleurectomy/decortication Adjuvant therapy vs. none	– +	Not given
Boutin *et al*. [12]	125	Prospective series: thoracoscopic diagnosis	Surgery vs. chemotherapy vs. talc pleurodesis vs. none	Univariate	Not given
Antman *et al*. [23]	180	Pathological database	Pleuropneumonectomy vs. chemotherapy	+	+
Chahinian *et al*. [11]	69	All patients from department	Partial or complete resection vs. none Radical resection and chemotherapy with response vs. no response	+ +	+
Thylen *et al*. [30]	100	Clinical data	Any (mainly chemotherapy) vs. none	+	Not given
Spirtas *et al*. [16]	1475	SEER database, positive histology by life	Any surgery, RT, or chemotherapy	+	Not given
Alberts *et al*. [24]	262	Histologically confirmed diagnosis	Therapy vs. none Individual treatments	+ –	+
Tammilehto *et al*. [21]	88	Pleural MM with adequate CT scan	Surgery vs. no surgery Hemithorax RT vs. none chemotherapy vs. not	Univariate Univariate –	+
Chailleux *et al*. [41]	167	From pathology files	Surgery, chemotherapy or talc pleurodesis vs. none	Univariate	Not given
Edwards *et al*. [13]	138	Consecutive patients	Surgical resection vs. surgical biopsy	–	+
De Pangher Manzini *et al*. [25]	80	Consecutive patients	Any surgery or intrapleural or intravenous chemotherapy vs. none	–	Univariate

Table 5.2 *Continued*

Reference	n	Patient selection criteria	Therapies compared	Statistical significance[a]	Prognostic significance of the performance score
Martin-Ucar *et al.* [26]	51	Patients who had palliative surgery	Pleurectomy vs. decortication	–	Only PS WHO 0 and 1 included
Fusco *et al.* [27]	113	All patients	Chemotherapy or palliative surgery vs. chemical pleurodesis	–	Not given
Merritt *et al.* [43]	101	'From a centre'	Pleurodesis, palliative radiotherapy, or chemotherapy vs. none	–	Not given
Ruffie *et al.* [32]	332	Pathological diagnosis, pleural MM	Chemotherapy vs. none Radical RT vs. palliative or no RT No surgery vs. palliative surgery vs. EPP	+ – –	Not given
Emri *et al.* [55]	40	Surgically obtained diagnosis	VATS vs. thoracotomy Chemotherapy vs. none	– –	Not tested
Pass *et al.* [17]	47	From PDT study	EPP vs. pleurectomy/decortication	–	Not given

[a] + Treatment effect proved statistically by multivariate analysis; –treatment effect not statistically significant.

[b] Univariate means that the significance could only be demonstrated by this statistical test. Similarly, a + in the column performance indicates that the performance score was a significant prognostic factor in multivariate analysis.

EPP, extrapleural pneumonectomy; MM, malignant mesothelioma; PDT, photodynamic therapy; PS WHO, performance score according to the WHO classification; RT, radiotherapy; VATS, video-assisted thoracoscopic surgery.

Interpretation of data on the prognostic impact of therapy for MPM is further hampered by different definitions of the 'treatment' between the different studies. This is also illustrated in Table 5.2. Depending on the patient population and the treatment offered in a particular centre, treatment might be defined as any tumour-directed therapy, which sometimes even includes talc pleurodesis [41], whereas in other centres this would be categorized as palliative therapy and not as tumour-directed therapy. Others evaluate different chemotherapy schedules, palliative radiotherapy or high-dose hemithorax irradiation, and surgery either separately or in different therapeutic combinations.

Despite these restrictions, it is likely that a therapy effect is present in malignant mesothelioma. Therefore a description of any tumour-directed therapy that has been given is an indispensable issue for a correct interpretation of survival data of these patients.

Time to diagnosis

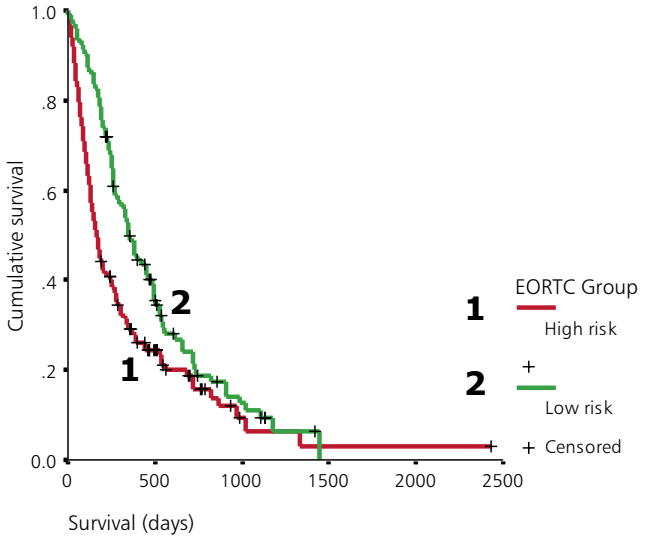
Some papers suggest that patients who have a longer interval between the presenting symptoms and the definite diagnosis have a better survival [23, 24, 41]. Although this phenomenon was not apparent in other studies [11, 12, 25, 32], some authors suggest that patients with a longer diagnostic delay may have a more slowly growing tumour [27]. Even if this statement holds, however, the value of diagnostic delay as a prognostic factor seems limited since it relies on the subjectivity of patients and physicians and seems only poorly reproducible.

Prognostic groupings

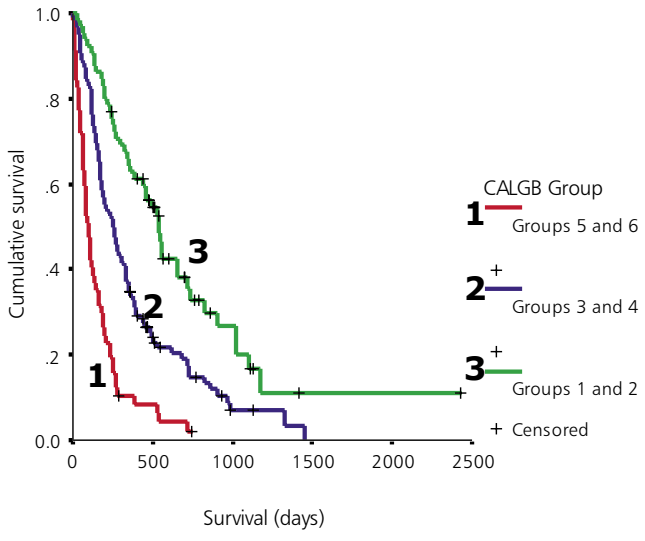
The CALGB [14] and the EORTC [15] have suggested prognostic scoring systems that discriminate between patients with a good and a poor outlook. The prognostic tree designed by the CALGB uses the performance score, age, haemoglobin, white blood cell count, presence of chest pain, and weight loss to define six patient groups with significantly different survival experiences [14]. They reveal a difference in median survival time from 1.4 to 13.9 months. The EORTC data divided the patients in two groups using the prognostic factors white blood cell count, performance status, histological subtype, probability of histological diagnosis, and gender [15]. The low-risk group comprised patients with no, one, or two poor prognostic factors; the high-risk group had three, four, or five poor prognostic factors. The median survival duration was 5.5 months and 10.8 months for the high-risk and low-risk groups, respectively.

These groupings probably reflect prognosis more precisely than the conventional anatomic staging systems that are available for MPM. The different parameters have the advantage over anatomic staging that they are simple laboratory and clinical indices, and therefore are easy to reproduce and available for all patients. Whether the prognostic factors that form the prognostic groupings mentioned above are optimal remains to be determined. The prognostic value of chest pain and weight loss has not been confirmed irrefutably, and the probability of the histological diagnosis is not documented systematically.

Scoring systems that include parameters other than tumour stage have been proposed for other tumour types, such as small-cell lung cancer [110]. Schemes including performance status and biochemical assessment reflected prognosis more precisely than the classical division between limited and extensive disease [111]. Analogous to this situation, the prognostic grouping proposals of the CALGB and the EORTC seem to reflect the prognosis of mesothelioma patients rather accurately [13, 112]. Figure 5.3 shows an updated analysis of the survival of the patient population from Leicester according to their prognostic grouping. The survival differences are strongly statistically significant.



(a)



(b)

Figure 5.3 Expanded and updated survival data from the series originally published by Edwards *et al*. [13]. The data are from 240 consecutive patients presenting in Leicester between 1988 and 2002 (a) Survival curves when the patients are stratified according to the EORTC prognostic groups (low risk, $n = 103$; high risk, $n = 147$; $P = 0.0018$). (b) Survival curves when the patients are stratified according to the CALGB prognostic groups (groups 1 and 2, $n = 65$; groups 3 and 4, $n = 118$; groups 5 and 6, $n = 57$; $P < 0.0001$). In the expanded patient population both scoring systems remain useful tools for the prognostic stratification of mesothelioma patients.

There is an obvious need for a simple reproducible prognostic grouping system. If internationally recognized, it will be valuable as an inclusion criterion in clinical trials and as a stratification factor in phase III clinical trials, will make comparison between trials easier, and will provide a better demarcation of the patient group to which the study results are applicable. Examination of the prognostic factors of patient groups may be sufficient to understand the survival differences across phase II trials [112]. Prognostic factors can also be of help in choosing the appropriate therapy for patients who are not eligible for any study protocol and are treated off-study.

Conclusion

Many prognostic factors have been described in MPM. Although only a limited number have been validated by prospective confirmatory studies, clinicians should be aware of their potential importance in deciding on patient management.

The most significant prognostic factors in MPM are histological subtyping, performance status, and tumour stage. Although the majority of studies confirm its prognostic value, tumour stage in general seems less useful clinically as an indicator of prognosis. Proper staging requires surgery and currently there is no consensus on the best staging system. Furthermore, surgical staging is only performed in a minority of patients suitable for radical surgery.

Other factors that are related to a poor prognosis, although the significance was not apparent in all papers, are old age, male gender, the probability of histological diagnosis, weight loss, and high platelet and leucocyte counts. Factors which can be measured simply, cheaply, and routinely in any laboratory, such as C-reactive protein, and which have been proven to be of prognostic significance in many other malignancies, might also be of value in malignant mesothelioma and are worth evaluating [113].

Special attention should be paid to the factor 'treatment'. A treatment effect has been demonstrated repeatedly by numerous, predominantly retrospective, studies. The retrospective studies are obviously biased by patient selection. The loss of statistical significance of the treatment effect when subjected to multivariate analyses illustrates this bias. Nevertheless, evidence of the beneficial effects of therapy on prognosis is growing. The perception that therapeutic interventions have little to offer should be dispelled and early diagnosis and treatment encouraged [9].

The biological prognostic factors need confirmation in larger prospective trials and should be analysed with respect to the existing prognostic factors. In future, they might guide the development of new therapeutic interventions.

The search for better treatments may profit from an accurate and simple prognostic scoring system that is universally accepted. This system will help to identify the individual patient who will benefit from a particular treatment, but will also improve the quality of reporting on clinical trials and facilitate the comparison of therapeutic interventions in mesothelioma [5, 114].

Acknowledgements

The authors would like to thank Dr R. A. M. Damhuis, Comprehensive Cancer Centre, Rotterdam, for helpful and stimulating discussions during the preparation of this chapter, and Ms R. de Wijk-van der Zalm and Ms C. Koopman for their skilful technical assistance.

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Chapter 4

4

A model system for optimising the selection of membrane antigen-specific human antibodies on intact cells using phage antibody display technology

Journal of Immunological Methods 262 (2002) 191-204

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Recombinant Technology

A model system for optimising the selection of membrane antigen-specific human antibodies on intact cells using phage antibody display technology

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Received 28 September 2001; received in revised form 20 December 2001; accepted 20 December 2001

Abstract

The functional expression of human antibody fragments on the surface of filamentous bacteriophage, and selection of phage antibodies (PhAbs) with antigens, has provided a powerful tool for generating novel antibodies. Applications of phage antibody display technology have increased over the past decade. Successful isolation of phage antibodies has been reported mostly using purified antigens. Isolation has proven to be more complicated with complex mixtures of antigens, such as intact cells. A given cell type contains thousands of different epitopes, each capable in theory of binding phage antibodies. Often antigens are not known or cannot be purified without disrupting their conformational integrity. To overcome problems involving phage antibody selections on intact cells, we have developed an experimental model system that allows for optimisation and comparison of various selection strategies. The model system comprises labelling of intact cells with the fluorescently labelled phospholipid fluorescein-DHPE. Upon incubation, this phospholipid is readily incorporated in the membrane of any cell type. Labelling intensity is regulated by varying the phospholipid concentration. After optimisation of key steps in the selection procedure, we were able to isolate fluorescein-DHPE specific phage from a synthetic library using intact cells. This model system can be applied to any cell type and we demonstrate that it can be used to efficiently compare and optimise selection strategies.

Keywords: Phage antibody display; Intact cells; Model system; Fluorescein-DHPE; Optimisation

Abbreviations: Amp, Ampicillin; BSA, Bovine serum albumin; EDTA, Ethylenediaminetetraacetic acid; ELISA, Enzyme-linked immuno sorbent assay; FBS, Foetal bovine serum; Fluorescein-DHPE, *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt; HBBS, Hanks' balanced salt solution; HRP, Horseradish peroxidase; Kan, Kanamycin; OD_{590}, Optical density at 590 nm; PBS, Phosphate-buffered saline; PEG, Polyethylene glycol; PhAbs, Phage antibodies; RT, Room temperature; scFv, Single-chain variable antibody fragment; Tet, Tetracycline.

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1. Introduction

Diagnosing cancer and distinguishing between different types of tumours can be a problem in histopathology due to lack of specific and well-validated tumour markers. With the development of antibody-fragment-display on the surface of phage, the possibilities for obtaining new antibody specificities have been greatly enhanced (Pereira et al., 1997b; Kupsch et al., 1999). Phage antibody display technology uses large libraries comprising the variable domains of antibody fragments that are fused to an envelope protein (pIII, pVI or pVIII) of filamentous bacteriophage (McCafferty et al., 1990). Phage antibodies (PhAbs) with specific binding properties are selected by binding to an immobilised target antigen and are retained, while unbound PhAbs are removed by washing. The bound PhAbs are then eluted and amplified in a bacterial host. Additional rounds of selection are performed to enrich for the desired binders (typically 2–5 rounds). As a consequence, even very rare binding specificities present in large libraries can be selected and amplified from a background of PhAbs with irrelevant binding specificities.

The phage antibody display technique makes it possible to recover human antibodies that are directed against new and previously unknown tumour-associated epitopes, which may be used as reagents for cancer research, detection and therapeutic applications (Nilsson et al., 2000). For example, anti-cancer PhAbs have been isolated with purified antigens as targets (Schier et al., 1995; Chowdhury et al., 1998; Henderikx et al., 1998; Mao et al., 1999) or with immune libraries constructed from rearranged V genes derived from the IgG mRNA of human B cells from patients (Cai and Garen, 1995; Chowdhury et al., 1997). There are few reports, however, of successful selections on complex mixtures of antigens, such as intact cells, by naive or synthetic libraries (de Kruif et al., 1995b; Van Ewijk et al., 1997; Huie et al., 2001) and immune libraries (Cai and Garen, 1995; Kupsch et al., 1995; Andersen et al., 1996; Siegel et al., 1997; Watters et al., 1997). Phage antibody display technology has been proven difficult for selecting PhAbs from naive or (semi-) synthetic libraries to targets on mammalian cells. The membranes of cells are supermolecular structures, comprised of polysaccharides, cholesterol, (glyco-) proteins and other types of molecules besides phospholipids. Furthermore, the number of target epitopes may be limited. During selection, PhAbs will bind to the entire multitude of molecules on the cell surface, resulting in a relatively high background of unwanted binders and a relatively low binding of desired PhAbs. The critical issue in the selection of desired PhAbs from naive or synthetic libraries by cell panning is the efficiency of both depletion of common binders and enrichment of specific binders.

The conformational integrity of the membrane antigens may be partially disrupted by isolation and coating procedures. To ensure the preservation of the native conformation of the antigen, phage antibody libraries should be panned on intact cells (Kettleborough et al., 1994). Furthermore, most tumour-associated antigens are not known a priori or not present in purified form. These factors thus have limited the number of studies that address the derivation of PhAbs from a naive or synthetic library to defined cell surface antigens. Therefore, in addition to the diversity of recognising library and the need for optimising affinity and folding efficiency of the phage-displayed antibodies, there should also be emphasis on developing model systems to improve experimental conditions for selection of PhAbs. This is especially true for selections with naive and synthetic libraries on living cells when the nature and concentration of the antigen are unknown. In an appropriate model system for selections on living cells, cell growth characteristics and protein expression should remain unchanged. The antigens should be continuously present and equally distributed on all cells. Furthermore, the model system should be widely applicable for any given cell type. A requirement is that the phage antibody specific for the antigen is present in the library. Under highest stringency conditions, undesired cells and target cells should be closely related, preferably differing by only a single antigen. Model systems described to date rely on theoretical models (Levitan, 1998) or on PhAbs directed against a predefined antigen expressed or induced on specific cells (Pereira et al., 1997a; Watters et al., 1997; Mutuberria et al., 1999).

In the method described here, we have used a phospholipid with a fluorescein-labelled head group (fluorescein-DHPE) that can easily be incorporated in different quantities in the membrane of any type of cell. The labelling is not harmful to cells and induces no detectable changes in growth characteristics and

protein expression. The amount of fluorescein incorporated into the cell membranes can easily be examined by flow cytometry or fluorescence microscopy. After isolating fluorescein-DHPE specific PhAbs using coated immunotubes, we optimised the experimental conditions for selection on cells. Using these optimised conditions, we isolated the fluorescein-DHPE specific phage from a large synthetic library by panning on intact-labelled cells. Furthermore, we used fluorescein-DHPE as a 'positive control' in immunohistochemistry and suggest the applicability of the procedure in other phage antibody-related techniques. Our results have demonstrated the usefulness of this model system for optimising phage antibody selections on cells.

2. Materials and methods

2.1. PMR-MM7 cell line

An immortal cell line, PMR-MM7, was derived from the pleural effusion of a 63-year-old man with malignant mesothelioma. Cells were cultured at 37 °C in RPMI 1640 medium (containing HEPES and Glutamax [Invitrogen, Breda, The Netherlands, Cat no. 72400]), supplemented with 50 μg/ml gentamycin (Invitrogen) and 10% (v/v) foetal bovine serum (FBS [Greiner Labortechnik, Alphen a/d Rijn, The Netherlands, Cat. no. 758093]) in a humidified atmosphere of 5% CO_2, 95% air. PMR-MM7 cells were extensively characterised and kept in long-term cell culture (>50 passages, 6 months of culturing) while using for phage antibody display technology.

2.2. Escherichia coli strain, phage antibody library and helper phage

E. coli XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]) was used for the production of PhAbs (Stratagene, La Jolla, CA, USA).

The semi-synthetic phage antibody library of human single-chain Fv (scFv) antibody fragments was kindly provided by Prof. Dr. T. Logtenberg (Crucell, Leiden, The Netherlands). The library consisted of a large collection of 49 cloned germline V_H gene segments combined with randomised synthetic heavy chain CDR3 regions and seven light J_H chains (de Kruif et al., 1995a). The phagemid pHEN1, derived from pUC119, was used for expression (Hoogenboom et al., 1991). The library, containing 3.6×10^8 different specificities of scFv genes, was displayed by fusion to the gene encoding capsid protein III (pIII) of filamentous bacteriophage. Superinfection with helper phage M13KO7 (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for rescue of PhAbs.

2.3. Isolation of fluorescein-DHPE specific phage antibody clones through solid phase selection

An immunotube (MaxiSorp™, Nunc, Roskilde, Denmark) was coated with 1.5 ml of 5 μg/ml fluorescein-DHPE (*N*-[fluorescein-5-thiocarbamoyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; F-362: Molecular Probes, Leiden, The Netherlands) in PBS. A control tube was coated with 3% (w/v) BSA (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands, Cat no. A-8327) in PBS. Both tubes were incubated overnight at 4 °C. After incubation, both tubes were washed three times with PBS. The fluorescein-DHPE coated tube was filled to capacity with 3% BSA in PBS and incubated for 2 h at room temperature (RT). A 0.5 ml aliquot of the phage antibody library (containing 5×10^{12} phage) was diluted to 1.5 ml with 3% BSA in PBS. The library was preblocked in the control tube for 2 h on a roller bench. The blocking solution was removed from the fluorescein-DHPE coated tube and replaced with the supernatant from the control tube. The tube was incubated at RT for 30 min with over-end rotation, then for 90 min at RT without shaking. Unbound phage was removed by washing the tube 20 times with 4 ml of 0.1% (v/v) Tween-20 in PBS and 20 times with 4 ml PBS. Bound phage was eluted by incubating with 1.5 ml of 100 mM triethylamine (Sigma-Aldrich Chemie) for 10 min at RT. The eluate was neutralised by adding 1.5 ml of 1 M Tris–HCl (pH 8.0). To determine the number of eluted phage, serial dilutions were made, starting with 10 μl of the eluate. These were added to 2 ml of log phase *E. coli* XL1-Blue in 2TY medium (3.4% [w/v] 2TY powder [Invitrogen] in water) supplemented with 12 μg/ml of tetracycline hydrochloride (Tet), 100 μg/ml of ampicillin sodium salt (Amp) and 5% (w/v) glucose (2TY-

TAG medium). The remainder of the eluate was used to infect 25 ml of log phase *E. coli* XL1-Blue for 30 min at 37 °C. Bacteria were then centrifuged for 15 min at 3000 × *g*, and pellets were resuspended in 150 μl of 2TY and plated onto 2TY-TAG plates (2TY-TAG medium supplemented with 1.5% [w/v] agar). All plates were incubated overnight at 37 °C. After incubation, polyclonal PhAbs were produced by scraping large quantities of bacterial colonies and subsequently purified on large-scale (Section 2.4) and/or small-scale (Section 2.5). Monoclonal PhAbs were produced from single-infected bacterial colonies. The selection procedure on fluorescein-DHPE coated immunotubes was repeated three times with large-scale produced polyclonal PhAbs.

2.4. Large-scale phage antibody production

Individual bacterial colonies were picked (monoclonal) or plates with bacterial colonies were scraped (polyclonal) with 3 ml of 2TY. One hundred microliters of the suspension was added to 50 ml of 2TY-TAG medium and grown at 37 °C to an OD_{590} of 0.5. Then, 2×10^{10} plaque-forming units (pfu) of M13KO7 helper phage were added and incubation was continued for 30 min at 37 °C without shaking followed by a 30 min incubation with shaking (150 rpm; Innova, New Brunswick Scientific, Edison, NJ, USA). The bacteria were pelleted at 3000 × *g* for 15 min, and the supernatant was removed. The pellet was resuspended in 50 ml of 2TY-TAK medium (2TY containing 12 μg/ml Tet, 100 μg/ml Amp and 25 μg/ml kanamycin monosulphate salt [Kan]), and the bacteria were grown overnight at 30 °C with shaking (225 rpm). After incubation, bacteria were pelleted by centrifugation at 3500 × *g* for 30 min at 4 °C. Phage in the supernatant was precipitated with 16 ml of cold 20% (w/v) PEG-6000/2.5 M NaCl and incubated 1 h on ice. Precipitated PhAbs were centrifuged at 4500 × *g* for 30 min at 4 °C, and the pellet was resuspended in 8 ml of PBS. After adding 2 ml of cold 20% PEG–NaCl, incubation was continued for an additional hour on ice. After centrifugation (4500 × *g*, 30 min, 4 °C), the pellet was resuspended in 850 μl of RPMI containing 2% BSA. The suspension was centrifuged at 13,000 × *g* for 2 min to pellet debris, and the supernatant was added to a tube containing 150 μl glycerol. PhAbs were stored at − 20 °C until further use in selection rounds or screening for specific binding analysed by flow cytometry and ELISA.

2.5. Small-scale phage antibody production

Individual bacterial colonies were picked or plates with bacterial colonies were scraped and transferred to a 96-well plate containing 160 μl of 2TY-TAG medium per well and grown overnight at 37 °C with shaking. After incubation, 15 μl of each suspension was transferred to a fresh 96-well plate containing 150 μl of 2TY-TAG medium per well and incubated at 37 °C for 2 h. Helper phage M13KO7 (5×10^8 pfu/well) was added and incubation was continued at 37 °C for 30 min without shaking followed by a 30 min incubation with shaking (180 rpm). The plate was centrifuged at 2500 × *g* for 15 min, and the supernatant was aspirated completely. The pellets were resuspended in 150 μl of 2TY-TAK medium and incubated overnight at 30 °C with shaking. The next day, bacteria were pelleted, and the supernatant stored at − 20 °C until further use in screening for specific binding as analysed by ELISA.

2.6. Analysis of fluorescein-DHPE specific phage antibody clones by ELISA

Specificity of monoclonal and polyclonal PhAbs was assessed by ELISA using plates coated with fluorescein-DHPE. For coating, fluorescein-DHPE was applied to the wells of a MaxiSorp™ immunoplate (Nunc) at a concentration of 5 μg/ml in PBS and incubated at 4 °C overnight. Excess antigen was removed by washing three times with PBS containing 0.1% Tween-20. Any remaining available sites on the plate were blocked with 3% BSA in PBS for 2 h at RT. All incubations were performed in PBS/1% BSA, with PBS/1% BSA/0.1% Tween-20 as wash buffer. The plate was washed, and serially diluted supernatants of single phage clones or polyclonal phage mixtures were applied to the plate and incubated for 1 h at RT. Bound phage was detected by adding 100 μl of horseradish peroxidase (HRP)-conjugated anti-M13 polyclonal antibody for 30 min (1:5000, Amersham-Pharmingen Biotech) at RT. Wells were stained with 100 μl of 3,3′,5,5′ -tetramethylbenzidine (TMB) substrate according to the manufacturer's recommenda-

tions (KPL, Gaithersburg, MD, USA). The peroxidase reaction was stopped with 50 μl of 2.5 N H_2SO_4. Plates were analysed at 450 nm in a microplate reader (Bio-Rad Laboratories, Hemel Hempstead, UK).

2.7. Labelling of cells with fluorescein-DHPE and analysis of phage antibody binding by flow cytometry

PMR-MM7 cells were harvested by treatment with 0.5 mM EDTA in PBS, washed, and counted. An aliquot of the cells was labelled with fluorescein-DHPE according to the manufacturer's instructions (F-362: Molecular Probes). In short, cells were washed in Hanks' balanced salt solution (HBBS: Invitrogen) and resuspended to a concentration of 10^7 cells/ml. For each ml of cells, 15 μl of fluorescein-DHPE, diluted 1:10 in HBBS, was added, mixed, and incubated for 10 min at RT in the dark. Cells were washed twice in RPMI 1640 culture medium (25 mM HEPES, Glutamax, 50 μg/ml gentamycin, 10% FBS). This medium was also used to block the freshly prepared polyclonal or monoclonal phage for 15 min. Approximately 5×10^5 cells were incubated with 10^{12} blocked phage for 1 h on ice, and then washed three times (wash buffer: PBS supplemented with 1% BSA and 0.01% NaN_3). Bound phage was detected using mouse anti-M13 antibody (Amersham Pharmacia Biotech, diluted 1:150 in wash buffer). Following three washes, the cells were incubated for 30 min on ice with 100 μl of 1:150 diluted phycoerythrin-conjugated $F(ab')_2$ fragments donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA, Cat no. 715-116-151). After three washes, cells were resuspended in 300 μl of wash buffer and flow cytometric analysis was performed on a FACS-Calibur (BD Immunocytometry Systems, Erembodegem, Belgium).

2.8. Optimisation the selection procedure for PMR-MM7 cells

2.8.1. Storage of PhAbs

To analyse the stability of the fluorescein-DHPE specific phage, a monoclonal PhAbs batch was aliquoted in RPMI containing 2% BSA. After storage for 1, 3 and 7 days at 4 or −20 °C, fluorescein-DHPE specificity was assessed on fluorescein labelled PMR-MM7 cells and analysed by flow cytometry.

2.8.2. Elution of PhAbs

Six different elution buffers (Table 1) were tested for their efficiency in removing monoclonal fluorescein-DHPE specific PhAbs from six identically treated T25 cm^2 flasks exhibiting confluent growth of fluorescein-DHPE labelled PMR-MM7 cells. After washing and elution, phage was enumerated by titrating the neutralised eluate. Titers were obtained by incubating PhAbs of appropriate serial dilutions with 2 ml of log phase *E. coli* XL1-Blue for 30 min at 37 °C without shaking. Bacteria were pelleted for 15 min at 3000 × *g*, resuspended in 150 μl of 2TY, and plated onto 2TY-TAG plates. After incubating the plates overnight at 37 °C, the number of colonies were quantified.

2.8.3. Blocking of non-specific binding by PhAbs

To reduce non-specific binding, three different blocking buffers were tested: 10% FBS, 1% BSA, and 4% dry milk powder, all in RPMI. Cell cultures, either labelled with fluorescein-DHPE or unlabelled, in T25 cm^2 flasks were blocked for 1 h at RT. Blocking buffer was replaced by blocking buffer containing fluorescein-DHPE specific phage (approximately 10^{12} phage) and incubated for 2 h at RT with horizontal shaking (100 rpm; IKA Labortechnik, Staufen Germany). After washing and elution, the number of phage was determined by infecting *E. coli* XL1-Blue, as described earlier.

2.8.4. Influence of selection volume

To determine the optimal volume for selection, cells from three identical (T25 cm^2) cultures, were labelled with fluorescein-DHPE and blocked in 1% BSA in RPMI for 1 h at RT. Unlabelled cells were

Table 1
Buffers used for elution of PhAbs

Buffer composition	Reaction conditions
(1) 50 mM CH_3COONa, 85 mM NaCl, 80 mM KCl, 0.1% BSA (pH 4.0)	2 min on ice
(2) 76 mM citric acid in PBS (pH 2.6)	5 min RT
(3) 100 mM triethylamine in water	10 min RT
(4) RPMI 1640 (without HEPES) (pH 3.0 by adding 1 M HCl)	2 min on ice
(5) 0.1 M glycine HCl (pH 3.0)	2 min on ice
(6) 0.1 M glycine HCl (pH 2.2)	2 min on ice

used as controls. The blocking buffer was then replaced with three different volumes (2, 5 or 50 ml) of blocking buffer, each containing 10^{12} fluorescein-DHPE specific phage, and incubated for 2 h at RT with horizontal shaking (100 rpm). After washing, phage was eluted with citric acid and phage titer was determined.

2.8.5. Wash conditions

Six T25 cm^2 flasks exhibiting confluent PMR-MM7 cells (three of which were fluorescein-DHPE labelled) were incubated in optimal blocking buffer for 60 min with horizontal shaking. Fluorescein-DHPE specific PhAbs (10^{12} phage per condition) were then added to the culture flasks. After 2 h incubation at RT with horizontal shaking, the cells were washed 5, 10 or 20 times using 5 ml of RPMI supplemented with HEPES and 1.5% BSA for each wash step. Phage was eluted with citric acid and titered on *E. coli* XL1-Blue.

2.9. Selection of desired phage antibodies using fluorescein-DHPE labelled PMR-MM7 cells

After blocking the phage antibody library (0.5 ml containing 5×10^{12} phage) with 2 ml of RPMI supplemented with 1% BSA, it was pre-adsorbed on a confluent monolayer of adherent PMR-MM7 cells (approximately 8×10^6) in a T75 cm^2 culture flask and incubated at RT for 60 min with horizontal shaking (75 rpm). Supernatant containing unbound phage was collected and transferred to a new flask containing PMR-MM7 cells for a second round of adsorption. After incubation, the phage supernatant was added to 3×10^7 PMR-MM7 cells (previously detached with PBS containing 0.5 mM EDTA) and incubated for 30 min with horizontal shaking. This suspension was then transferred to a T25 cm^2 culture flask confluent with fluorescein-DHPE labelled PMR-MM7 cells (approximately 2.5×10^5). After incubation for 2 h at RT with horizontal shaking (100 rpm), cells were washed 10 times, using 5 ml of RPMI containing 1.5% BSA for each wash step. Phage was eluted using 300 μl of PBS and 450 μl of 76 mM citric acid and incubated for 5 min at RT. The supernatant was neutralised by transferring the eluted phage to a tube containing 300 μl of 1 M Tris–HCl (pH 8.0). Phage was titered and amplified by infecting 10 ml of log phase *E. coli* XL1-Blue (OD_{590} of 0.4–0.6) in 2TY medium with 5% (w/v) glucose and 12 μg/ml Tet. After 30 min of infection, bacteria were pelleted ($3000 \times g$ for 15 min), resuspended in 150 μl of 2TY medium and plated onto 2TY-TAG plates. Plates were incubated overnight at 37 °C. Phage was rescued as described in Section 2.4. This procedure was repeated for three additional rounds.

2.10. Immunocytochemistry with fluorescein-DHPE specific phage antibodies

Cytocentrifuge preparations were made using 5×10^4 unlabelled or fluorescein-DHPE labelled PMR-MM7 cells per 40 mm^2 adsorbed on poly-L-lysine (Sigma-Aldrich Chemie) coated slides and air-dried for 2 h. Alternatively, cells on cytospin preparations were labelled by incubating with 100 μl of fluorescein-DHPE diluted 1:1000 in HBBS for 10 min and then washed twice with PBS. Slides were fixed in 70% ethanol for 10 min at RT and washed once in PBS, followed by two rinses for 15 min each with PBS containing 1% BSA. A fluorescein-DHPE specific phage antibody clone was precipitated with PEG as described in Section 2.4 and resuspended at a concentration of 10^{13} phage/ml in PBS containing 1% BSA. The suspension (100 μl) was added to the slides and incubated for 60 min at RT. After washing three times with PBS, PhAbs were detected by incubating with a 1:150 dilution of HRP-conjugated anti-M13 (Amersham Pharmacia Biotech) for 30 min at RT. Slides were washed twice in PBS and developed with 3-amino-9-ethylcarbazol (AEC) substrate for 30 min at RT. Finally, sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and mounted in Kaiser's glycerol gelatin (Merck). In addition to PhAbs, periplasmic fractions containing soluble scFv fragments were tested for potential use in immunocytochemistry. Periplasmic fractions were obtained from 25 ml cultures of *E.coli* XL1-Blue bacteria infected with fluorescein-DHPE specific phage grown overnight in LB-TAI (2% Lennox LB medium [Invitrogen] containing 12 μg/ml Tet, 100 μg/ml Amp and 1 mM isopropyl β-D-thiogalactoside [IPTG]). Bacteria were centrifuged for 15 min at $3500 \times g$. Pellets were resuspended in 250 μl of ice-cold TES (0.2 M Tris–HCl [pH 8.0], 0.5 mM EDTA, and 0.5 M sucrose) followed by the addition of 375 μl

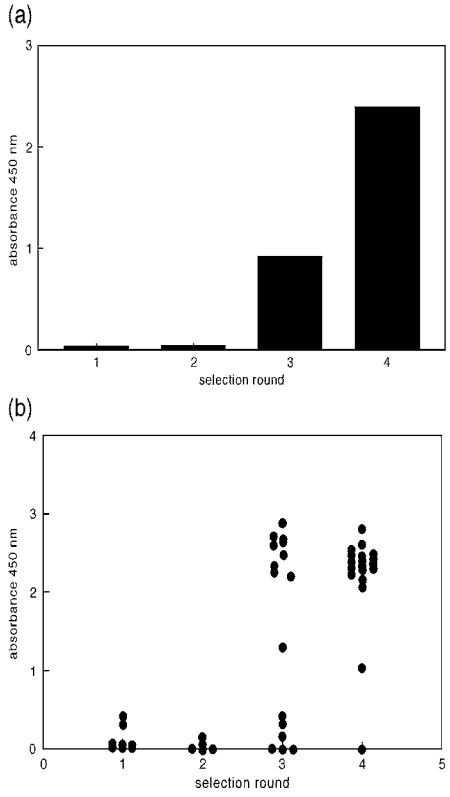

Fig. 1. Analysis of phage antibody binding after four rounds of solid-phase selection by fluorescein-DHPE coated ELISA. (a) Polyclonal PhAbs from each selection round. (b) Dots represent 23 monoclonal PhAbs from each selection round. Data were corrected for non-specific binding with PBS containing 3% BSA-coated well.

of ice-cold 1/4 TES (1 volume of TES plus 3 volumes of water) and incubated for 60 min on ice. The suspensions were centrifuged at 10,000 × *g* for 30 min at 4 °C, and the clear supernatants were used as primary reagents. The staining procedure was carried out as described above with the following modification. ScFv fragments were detected using the 1:1 diluted hybridoma supernatant of the mouse antibody 9E10 (American Type Culture Collection), which recognises the C-terminal Myc peptide tag. Anti-mouse IgG Fc-specific HRP conjugated antibody (Amersham Pharmacia Biotech) or EnVision HRP labelled polymer (DAKO, Glostrup, Denmark) was used as secondary reagent in the detection protocol.

3. Results

3.1. Selection of fluorescein-DHPE specific PhAbs

The use of fluorescein-DHPE as a model antigen required the presence of phage specific for this antigen in the library. Therefore, four rounds of a solid-phase selection on fluorescein-DHPE coated immunotubes were performed. Fig. 1 demonstrates the enrichment and screening of polyclonal PhAbs (A) and monoclonal PhAbs (B) from the selection rounds when tested in an ELISA using fluorescein-DHPE coated plates. Twenty-three monoclonal phage anti-

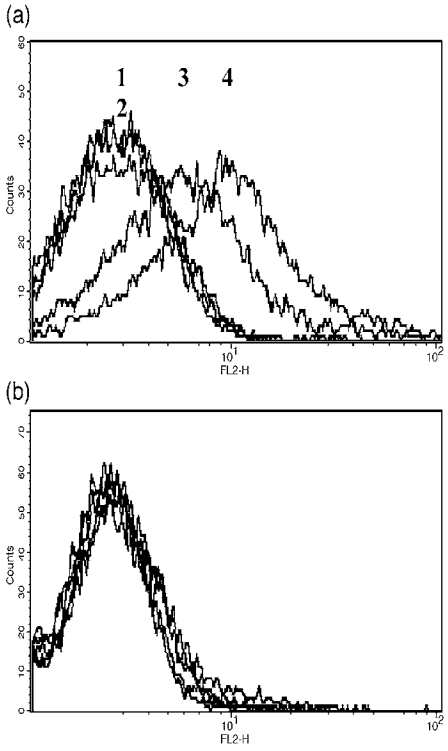

Fig. 2. Analysis of phage antibody binding to fluorescein-DHPE labelled PMR-MM7 cells by flow cytometry. Fluorescein-DHPE labelled cells (a) and unlabelled PMR-MM7 cells (b) were incubated with polyclonal phage from each selection round. Cells without phage served as negative control (dashed line). Numbers correspond to the selection round of polyclonal PhAbs on fluorescein-DHPE coated immunotubes.

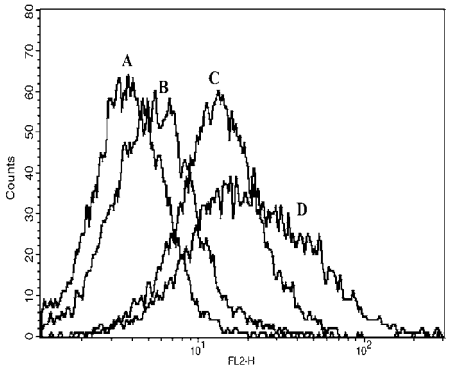

Fig. 3. Analysis of phage antibody binding after preservation of phage for 7 days at different temperatures on fluorescein-DHPE labelled PMR-MM7 cells by flow cytometry. PhAbs were stored for 7 days at 4 °C (B) or − 20 °C (C) compared to freshly prepared (D) and no PhAbs (A).

body clones were isolated per selection round, but this can easily be increased following the described fluorescein-DHPE ELISA protocol. At least three rounds of panning were necessary to remove most of the unwanted clones and enrich for the desired ones. Ninety-five percent of the PhAbs from the fourth round of selection recognised fluorescein-DHPE in ELISA.

3.2. Phage antibody binding to PMR-MM7 cells

The specificity of polyclonal PhAbs obtained through solid-phase selection on fluorescein-DHPE-coated immunotubes was demonstrated using flow cytometry with the mesothelioma cell line PMR-MM7. PMR-MM7 cells were labelled with fluorescein-DHPE, and binding with polyclonal PhAbs from each selection round was analysed by flow cytometry. When compared to unlabelled cells, fluorescein-DHPE labelled cells incubated with polyclonal PhAbs from third to fourth rounds of selection produced a shift in mean fluorescence signal, indicating the specificity of binding (Fig. 2). Higher percentages of fluorescein-DHPE specific phage in a polyclonal PhAbs mixture increased the mean fluorescence of intensity, indicating more phage antibody binding per cell.

3.3. Optimisation of the selection procedure for fluorescein-DHPE on PMR-MM7 cells

Phage particles stored at 4 °C lose some of their specificity even after 1 day, but remained stable for at least 1 week at − 20 °C (Fig. 3). However, periplasmic fractions containing soluble scFv fragments lose

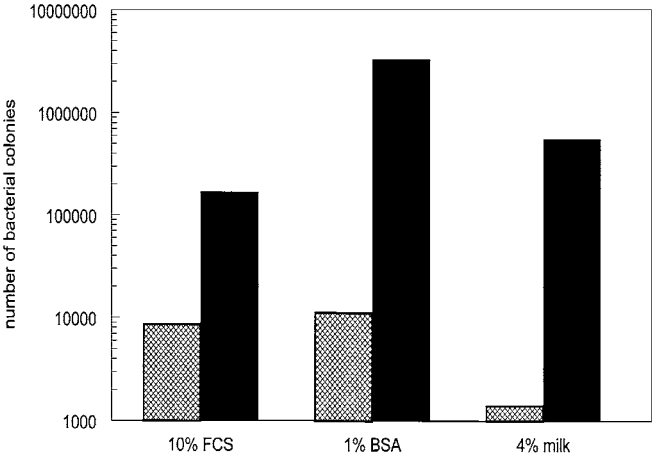

Fig. 4. Effect of blocking buffers 10% FBS in RPMI, 1% BSA in RPMI, and 4% milk powder in RPMI were tested for reduction of non-specific binding. PMR-MM7 cells either unlabelled (chequered bars) or labelled with fluorescein-DHPE (filled bars), were blocked for 1 h at RT. Supernatant was replaced by blocking buffer containing fluorescein-DHPE specific phage and incubated at RT for 2 h with horizontal shaking. After washing and elution with citric acid, the number of phage was determined by titering on *E. coli* XL1-Blue.

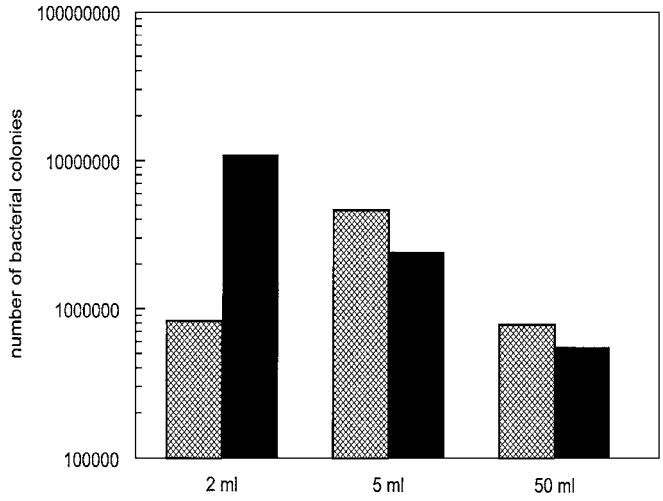

Fig. 5. Effect of selection volume in phage antibody display selections. Unlabelled cells (chequered bars) and fluorescein-DHPE labelled cells (filled bars) were incubated with phage for 2 h in 2, 5, or 50 ml of 1% BSA in RPMI, then washed. Phage was eluted and titered on *E. coli* XL1-Blue.

their specificity at both 4 and −20 °C after 2 days and must be prepared freshly before use (data not shown).

We compared six different buffers for their phage elution efficiency. Acetate buffer (pH 4.0), glycine HCl (pH 2.2) and RPMI (pH 3.0) were relatively inefficient while citric acid (pH 2.6), TEA and glycine HCl (pH 3.0) were highly efficient at eluting phage. To assess the effect of different blocking buffers on their efficiency for preventing non-specific binding,

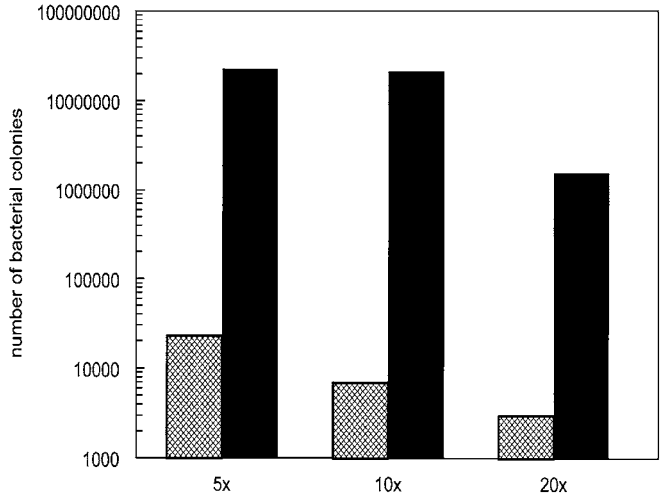

Fig. 6. Effect of wash steps on phage antibody display selections were determined after incubating unlabelled cells (chequered bars) and fluorescein-DHPE labelled cells (filled bars) with PhAbs in a T25 cm^2 culture flask. Cells were washed 5, 10, or 20 times with 5 ml of buffer. Phage was eluted and titered on *E. coli* XL1-Blue.

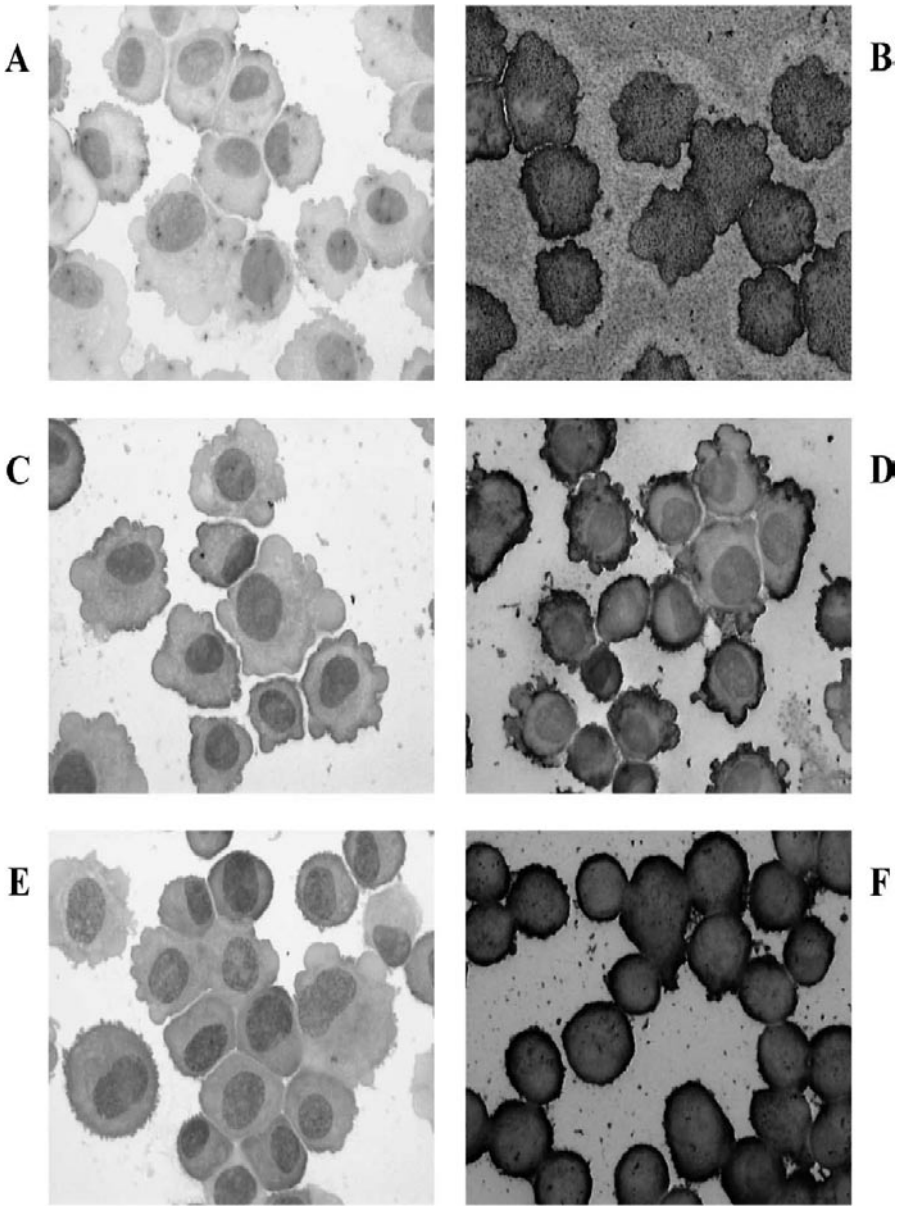

Fig. 7. PhAbs and scFv fragments immunocytochemistry on unlabelled and labelled PMR-MM7 cells. Cytocentrifuge preparations of 5×10^4 cells/40 mm^2 of unlabelled cells (left column) or fluorescein-DHPE labelled cells (right column), were stained with PhAbs or scFv fragments. PhAbs were detected by HRP-conjugated anti-M13 (A, B). Soluble scFv fragments were detected using 9E10, followed by anti-mouse IgG conjugated with HRP (C, D) or Envision HRP labelled polymer (E, F). Slides were developed with 3-amino-9-ethylcarbazol as substrate and counterstained with Mayer's hematoxylin.

we incubated unlabelled and fluorescein-DHPE labelled cells with a monoclonal fluorescein-DHPE specific phage clone under identical conditions. RPMI supplemented with either 10% FBS, 1% BSA or 4% milk powder was used to prevent cell detachment from the culture flask. RPMI containing 10% FBS gave the lowest signal-to-noise ratio while RPMI containing 1% BSA gave the highest (Fig. 4).

Little increase in specific binding occurred when selections were performed in 50 ml. Optimal results were obtained using small selection volumes during a 2 h incubation period (Fig. 5).

Different wash regimens were performed to determine the minimal number of washes for reducing background. When T25 cm^2 culture flasks with confluent PMR-MM7 cells were washed 10 times using 5 ml of wash buffer per step, there was little increase in specificity compared to five wash steps (Fig. 6). After 20 washes, even the specific bound phage was removed.

3.4. Selection of fluorescein-DHPE specific PhAbs on intact cells using the optimised procedure

Using the optimised conditions, specific PhAbs were selected on viable PMR-MM7 cells labelled with fluorescein-DHPE. After four rounds of selection, there was an increase in the number of phage recognising fluorescein-DHPE, thus indicating successful enrichment for fluorescein-DHPE specific phage. Four out of 96 individual monoclonal PhAbs were specific for fluorescein-DHPE in ELISA (data not shown).

3.5. Using fluorescein-DHPE as positive control in phage-related techniques

To demonstrate the usefulness of fluorescein-DHPE as a positive control in phage antibody-related techniques, we used our model system for immunocytochemical studies. Cytocentrifuge preparations of unlabelled or fluorescein-DHPE labelled PMR-MM7 cells were stained with a PEG-precipitated fluorescein-DHPE specific phage antibody clone or with soluble scFv fragments derived from the same phage clone (Fig. 7). Soluble scFv fragments were more suitable in immunocytochemistry because they gave lower background and a more uniform staining pattern.

4. Discussion

Phage antibody display technology is a powerful tool for the discovery of novel tumour markers by direct selection on intact cells. However, phage antibody selections are difficult to achieve with complex mixtures of epitopes, such as cell membranes, when compared to purified antigens. Because PhAbs bind to the entire array of molecules on the cell membranes, selection yields a relatively high background binding due to unwanted binders and a relatively low binding of desired PhAbs. To reduce the selection of PhAbs to irrelevant antigens, researchers have exploited different depletion and/or subtraction selection strategies (Hoogenboom et al., 1998). Techniques such as panning on dishes, tubes or columns, cell-sorting using flow cytometry, BIAcore or latex/magnetic-bead systems have been described and are reviewed elsewhere (Winter et al., 1994; Hoogenboom, 1997). Selection procedures on target cells are often preceded by an adsorption step in which the library is incubated with undesired cells to remove undesired specificities. These approaches have yielded interesting antibodies in only a few cases due to the problem of enrichment of PhAbs specific for non-relevant antigens. Because results reported by different investigators are difficult to compare, defining an optimal selection strategy is often complicated. This is due to differences in type (naive, synthetic or disease-related immune library), format (Fab or scFv fragments, pIII or pVIII fusion), size and quality of the antibody library, the nature and concentration of the target antigen, the selection procedure, wash and elution conditions employed. We used a semi-synthetic library (de Kruif et al., 1995a) containing 3.6×10^8 different scFv specificities. Libraries with greater scFv and Fab specificities have been described (Griffiths et al., 1994; Vaughan et al., 1996), with emphasis either on optimising functional affinity and/or folding efficiency to improve the expression of antibody fragments on the phage (Pluckthun and Pack, 1997; Cloutier et al., 2000).

Rotterdam has a relatively high incidence of malignant mesothelioma accompanying diagnostic problems and no curative treatments (Hoogsteden et al., 1997). We were interested in recovering PhAbs directed against novel mesothelioma-associated epitopes for potential use in cancer detection and immunotherapy. We optimised our selection procedure using the uniclonal mesothelioma cell line PMR-MM7.

The successful approach described for melanoma (Cai and Garen, 1995; Chowdhury et al., 1997), where immune libraries are constructed from mRNA of B cells from patients, cannot be applied for mesothelioma due to lack of autoantibodies in most patients.

Often antigens are not known or are difficult to purify without losing their conformational integrity (de Kruif et al., 1996). Selection using a large scFv library on the final intended target, such as intact cells, is thus important (Kettleborough et al., 1994; Chowdhury et al., 1997).

To improve phage antibody selections for surface antigens on intact cells, we developed an experimental model for optimising the important parameters for selection. In this article, we describe a fluorescein-labelled phospholipid (fluorescein-DHPE) as a model antigen that can easily be incorporated in different quantities into the membrane of any type of cell. We used fluorescein-DHPE in membranes of living tumour cells as a model system to study the feasibility of PhAbs selection from a large semi-synthetic library on complex impure antigens, and to optimise the selection procedure. This knowledge may potentially broaden the scope of phage technology for the selection of antibodies against novel antigens anchored on the cell membrane. After adsorption with unlabelled cells, panning was carried out on adherent cells with fluorescein-DHPE incorporated in their membranes as targets. The fluorescein-DHPE model satisfies the essential requirements. It is in accordance with the desired screening method and induces no detectable changes in growth characteristics and protein expression. There is a continuous presence and equal distribution of the antigen on cells. Undesired cells used for pre-adsorption and target cells differ in only one antigen, and the phage antibody specificity for fluorescein-DHPE is present in the library used. While results from the optimisation steps are derived with PMR-MM7 cells under the described conditions (type, size and shelf-life of library, cells, panning aspects, amplification in bacteria and renormalisation, etc.), they demonstrate the range of possibilities for optimising parameters for selection. Other conditions may have their own unique features and should be individually optimised.

In this study, we demonstrated the isolation of fluorescein-DHPE specific PhAbs through solid-phase selection with coated immunotubes. We used ELISA for phage antibody screening because of its high throughput potential. After the first and second selection rounds, we detected no fluorescein-DHPE specific phage, presumably due to our sampling of only a small fraction of the bacterial colonies. After four selection rounds with coated immunotubes, 95% of all phage antibody clones recognised fluorescein-DHPE. The high recovery in the fourth selection round may be due to a combination of efficient coating, possible epitopes per antigen, and reduction of non-specific binding by blocking. Fluorescein-DHPE incorporated into the membrane of cells was also recognised by the PhAbs. Although phage particles themselves are relatively resistant to harsh conditions such as high temperatures, detergents and acidic pH, PhAbs are more labile. PhAbs stored at 4 °C appear to lose their specificity even after 1 day. They are stable for at least 1 week when stored at − 20 °C. Citric acid and TEA were most efficient in eluting phage that were still capable of infecting bacteria. Although cell lysis occurred with TEA, it did not preclude phage recovery. While non-fat dry milk appears to be the most frequently used blocking agent in this field, we, as well as the others (Siegel et al., 1997), have found that milk-containing buffers were not optimal. In our hands, RPMI supplemented with 1% BSA was the most effective blocking buffer. Selections performed in small volumes for a 2 h incubation period yielded the best results, presumably due to the close proximity of target and phage. Steric hindrance in small volumes is obviously a minor problem. Perhaps the low efficiency of larger selection volumes can be compensated with longer incubation times. Exhaustive washing had a profound negative effect on the results. After 20 washes, even the specific bound phage was removed, perhaps due to cell detachment from the culture flask. It appears that washing does not reduce background binding, but rather dilutes the unbound phage and can even remove specifically bound phage. Using these optimised conditions, we have successfully isolated specific PhAbs from a semi-synthetic library that recognise fluorescein-DHPE in the membrane of living cells. Our data suggest the usefulness of the fluorescein-DHPE model for optimising and comparing selections using living cells. It is of interest to compare subtraction selection strategies as, for example, flow cytometry (de Kruif et al., 1995b) and magnetically activated cell sorting (Siegel et al., 1997) to panning on cells grown in monolayers.

While results are subjected to the experimental conditions described here, the fluorescein-DHPE model system offers several advantages compared to other model systems. It is easy to perform (labelling takes 20 min), requiring no complex (molecular) techniques, and it is suitable for all cell types. The efficiency of the selection procedure depends on the nature of the antigen, the antigen-cell surface density, total antigen concentration, and on antigen accessibility. Fluorescein-DHPE can be incorporated into the membrane in different quantities, and the fluorescein head group extends out of the membrane. The relation between antigen density and selection efficiency can be determined by changing the amount of antigen in the membrane or by titrating fluorescein-DHPE specific phage into the library. This model can be used to optimise phage-related techniques and provides a positive control, as we have shown by immunocytochemistry. The use of this model allows comparison of selection and optimisation conditions for every cell type, thereby expanding the utility of phage antibody display technology.

Acknowledgements

This work was financially supported by the University Hospital 'Dijkzigt' (Rotterdam). We thank Annabrita Hemmes, Rogier Kersseboom and Femke Muskens for advice and technical assistance.

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Chapter 5

5

Proteomics of pleural effusions

Humana Press Inc.
Proteomics of human body fluids: principles, methods, and applications
Editor Visith Thongboonkerd

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Proteomics of pleural effusions

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A pleural effusion is the abnormal accumulation of fluid between the two layers of pleura that line the chest cavity and surround the lung. Pleural effusion can be the result of several causes. Proteomic analysis may be useful in indicating the pathogenic mechanism involved in pleural fluid accumulation and might pinpoint specific diagnosis. DIfferential Gel Electrophoresis (DIGE) has been employed to directly compare the proteomic profile of serum and pleural effusion of mesothelioma patients to identify unique proteins by observing concentration changes and modifications on single protein level. This technique involves the pre-electrophoretic labeling of complex protein samples using different cyanine-based fluorescent tags prior to carrying out separation by 2D polyacrylamide gel electrophoresis. Several proteins were found to be differentially or uniquely epressed in the serum or in the pleural effusion.

1. INTRODUCTION

1.1 Pleural effusion

The human lung is surrounded by an outer parietal layer and inner visceral layer of pleura (Figure 1A). In the pleural cavity, which is the space between these two layers, a small amount of fluid is present under normal physiological conditions consisting of a few ml (approximately 0.3 ml per kg body mass). The forces operating on the pleura with respect to movement of liquid are (1) the oncotic pressures exerted by the blood in the pleural capillaries and by the liquid in the pleural space and (2) the hydrostatic pressures within pleural capillaries and in the pleural space. The fluid is produced continuously and reabsorbed mainly through the lymphatic system [1, 2]. Its function is to reduce friction of the lungs during respiratory movements. In abnormal conditions, the pleural space can be filled with air, blood, plasma, serum, lymph, or pus. This expansion of the pleural space can compress the underlying tissue and causes partial collapse of the lung. There are 2 main types of pleural effusions: transudates and exudates. Pleural transudates pass membranes or squeeze through tissue into the extra cellular space when imbalances in hydrostatic or oncotic pressures occur. In contrast, pleural exudates are slowly discharged from blood vessels as a result from an alteration in vascular permeability. Pleural effusions can have several causes, such as with congestive cardiac failure or from low protein in the blood, as in liver disease, severe malnutrition, and in certain kidney conditions. Physical trauma, infection, blockage of blood supply to the lung, and cancer can also result in accumulation of fluid in the pleural space (Figure 1B).

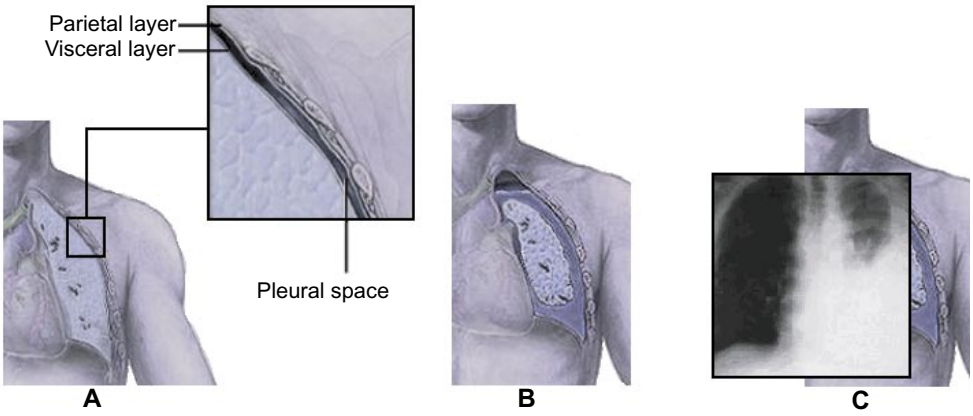

Figure 1: Human lung under normal conditions (A) and with the accumulation of pleural fluid between the layers that lines the lungs and chest cavity (B). Chest radiography showing presence of pleural effusion (C).

1.2 Diagnosing pleural effusions

Pleural effusions can usually be seen on an X-ray image of the chest (Figure 1C). For diagnostic purposes, the fluid is removed with a fine needle aspiration inserted in the pleural cavity. Numerous biochemical criteria with different cut-off values have been used to separate pleural transudates from exudates, but most clinicians use the three criteria proposed by Light et al. [3] to determine if a pleural fluid is an exudate. These criteria include: (1) a pleural fluid to serum protein ratio > 0.5; (2) a pleural fluid to serum lactate dehydrogenase (LDH) ratio > 0.6; and (3) pleural fluid LDH concentration > 200 IU/l. Most transudates result from congestive heart failure with the next most common cause being hepatic hydrothorax. Defining an effusion as a transudate narrows the differential diagnosis to a small number of disorders. It also ends the need for further diagnostic evaluation of the pleural effusion itself [4]. Exudates have a much larger differential diagnosis of over 50 causes, predominantly caused by infectious conditions, lymphatic abnormalities, inflammatory processes, and malignant conditions. The most frequent etiology of malignant pleural effusion is bronchogenic carcinoma, which causes over one-third of all such cases. Other frequent causes of malignant pleural effusion include metastatic breast cancer, lymphoma, mesothelioma, gastric or esophageal cancer, and ovarian carcinoma. The diagnosis of a malignant pleural effusion is established by demonstrating malignant cells in the pleural fluid or in the pleura itself. Numerous papers have recommended various diagnostic tests, such as cytological and chromosomal analysis of pleural cells, measurement of pH, glucose, amylase, or measurement of proteins as carcinoembryonic antigen (CEA) or LDH in the effusions to discriminate malignant from non-malignant pleural exudates. However, the diagnosis of disease based on a pleural effusion is often difficult, and to confirm the cause, or to rule out other possible causes, proteomics may be useful in indicating the pathogenic mechanism involved in the production of the effusion.

1.3 Complexity of proteomic studies

Proteins that are over-expressed and shed into pleural effusions have been studied for many decades for diagnosing the specific cause of their formation. We utilized proteomics to analyze pleural effusions in order to discover changes in expression of pleural proteins and to elucidate the basic molecular mechanisms that either cause, or result from, malignant mesothelioma. Malignant mesothelioma is a tumor of mesodermally derived tissue lining the coelomic cavities accompanied by diagnostic problems and for which no satisfactory curative treatment is available [5]. Mesothelioma cells release proteins into the pleural effusion that may have diagnostic value as biomarkers on their own and will provide further insights into pathological processes. Ultimately, these proteins could be valuable in cancer research e.g. as targets for the design of drug treatments. However, tumor-associated proteins are of low-abundance and therefore difficult to detect. A low total-protein concentration, a high amount of albumin and immunoglobulins (IgG), and a wide dynamic range (several orders of magnitude) of protein concentration cause several difficulties in the identification of tumor-associated proteins. It is also apparent that in most diseases, proteins are subjected to numerous changes including post-translational modifications and/or proteolytic cleavage. Proteins present in effusions are produced and secreted by many different types of human cells (e.g. inflammatory cells, tumor cells), each of which contains at least 2,000 to 6,000 different primary proteins [6, 7]. Post-translational modifications such as glycosylation, phosphorylation, acetylation, nitration, and ubiquitation multiplies this number [8-11]. Therefore, it is not surprising that no approaches currently come close in detecting all proteins present in complex biological systems. Often the highly expressed proteins ("housekeeping proteins") are detected but the expression and modification changes of less abundant proteins may be most interesting. Therefore, the development of quantitative proteomics such as differential gel electrophoresis has widened the applicability to detect proteins that undergo modification in order to produce a phenotypic change.

1.4 Differential gel electrophoresis

A powerful quantitative technique currently available is DIfferential Gel Electrophoresis (DIGE) [12-16]. This technology is commercially available (GE Healthcare, formerly Amersham Biosciences). It has the potential to overcome many of the limitations of proteomic studies by allowing the direct comparison in proteomic profile of different samples at a particular time, under a particular set of conditions (table 1) [17]. DIGE encompasses a simple strategy involving three molecular weight- and charge-matched cyanine dyes (Cy2, Cy3, and Cy5) possessing unique absorption and emission spectra. The dyes are used to fluorescently label up to three different protein samples prior to mixing them together and running them si-

multaneously on the same 2D gel [18, 19]. The fluorescent dyes bind to the terminal amino group of lysine side chains in proteins with no change in protein charge and add only 0.5 kDa to the mass of the protein, thereby, minimizing dye-induced shifting during electrophoresis. Due to a minimal labeling (only 2-5% of the total number of lysine residues are labeled), binding of the dye to the protein appears to have no effect on mass spectrometry analyses. Two different samples are labeled with Cy3 and Cy5 and a third sample, labeled with Cy2, is introduced as an internal control for each gel. The internal control is often a pooled sample comprising equal amounts of each of the samples within the study. This allows normalization and both inter- and intra-gel matching of proteins and is imperative for accurate protein quantification. Once labeled, samples are mixed and isoelectrically focused on an immobilized pH gradient (IPG) strip and co-electrophoresed on a 2D polyacrylamide gel (2D PAGE) under denaturing conditions. Each dye is then scanned using different emission filters and images are analyzed with DeCyder Differential In-gel Analysis software. This software allows accurate protein alignment and quantification between scanned images. Spots may be directly picked through an automated system. When DIGE is combined with mass spectrometry, proteins undergoing relevant changes in the context of development, pathology, and experimental manipulation can be detected and identified (Figure 2).

2. METHODS

2.1 Sample collection and preparation

Serum and pleural effusion samples were collected from mesothelioma patients who presented with large pleural effusions. Removal of effusion was performed to treat patient's shortness of breath. Prior to the pleural fluid removal procedure, patients were given a local anesthetic (Lidocaine 1%). After introducing a metallic needle in the pleural cavity, fluid was gently aspirated and collected in sterile tubes without anticoagulant or other additives. Because many components of biological samples may interfere with analysis, they were removed before storage. Insoluble substances were removed by centrifugation at 400xg for 10 min at 4°C. The supernatant was then subjected to a second centrifugation at 3000xg for 20 min at 4°C and the resulting supernatant was stored in aliquots at –80°C until further analysis.

Abundant proteins, such as albumin and immunoglobulins (IgG), were removed by using the ProteoPrep Blue Albumin Depletion Kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. In this procedure, the samples were applied on a highly specific albumin and IgG binding medium in small spin columns. After incubation, non-binding proteins were washed off in a small volume of washing buffer. Samples were further purified by removal of salts, lipids, and other interfering substances and concentrated by using the 2D clean-up kit (GE Healthcare, Fairfield, CT, USA). Before applying samples in 2D electrophoresis, the protein concentration was determined using the 2D Quant kit (GE Healthcare). First, proteins were precipitated and then resuspended in a copper-containing solution. Unbound copper was visualized with a colorimetric agent and the color density measured on a spectrophotometer at wavelength 480 nm. The color density is thus inversely related

Table 1: Characteristics of DIfferential Gel Electrophoresis (DIGE)

Advantages	Disadvantages
Application of up to 3 samples on one 2D gel	Mass shift of ~500 Da, impractical for subsequent MS (post-staining required especially for low-molecular weight proteins)
Four orders of magnitude dynamic range and good correlation between spot density and protein content	Labeling dependent on lysine content
Internal standard allows for quantitative comparison of multiple gels	Gel spots only visible under fluorescent light, equipment required for visualization and spot excision
Compatible with mass spectrometry	

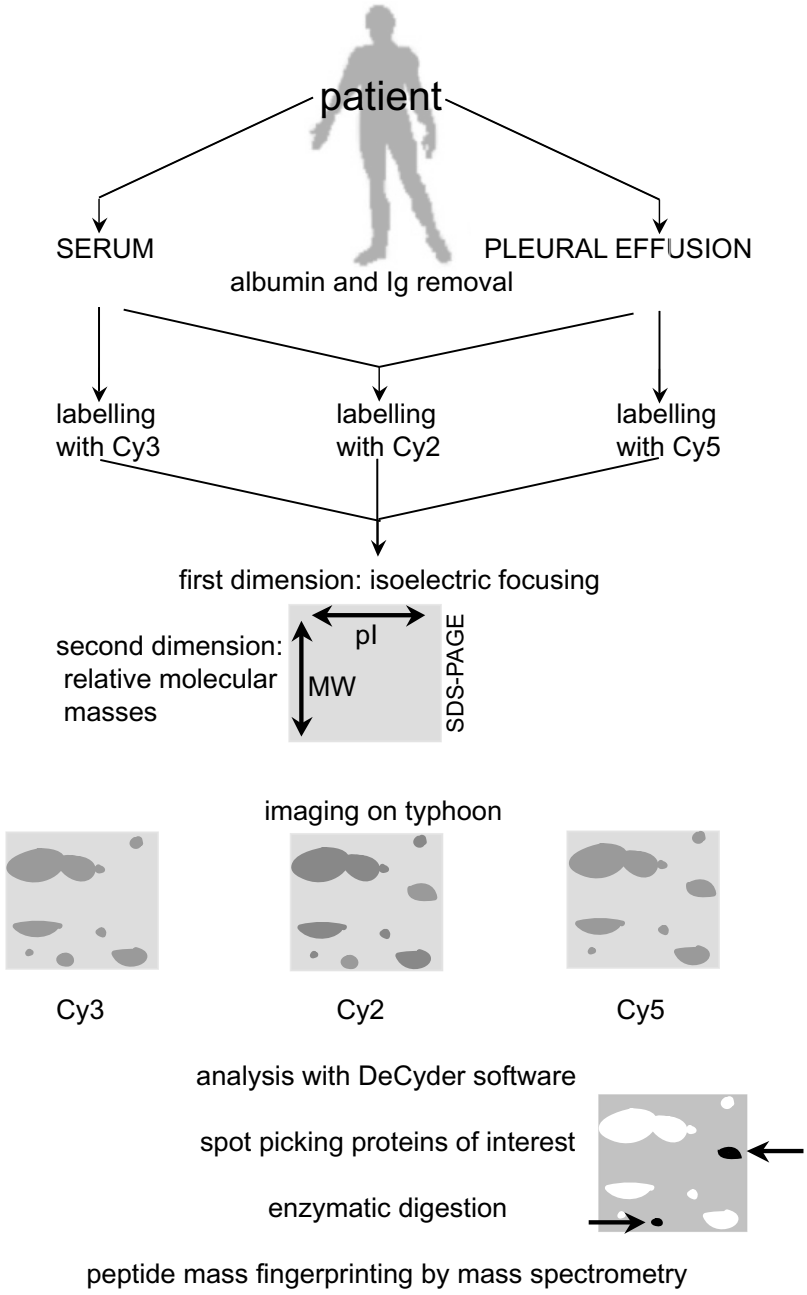

Figure 2: Flowchart of DIGE analysis of serum and malignant pleural fluid derived from a patient suffering from malignant mesothelioma. Samples to be compared are labeled with either Cy3 or Cy5, whereas the Cy2 is employed to label a pooled sample comprising equal amounts of serum and effusion within the study. The labeled samples are combined and then run on a single 2D gel. Proteins are detected using a dual laser-scanning device equipped with different excitation/emission filters in order to generate three separate images. The images are matched by computer-assisted overlay method, signals are normalized using the corresponding Cy2 spot intensities, and spots of interest are excised and analyzed by mass spectrometry. Differentially expressed proteins in pleural effusions can be useful in diagnosis or for the detection of biomarkers in cancer.

to the protein concentration. This assay has the advantage that it is compatible with samples containing reagents that are often used in protein sample preparation, like detergents, reductants, chaotropes and carrier ampholytes.

2.2 2D-electrophoresis

Two-dimensional electrophoresis (2-DE) was used to separate the proteins of the samples in two ways, according to their isoelectric point (first dimension) and to their molecular mass (second dimension). For the first dimension the samples were dissolved after precipitation in rehydration buffer consisting of 8 M Urea, 2% (w/v) Chaps, 0.5% (v/v) IPG buffer pH 4-7 containing carrier ampholytes (GE Healthcare) and 2.8 mg/ml dithiotreitol (DTT). Samples were applied onto 18 cm Immobilin Drystrips pH 4-7 (GE Healthcare) and focused in the dark using an IPGphor isoelectric focusing unit (GE Healthcare). This flatbed system uses thermally conductive ceramic stripholders with built-in platinum electrodes as focusing and rehydration chambers. After rehydration of the strips for 12 hours at 30V, a program was run that applied 1 hour 500V, 1 hour 1000V and then slowly ramped up to 8000V for 3 hours and then maintained at 8000V for an additional 2 hours to achieve > 35 000 Vhrs. Following focusing, the strips were incubated for 15 minutes in an equilibration buffer containing 6 M Urea, 50 mM Tris-HCl pH 8.8, 20% (v/v) glycerol and 2% (w/v) sodium dodecyl sulphate (SDS), 10 mg/ml DTT was added as a reductant. A second incubation of 15 minutes was performed in the same solution, but replacing DTT by 25 mg/ml iodoacetamide. This step alkylates the proteins, preventing reoxidation during electrophoresis. After equilibration, the strips were transferred onto 12% uniform SDS polyacrylamide gels for resolution in the second dimension. Gels were poured between low fluorescent glass plates of which the inner side of one plate was treated with Bind-Silane (GE Healthcare) so that the gel remained attached to this plate after electrophoresis and disassembly of the gel sandwich. This minimizes gel distortion and simplifies handling during subsequent fixing, staining, imaging and automatic spot picking steps. Gels were run on the Ettan Dalt system (GE Healthcare) at a constant power of 180 W at room temperature (RT) until the dye front had reached the bottom of the gel.

2.3 Gel staining and visualization

For visualization of the separated proteins, gels were stained using SYPRO Ruby protein gel stain (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's instructions. Gels were scanned on the Typhoon 9410 laser-scanner (GE Healthcare) using 457 nm as excitation wavelength and 610 nm band pass (BP) 30 nm as emission filter.

2.4 2D DIGE

Two-dimensional fluorescence difference gel electrophoresis (2D DIGE) allows detection and quantitation of differences in protein abundances between different samples using cyanine dye labeling with spectrally resolvable CyDye DIGE Fluor minimal dyes (GE Healthcare). This technique was used to detect differences in protein profiles of serum and pleural fluid of mesothelioma patients.

For this procedure, samples were prepared and protein concentration was determined as described in chapter 2.1. Typically, 50 µg of sample was minimally labeled with 400 pmol of either Cy3 or Cy5 freshly dissolved in anhydrous dimethyl formamide. Labeling reactions were performed on ice in the dark for 30 min and then quenched with an excess of free Lysine (1 µl of 10 mM L-Lysine solution [Sigma-Aldrich]) for 10 min on ice. A pool of all samples was also prepared and labeled with Cy2 to be used as a standard on all gels to aid image matching and cross-gel statistical analysis. Differentially labeled samples that were going to be run on one 2D gel were mixed, 50 µg of Cy2 labeled pooled samples was added to 50 µg of Cy3 labeled sample (serum) and 50 µg Cy5 labeled sample (pleural effusion). In preparative gels, 350 µg of pooled unlabeled proteins were additionally loaded. The final volume was adjusted to 350 µl rehydration buffer, the volume needed for loading of an 18 cm IPG strip. Isoelectric focusing and second dimension gel electrophoresis were performed as described in chapter 2.2. Gels were scanned on a Typhoon 9410 laser scanner for visualization. The following settings were used: 100 µm resolution, PMT values between 500 and 520 and the laser settings for the CyDyes (laser [nm], emission filter [nm]): Cy2 (488, 520BP40), Cy3 (532, 580BP30), and Cy5 (633, 670BP30). After scanning, one glass plate was removed and the gel was fixed in 10% (v/v) methanol, 7.5% (v/v) acetic acid overnight. A post-staining was performed with SYPRO Ruby dye for 4 h at RT and gel was scanned at 457 nm with emission filter 610 nm with a band pass of 30nm. Gel analysis was performed using DeCyder DIA V5.02 for intra-gel comparison and for inter-gel matching the DeCyder BVA V5.02 software (GE Healthcare) was used.

2.5 Automatic spot picking
Fluorescently stained protein spots of interest were excised from 2D gels using an automated Ettan spot picker ([Figure 3] GE Healthcare) following the manufacturer's instructions. This robotic system automatically picks selected protein spots from gels using a pick list from the image analysis, and transfers them into 96-well low protein binding microplates (Nunc A/S, Roskilde, Denmark).

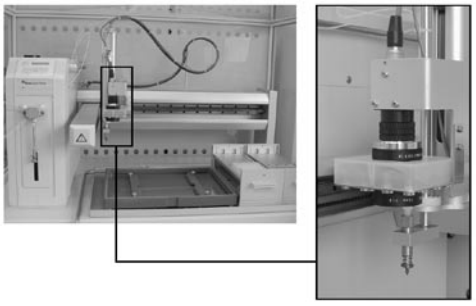

Figure 3: The automated Ettan spot picker instrument (GE Healthcare) with the picker head enlarged.

2.6 Tryptic in-gel digestion
The excised plugs were washed for 5 min with milli-Q. Following this washing step, gel plugs were alternately treated twice for 5 minutes each with Milli-Q and acetonitrile. Gel plugs were dried in a rotary evaporator (Savant, Farmingdale, NY, USA) for 30 min. Proteins were digested overnight at RT in 4 µl of 100 µg/ml sequencing grade modified trypsin (Promega, Madison, WI, USA).

2.7 MALDI-tof mass spectrometry
After digestion, 7 µl of 0.7% trifluoroacetic acid in 30% acetonitrile was added to the gel plugs. 1 µl of this mixture was added to 2 µl of 2 mg/ml ionization enhancing material a-cyano-4-hydroxy-trans-cinnamic acid (Bruker Daltonics, Billerica, MA, USA) in acetonitrile. Of the sample-matrix mixture, 1 µl was applied onto a 600 µm 384-spot metal anchor chip plate and crystallized in air. Peptide mass spectra were acquired on a MALDI-tof mass spectrometer (MS) equipped with a 337 nm nitrogen laser (ULTRAFLEX, Bruker Daltonics). The instrument was calibrated with a peptide calibration standard (Bruker Daltonics).

2.8 Database search
Peptide mass fingerprinting is based on mass measurement of peptide fragments derived from a single protein, digested with trypsin. The anticipated mass values of peptides in virtual digests of all proteins are calculated and listed in the MSDB database of the National Center for Biotechnology Information (NCBI).
A mass list of peptides, from each sample analyzed on the MALDI-tof MS, was generated in the Flexanalysis software (Bruker Daltonics). Peaks obtained from autolytic fragments of trypsin were omitted from the spectra. The mass lists were submitted to Matrix Science Mascot UK software to identify the proteins in the MSDB database. The criteria we used for the search in the database were as follows: (a) maximum allowed peptide mass error of 200 ppm, (b) at least five matching peptide masses, (c) molecular weight of identified protein should match estimated values by comparing with marker proteins, (d) top-scores given by software should be higher than 61 ($p<0.05$).

2.9 Western Blotting
For detection of apolipoprotein spots in the 2D PAGE, proteins were electroblotted onto Immobilon-P membranes (Millipore Corp, Billerica, MA, USA) with the Criterion Blotter (BioRad Laboratories, Hemel Hempstead, UK). To saturate nonspecific protein binding sites, the membranes were incubated for 1 h with blocking buffer (TBST containing 5% (w/v) low fat milk powder) and then incubated overnight with 1:10.000 diluted rabbit anti-human apolipoprotein A1 (Calbiochem, San Diego, CA, USA) in blocking buffer. Blots were washed and incubated with 1:1000 diluted horseradish peroxidase-conjugated swine anti-rabbit (DAKO, Glostrup, Denmark) and visualized by the SuperSignal West Pico chemiluminescent substrate (Pierce Perbio, Rockford, IL, USA) according to the manufacturer's instructions.

3. APPLICATIONS

Evaluation of pleural effusion is useful for improving the diagnosis and research of several inflammatory diseases and malignancies. This chapter describes a strategy for the comparative analysis of serum proteome and pleural effusion proteome to elucidate the basic molecular mechanisms that either cause, or result from, cancerous disorders. Pleural effusion and serum obtained from the same patients with malignant mesothelioma were analyzed using a strategy that combined analytical techniques in electrophoresis, mass spectrometry, and western blotting (Figure 2). The resulting data provide fundamental information on the composition and difference of protein contents in these body fluids.

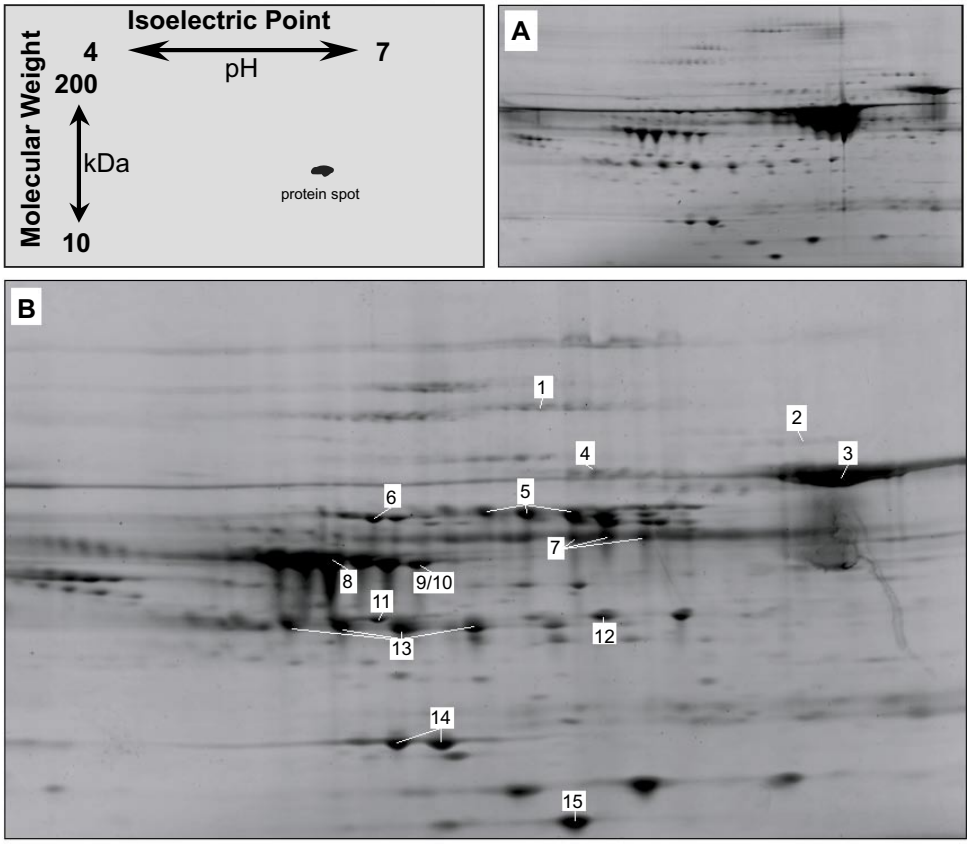

Figure 4: Human pleural effusion was processed with the ProteoPrep albumin/IgG removal kit and proteins from the unbound fractions were collected. 2D map using equal protein amounts for crude human pleural effusion (A) and after depletion of albumin and IgG (B) were obtained. Pleural proteins (50 µg) from a cancer patient were resolved by 2DE using a pH 4-7 gradient IPG strip in the first dimension and an 4-12% gradient polyacrylamide SDS gel in the second dimension. Gels were stained with SYPRO Ruby and imaged using a Typhoon 9410. Numbers correspond to the excised protein spot and analyzed by mass spectrometry (table 2).

After informed consent of the participants, serum and pleural effusions were collected according to special procedures for a reliable and consistent sample collection. Many factors, such as time of collection, containers used, preservatives and other additives, transport to the laboratory and storage, affect the quality of the samples and the stability of the proteins of interest and must be considered at the initial collection stage. For comparative studies between patients, it is crucial that body fluids are handled and stored consistently throughout the study.

First, pleural effusions were separated by 2D electrophoresis using isoelectric focusing (IEF) as the first dimension followed by SDS-PAGE for the second dimension. Because the majority of the proteins will focus within the pH 4-7 range, narrow-range immobilized pH gradient (IPG) strips were used. Figure 4a demonstrates a typical 2DE pleural effusion profile evidenced by SYPRO Ruby staining. More than 300 individual protein spots were detected in the molecular mass range of 20 to 200 kDa. The albumin smear at around 67 kDa and IgG fragments are the major protein components of pleural effusions, representing 50 to 70% and 10 to 20% of the total pleural proteins, respectively (mg/ml range). Together with transferrin, fibrinogen, complement components and a few other proteins, the top 20 proteins are responsible for about 99% of the protein mass. Pleural effusions contain comparatively small quantities of cytokines detected by cytokine arrays and ELISA, such as transforming growth factor-b (TGF-b), interleukins (IL-1, IL-6, IL-8, and IL-10) or vascular endothelial growth factor (VEGF), but these are in the ng/ml to pg/ml range,

a difference of nine orders of magnitude or more. Therefore, removal of abundantly expressed proteins is a key element of proteome research to allow the visualization of co-migrating proteins on 1DE and 2DE gel and to allow a higher sample load for improved visualization of lower copy number proteins. A convenient approach to remove high-abundance proteins from body fluids is affinity chromatography with resins carrying highly efficient and specific ligands for these proteins. Removal of albumin and IgG using the commercially available ProteoPrep kit (Sigma-Aldrich) clearly improved resolution and increased spot count in depleted samples (Figure 4b).Mass spectrometry can be used to identify proteins in a sample by providing the molecular mass to electric charge (m/z ratio) of peptides in the femtomole to attomole range with an accuracy of < 10 ppm. With the completion of the human genome project [20], it is now possible to identify proteins by using search algorithms that interrogate protein sequence databases in an automated fashion. A panel of 15 spots (numbered spots in Figure 4b) were exemplary selected and subjected to protein identification by trypsin digestion, MS analysis, and database search (table 2). All these proteins have been described to be present in pleural fluid and were likely to have originated from serum [21-27]. The large number of spots in a 2D gel is partly due to post-translational and proteolytic modifications of proteins: one protein may, therefore, be present in several locations in the gel (Figure 4b).

This approach has recently been published for a composite pleural effusion sample from seven lung adenocarcinoma patients. This study revealed at least 472 silver-stained protein spots to be present in a 2DE map, half of which could be identified by liquid chromatography-tandem mass spectrometry [21]. Although the results of these studies provide information for a basic understanding of the protein composition of pleural effusions, the value for clinical medicine is limited. The approach is time consuming and the interpretation of results is hampered by additional factors that are introduced by variables in experimental parameters. For example, difficulties in the detection of low-abundance proteins due to limitations in dynamic range, diversity within a complex biological sample, and the typical changes associated with different causes of effusion and with time are variables that impede the interpretation. Many of the proteins present in pleural fluid are likely to have originated from serum. Of interest are the proteins that have not previously been reported in the literature to be present in serum. These proteins could orginate from infiltrating cells or from the parenchymal interstitial linings of the lung. They also represent potential candidates for useful biomarkers concentrated or only measurable in pleural effusions. To discover the proteins of interest, we used a strategy of comparative analysis of serum proteome and pleural effusion proteome from the same mesothelioma patient using the DIGE technology (Figure 2). Comparing body fluids of the

Table 2: Identified proteins from the human pleural effusion proteome (* scores higher than 61 were significant ($p < 0.05$)

No.	Protein	Accession number	Nominal mass (kDa)	Coverage	Score*
1	Complement factor H	NBHUH	139.034	13%	**95**
2	Complement factor B	BBHU	86.847	27%	**152**
3	Transferrin	TFHUP	77.000	36%	**182**
4	Fibrinogen precursor	FGHUA	69.756	40%	**111**
5	Hemopexin	CAA26382	51.643	18%	**93**
6	Alpha-1B glycoprotein	OMHU1B	52.479	38%	**100**
7	Fc α Receptor I	MGC27165	53.358	17%	**104**
8	Alpha-1 antitrypsin	1THU	46.707	56%	**209**
9	Vitamin D-binding protein	AAA61704	54.513	60%	**228**
10	Antithrombin	1AZXI	47.656	35%	**85**
11	Apolipoprotein A4	Q13784	28.141	38%	**68**
12	Chain of fibrinogen	1FZAB	36.331	81%	**267**
13	Haptoglobin	HPHU1	38.941	36%	**133**
14	Apolipoprotein A1	CAA00975	28.061	69%	**192**
15	Transthyretin	2ROXA	12.996	86%	**124**

same patient gives less individual variation of course, as the genetic component is no longer a variable and samples are completely matched for age, sex, ethnic origin and other parameters (smoking, alcohol consumption, medication and many others). The spectrally distinct dyes allow co-separation of different CyDye DIGE fluor-labeled samples in the same gel and ensure that all samples will be subject to exactly the same first- and second-dimension electrophoresis running conditions. This limits the experimental variation and thus ensures accuracy within gel matching. Samples were differentially labeled with spectrally different fluorescent dyes: serum was labeled with the cyanine dye Cy3 and pleural fluid with cyanine dye Cy5. A Cy2-labeled pool of samples was used as standard with all Cy3- and Cy5-labeled sample pairs to facilitate cross-gel quantitative analysis. Once labeled, all samples (Cy2, Cy3, and Cy5) were mixed. For mass spectrometry a preparative gel was run. Therefore, 350 µg unlabeled pooled sample was added to the CyDye labeled mixture. The protein mixture was isoelectrically focused on an IPG strip and co-electrophoresed on a 2D polyacrylamide gel (2D PAGE). Each dye was then scanned using a Typhoon gel scanner equipped with different emission filters and quantitation of protein expression differences and analyzed with DeCyder DIGE Analysis software (Figure 5).

In DeCyder analysis, the Cy2, Cy3, and Cy5 images were merged for each gel and spot boundaries were detected for the calculation of normalized spot volumes/protein abundances. At this stage, dust particles, scratches, and other features resulting from non-protein sources were filtered out. The analysis was used to rapidly calculate abundance differences between serum and effusion run on the same gel. The 2D DIGE map is shown in Figure 5; 1436 spots of various intensities were detected by the software in this map. Statistical analysis was performed of each protein spot using a 2-fold change ratio. The majority of the spots (1304 spots, 90.8% of total) showed small changes between effusion and serum. The standardized volumes of 76 spots (5.3%) were decreased and 56 spots (3.9%) had volumes increased by more then 2-fold, i.e. the change in protein level in pleural effusion compared to serum proteins (Figure 6). To ensure consistency in the observed DIGE profile, the whole experiment was repeated a second time to eliminate confounding factors that may arise during the practical procedure. Although the total amount of spots detected differed, there was no substantial variation in the profile of the protein spots when the whole experiment was repeated using the same samples on a different day. Expression differences identified by 2D DIGE can therefore be confidently assigned to biological differences and are not due to system variability. Serum and pleural effusion from other mesothelioma patients were analyzed to confirm consistency and relevancy of the differentially expressed proteins in serum and pleural effusion using Cy2 labeled pooled sample as cross gel standard. Every difference is assigned a statistical confidence value. The DeCyder BVA software allows protein alignment and quantification between scanned images.

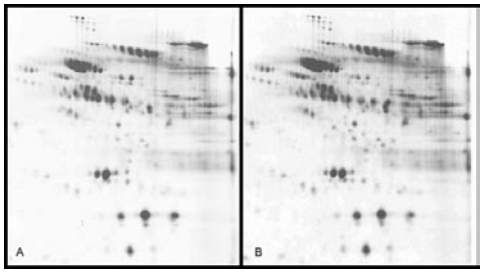

Figure 5: Images from a pH 4-7 2D-DIGE gel of two protein samples labeled with minimal CyDye DIGE fluors. The Cy3 image (A) corresponding to serum; the Cy5 image (B) corresponding to pleural effusion.

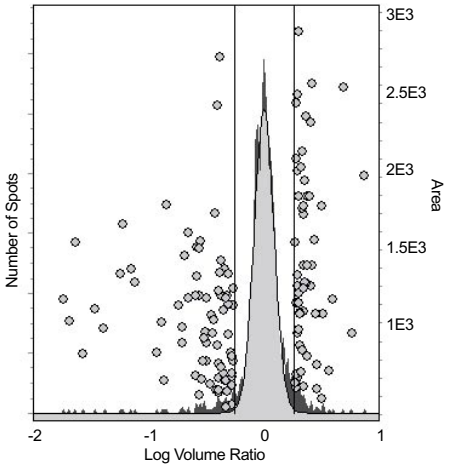

Figure 6: DeCyder software statistical output showing 2-fold changed protein spots. Dots on the left are decreased in pleural effusion; dots on the right are increased in effusions compared to serum of the same patient. Spots can be highlighted individually for detailed information display, e.g. 3D intensity view and table view of quantitative data.

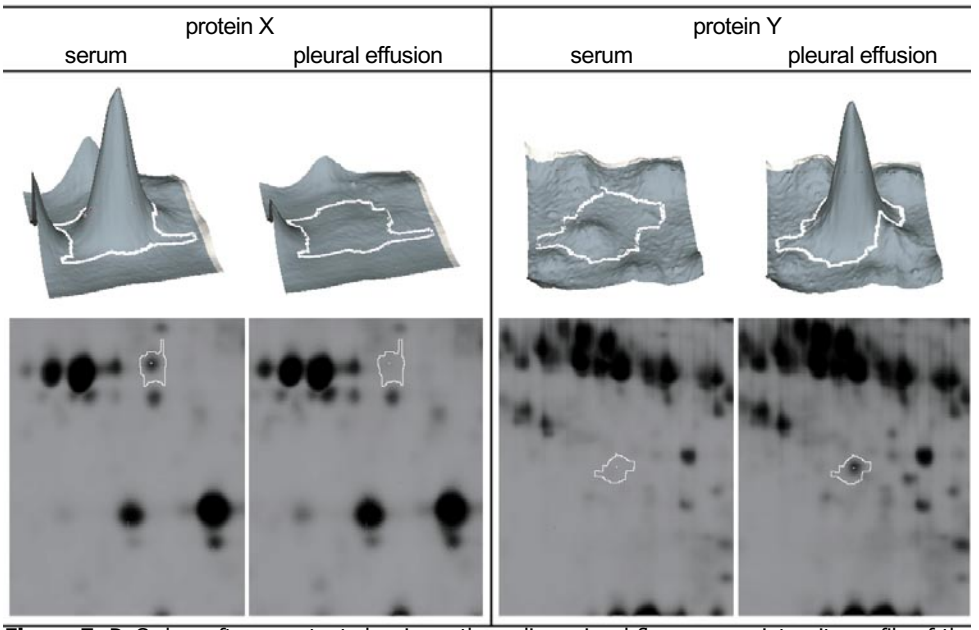

Figure 7: DeCyder software output showing a three-dimensional fluorescence intensity profile of the circled spots in the magnified gel image with Cy3 labeled serum and Cy5 labeled pleural effusion. 3D Simulation of the protein spots allows an objective view for the comparison of spot intensity between the two images. Protein X is increased in serum, protein Y is decreased in serum compared to pleural effusion, both body fluids were obtained from the same patient.

Over-expression of proteins by mesothelioma cells can result in their shedding in the pleural effusion and will lead to enhanced intensity spots compared to serum of the same patient. Absence of proteins in the effusion may be caused by specific proteolysis or by specific absorption from the circulation by tumor cells. A protein spot with the approximate molecular weight of 30 kDa and pI of 5.5, significantly expressed in the serum but not in the effusion, was selected for identification and further analyses (Figure 7, protein X). Selection of proteins for excision requires post-staining of gels with SYPRO Ruby, because the majority of the protein will not exactly co-migrate with the CyDye labeled protein (dyes add ~0.5 kDa to the total molecular mass). Thus the SYPRO Ruby stained proteins of interest rather than the CyDye labeled protein spots were excised from the gel through an automated system using a pick list (Figure 8). Proteins were used for MALDI-tof MS analysis using peptide mass fingerprinting and database searching. When DIGE is combined with mass spectrometry, proteins undergoing relevant changes in the context of development, pathology, and experimental manipulation can be detected and identified.

The resultant mass fingerprinting spectra of the tryptic digest was used for protein search in the MSDB database of the National Center for Biotechnology Information (NCBI). Nineteen matched peptides with a total coverage of 75% are the basis of the

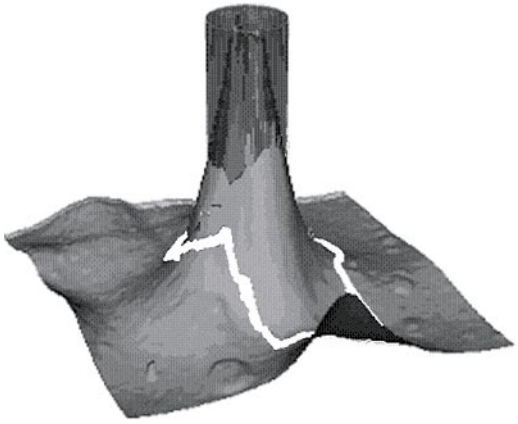

Figure 8: 3D intensity plot of a protein selected for MS identification, showing the area used in the analysis (white line) and the selected area for automated spot picking (circular volume).

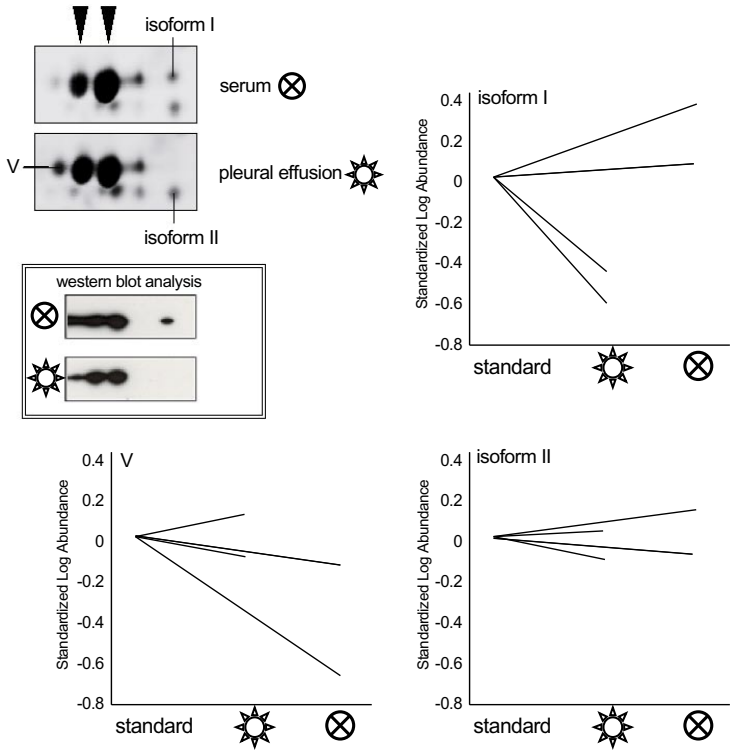

Figure 9: A magnified gel image showing Cy3 labeled serum and Cy5 labeled pleural effusion. Isoform I represents only a small fraction of the total serum apolipoprotein A1 (arrows) but was not detected in pleural effusion. This was confirmed by 2DE western blot analysis. DyCyder BVA output showing graphs of standardized log abundances (y-axis) for the isoforms (I and II) and protein V in sera (⊗) and pleural effusions (☼) after intra- and inter-gel matching.

identification of the decreased spot in effusions as apolipoprotein A1 (accession number CAA00975), a protein with a molecular mass of 28.061 Da and pI of 5.27. This spot (isoform I) migrates differently from the major apolipoprotein A1 spots (arrows, Figure 9) and represents a small fraction of the total serum apolipoprotein A1 but is absent in pleural effusion. This directly illustrates the advantage of 2D gel-based approaches in visualizing changes in the molecular weight and pI of a protein. The different pI and slightly different molecular mass reflects biological significant processing and charge-altered post-translational modifications such as possible phosphorylation, sulfation or (de-)acetylation. Thus comparison of the protein spots from serum and effusion by 2D-DIGE provides a very striking quantitative picture of proteins absorbed or shed into body fluids. The identity of apolipoprotein A1 was further confirmed by 2DE western blot analysis using a rabbit anti-human apolipoprotein A1 (Figure 9). The smaller isoform II was not detected in the western blot analysis. However, this isoform was identified by MS with a total of 13 matched peptides that covered 57% of full lengths, unprocessed apolipoprotein A1 as shown in figure 10. The matched peptides are clustered in the C-terminal region of the protein. A cleavage site before amino acid 27 can produce a protein with a smaller molecular weight, consistent with the different migration in the 2D gel. The absence of the smaller isoform II in western blotting is probably caused by the N-terminal binding of the antibody to apolipoprotein A1. This truncation product has not been reported previously and it is not known whether the fragmentation was due to in vivo biological processing or due to protease activity. It may be the product of cleavage by one or more proteases, including kallikrein or matrix metalloproteases. These results highlight one of the advantages of DIGE using 2D SDS PAGE as the proteins are separated according to their pI and molecular

Apolipoprotein A1 isoform I

1	**DEPPQSPWDR**	VK**DLATVYVD**	**VLK**DSGR**DYV**	**SQFEGSALGK**	QLNLK**LLDNW**
51	**DSVTSTFSK**L	R**EQLGPVTQE**	**FWDNLEKETE**	**GLR**QEMSKDL	EEVKAK**VQPY**
101	**LDDFQK**K**WQE**	**EMELYR**QKVE	PLR**AELQEGA**	**RQKLHELQEK**	**LSPLGEEMR**D
151	RAR**AHVDALR**	**THLAPYSDEL**	**R**QLAARLEA	LKENGGAR**LA**	**EYHAKATEHL**
201	**STLSEKAKPA**	**LEDLRQGLLP**	**VLESFKVSFL**	**SALEEYTK**KL	NTQ

Apolipoprotein A1 isoform II

1	*DEPPQSPWDR*	*VKDLATVYVD*	*VLK*DSGR**DYV**	**SQFEGSALGK**	QLNLK**LLDNW**
51	**DSVTSTFSK**L	R**EQLGPVTQE**	**FWDNLEKETE**	**GLR**QEMSKDL	EEVKAK**VQPY**
101	**LDDFQK**K**WQE**	**EMELYR**QKVE	PLR**AELQEGA**	**RQKLHELQEK**	**LSPLGEEMR**D
151	RAR**AHVDALR**	**THLAPYSDEL**	**R**QLAARLEA	LKENGGAR**LA**	**EYHAKATEHL**
201	**STLSEKAKPA**	**LEDLRQGLLP**	**VLESFKVSFL**	**SALEEYTK**KL	NTQ

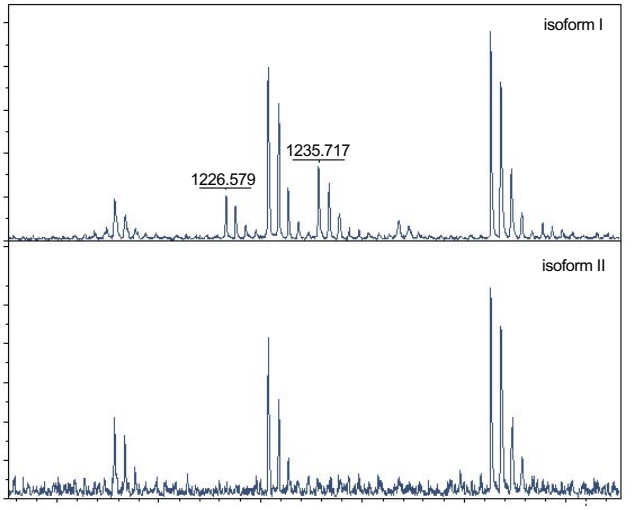

Figure 10: Identification of apolipoprotein A1 by MS. The amino acid sequence of apolipoprotein A1 isoformes are shown. Peptides derived from isoform I and isoform II were matched by mass fingerprinting and printed bold. In the smaller isoform II, the peptides DEPPQSPWDR (MW 1226.579) and peptide DLATVYVDVLK (MW 1235.717) were not detected (depicted in italic) as shown in TOF MS spectra.

weights. With regard to the apolipoprotein A1 isoform I identified in this study, it is difficult to speculate on the physiological meaning of the change in their relative abundance between the serum and pleural effusion of the same patient. Reductions in the serum levels of apolipoprotein A1 have been correlated with hepatitis B virus induced diseases [28-30]. An isoform of apolipoprotein A1 was detected by 2DE in serum obtained from individuals with high risk for the development or who were diagnosed with hepatocellular carcinoma [31]. Apolipoprotein A1 is a potential marker of the aggression in colonic adenocarcinoma [32] and is upregulated in primary carcinoma tissue of the vagina [33]. However, a down-regulation of apolipoprotein A1 in serum is described in early stage ovarian cancer [34, 35]. The isoform described in this study could be induced by posttranslational modifications but experiments, using both biochemical and biological approaches, are necessary to further assess the role of apolipoprotein A1. Post-translational modifications (e.g. phosphorylation, glycosylation) play a crucial role in cell signaling and protein function [8] and more than 200 different protein modifications have already been described [10, 11]. We are currently trying to identify the additional proteins whose expression is significantly altered, as revealed by DeCyder analysis.

4 FUTURE DIRECTIONS

Novel proteomic methods have the advantage that the results are less biased by the theories or beliefs of the investigator and is only limited by the sensitivity of the method and can give rise to new discoveries and can generate new hypotheses. This is in contrast to the traditional reductionist one-stimulus one-protein investigations, e.g. an ELISA or Western blot, where the researcher has to decide beforehand on which antibodies to use.

DIGE technology is gaining acceptance in the field of proteomics and has enabled the detection of more subtle changes in protein expression than the conventional 2D-PAGE [36]. Quantitative comparison by 2D DIGE and protein profiling techniques allow the rapid comparison of different complex samples making a study of specific diseases or biological processes under clinically relevant conditions possible. Recent DIGE studies comparing the difference between normal and cancerous tissue were successful in demonstrating changes in protein expression levels [37-39]. Here we provide a brief example of comparing serum and pleural effusion of the same mesothelioma patient to identify unique proteins by observing concentration changes and modifications on a single protein level. Several proteins were found to be differentially or uniquely expressed in the serum or in the pleural effusion. In our study, the number of patients is too small to permit conclusive associations between disease biomarkers and the unique mode of biosynthesis and processing of e.g. isoform I of apolipoprotein A1. Nonetheless, the results are provocative and further work into the function and expression of this protein and other differentially expressed proteins (either up- and down-regulated) are thus encouraging. New possibilities with the DIGE technology can range from single variable comparisons to complex multivariable comparisons and/or time course studies (e.g. transudates vs. exudates, mesothelioma effusions vs. effusions of other origin).

In conclusion, we show that 2D DIGE and DeCyder analysis is a sensitive, MS-compatible technique for identifying statistically significant differences in the protein expression profiles of multiple samples. The ongoing rapid development in separation techniques, sample comparison and protein quantification, mass spectrometry, and bioinformatics will continue to stimulate the investigation of pleural effusions and will lead to new insights into the mechanisms of disease in the near future. The area of clinical proteomics and the study of the mechanism of disease will have a major impact on the way diseases are diagnosed and treated.

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Chapter 6

6

Protein profiling of pleural effusions to identify malignant pleural mesothelioma using SELDI-TOF mass spectrometry

Submitted for publication

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PROTEIN PROFILING OF PLEURAL EFFUSIONS TO IDENTIFY MALIGNANT PLEURAL MESOTHELIOMA USING SELDI-TOF MASS SPECTROMETRY

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The objective of this study was to detect protein profiles that could be used to identify maligant pleural mesothelioma (MM) with surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry (MS). Pleural effusions were collected from patients with confirmed mesothelioma (n=54) and from patients with effusions due to other causes ([n=54] cancerous and non-cancerous). Samples were fractionated using anion exchange chromatography and then bound to different types of ProteinChip array surfaces. Peak intensity data were subjected to classification algorithms in order to identify potential classifier peaks that could be used to discriminate between mesothelioma and non-mesothelioma samples. One such protein peak at m/z 6614 was characterized as apolipoprotein (Apo) CI and was decreased in pleural effusions due to mesothelioma. In this setting, the sensitivity and specificity of this potential biomarker was 76 % and 69 %, respectively. Studies are underway to further investigate the possible role and function of the decrease of this protein in the oncogenesis of mesothelioma. The inclusion of pleural effusion Apo CI protein values in a multimarker panel for effective diagnosis of pleural mesothelioma is suggested as a future application.

1. INTRODUCTION

Malignant mesothelioma is a highly aggressive neoplasm arising from the mesothelial surfaces of the pleural cavities and less frequently in the peritoneal cavities, the pericardium, or the tunica vaginalis testis. Asbestos is the major causative agent but a few other generally recognized causes of MM exists: endemic erionite exposure in Turkey (1), ionizing radiation (2), and chest injuries (3). Also the potent oncogenic polyoma virus, simian virus (SV)-40, has been implicated as a cofactor in the causation of MM (4-10) but this remains controversial (11-14). With median survival durations of 6-10 months from onset of symptoms, the prognosis is poor (15, 16). Recent advances in both surgical and chemotherapy have improved survival, but the treatments remain toxic and selection of appropriate patients for these therapies is difficult (17-19). Several imaging techniques such as chest radiography (X-ray), computed tomography (CT) or magnetic resonance imaging (MRI) have proven useful when MM is suspected due to the presence of pleural effusion (in 95% of the patients at some time during the course of the disease) combined with a history of occupational or secondary asbestos exposure. Patients who present with large pleural effusions will have a thoracentesis to confirm the presence of cancerous cells using (electon) microscopic and/or immunohistochemistry (IHC) techniques. In spite of intensive research efforts, no single immunostain exists that is entirely conclusive for MM, and for most commercially available antibodies recorded in the literature both the diagnostic value of each one or their various combinations in immunohistochemical panels are still under debate (20-22). A panel of immunohistochemical stains is necessary to diagnose MM, consisting of positive immunostains for MM and immunostains specific for cancers that can spread to the pleura. Only in 32% of the cases diagnostic cytologic evidence is found in the pleural fluid (23). Main problem areas in the pathologic assessment of specimens submitted with a clinical suspicion of MM are the distinction with adenocarcinoma, metastatic sarcoma or sarcomatoid carcinoma, and distinction between malignant and benign mesothelioma, even by experienced cytopathologists (24). Therefore, more objective routes were pursued for investigation of informative diagnostic content: correlation of

specific protein expression levels with the MM pathology were investigated in serum and pleural effusion for osteopontin (25), mesothelin (26), and soluble mesothelin-related proteins (SMRP) (27). SMRP is the only in vitro diagnostic commercial kit available for differentiating epithelioid MM (rather than for the mixed subtype and sarcomatoid subtype, because only the epithelioid subtype is positive for mesothelin staining(28)) from other neoplasms. Initial SMRP serum measures reported by Robinson and coworkers (27) had a sensitivity of 84% and specificity approaching 100% compared with pleural metastasis of other tumors but later reports could not confirm this (sensitivity 58.3%, specificity 73.3%) (29). SMRP levels in pleural fluid are better in differentiating MM from pleural metastatic carcinomas compared to serum levels (29). The diagnosis of MM, if definite, leads to a discouraging prognosis for the patient but without it treatment cannot be planned, and the patient's legal position in terms of compensation remains unclear.
Research into novel biomarkers for MM may yield more information that could contain potential to guide therapeutic decisions in the near future. The objective of this study was to detect novel protein profiles in supernatant of pleural effusions that could be used as biomarker(s) to further differentiate MM from other pulmonary disease states. The advantages of studying pleural effusions are (I) in the case of MM it is in close proximity to the tumor; (II) they are removed for patient comfort, pursuing pressure release or diminishing dyspnea, and (III), they are relatively easy to obtain in large amounts. Surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry (MS) was used with different Protein Chip arrays (IMAC30, H50, and CM10) in a total of 108 pleural effusion samples. Using an artificial intelligence classification algorithm, several novel biomarkers were selected to facilitate separation of MM from other cancers or inflammatory conditions. Given the probable rise in the worldwide incidence of this disease during the next few years, increasing the sensitivity and specificity for mesothelioma for individual cases will be very relevant.

2. METHODS

Mesothelioma patients and controls
Pleural fluid was collected after informed consent from patients who presented with large pleural effusions. Removal of effusion was performed to treat patients' shortness of breath. Pleural effusions were due to the following conditions: 41 patients with mesothelioma and 48 age-matched patients with effusions due to other causes (non-infective inflammatory exudates (n=3), transudates (n=3), a still unknown cause (n=2), and other malignancies (n=40)). The characteristics of the patients are reported in Table 1. All mesothelioma patients were cytologically or histopathologically proven by pathologists and many of these patients had a past exposure to asbestos, mainly occupational by working in shipbuilding plants, docks, or industrial plants. Therefore, 95% of our mesothelioma patients were men (the two women developing mesothelioma were living with asbestos workers). The non-mesothelioma cancers with pleural involvement can be divided into carcinoma (28 metastasis of distant tumor and 6 non-small cell lung carcinoma), sarcoma (3), lymphoma (2), and melanoma (1). From some patients, effusions were taken at multiple time-points. We obtained written or oral informed consent from all participants. The study was approved by the Medical Ethics Review Committee (METC) of the Erasmus MC, Rotterdam, The Netherlands.

Antibodies and proteins
All antibodies and calibrator proteins were purchased from US Biologicals (Swampscott, MA, USA): goat anti-human apolipoprotein (Apo) CI (cat # A2299-61); human Apo CI purified from plasma (cat # A2290-60) . ProteinA HyperD beads were purchased from BioSepra (Cergy-Saint-Christophe, France).

Processing of pleural effusions
Irrespective of cause, all effusions were collected, processed, and stored in the same way. Prior to the pleural fluid removal procedure, patients were given a local anesthetic (Lidocaine 1%). After introducing a metallic needle in the pleural cavity, fluid was gently aspirated and collected in sterile tubes without anticoagulant or other additives. Total volumes varied from 10 ml to 4 L. Pleural cells were removed by centrifugation at 400xg for 10 min at 4°C and supernatant was then subjected to a second centrifugation at 3000xg for 20 min at 4°C and the resulting supernatant was stored in aliquots at –80°C until further analysis. No infectious agents were observed in the pleural fluid and bacterial cultures were negative in all cases.

Protein expression profiling
Pleural effusions were fractionated using anion exchange chromatography according to the protocol described by Gilbert et

Table 1: Patients characteristics

	Group 1. Mesothelioma effusions	Group 2. Other effusions
Number of patients	41	48
1 x thoracentesis	34 patients	44 patients
2 x thoracentesis	3 patients	2 patients
3 x thoracentesis	2 patients	2 patients
4 x thoracentesis	2 patients	0 patients
Number of samples	54	54
Age range (mean)	44 - 80 (62.5) yr	31 - 84 (61.1) yr
Sex M : F	39 : 2	20 : 28
Volume effusion range (mean)	10 – 4000 (1077) ml	10 – 2000 (603) ml
Diagnose (number of patients)	Exudative pleural effusion Malignancy Pleural mesothelioma (41)	Exudative pleural effusion Malignancy Metastatic carcinoma (28) NSCLC (6) Sarcoma (3) Non-hodgkin lymphoma (2) Melanoma (1) Lymphatic abnormalities (3) Undiagnosed effusions (2) Transudative pleural effusion Renal failure (2) Pulmonary embolism (1)

al (30). Briefly, pleural effusion was applied to Q HyperD F anion exchange resin, and fractions were eluted using a descending stepwise pH gradient. Each of these fractions was applied to IMAC30, H50, and CM10 ProteinChip arrays. The arrays were read in PBSIIc ProteinChip reader, a time-lag focusing, linear, laser desorption/ionization- time of flight mass spectrometer. All spectra were acquired in the positive-ion mode. Time-lag focusing delay times were set at 400 ns for low-mass scans and 1900 ns for high-mass scans. Ions were extracted using a 3 kV ion extraction pulse and accelerated to a final velocity using 20 kV of acceleration potential. The system employed a pulsed nitrogen laser at repetition rates varying from 2–5 pulses per second. Typical laser fluence varied from 30–150 mJ/mm^2. An automated analytical protocol was used to control the data acquisition process in most of the sample analysis. Each spectrum was an average of at least 50 laser shots and externally calibrated against a mixture of known peptides or proteins.

Data analysis

Data preprocessing was performed in CiphergenExpress version 2.1. Spectra were baseline subtracted using a fitting window of 8 times expected peak width. Data were normalized using an external coefficient of 1, and peak detection was performed using the automated Biomarker Wizard algorithm. Univariate analysis was performed using the Mann-Whitney test for each pairwise comparison. Multivariate analysis was performed using PAM (Prediction Analysis for Microarrays) (31). It is a nearest shrunken centroid method that can be used in high-dimensional classification problems. Each prediction variable (i.e. peak) is standardized by the within-group standard deviation so that higher weight is given to the peaks whose intensity is homogeneous within the same group. This algorithm shrinks the class/group centroids toward the overall centroid. The optimal amount of shrinkage is determined by cross-validation. This method can be used to perform feature selection and classification simultaneously. In this study, we used the feature selection functionality. Permutation testing was performed to determine the threshold for calling a peak significant based on random class assignments. By assuming that each of those test statistics is to occur equally likely, the original test statistic (without any permutation) is compared with the test statistics distribution and the significance probability is calculated. Hothorn *et al* (32)

described the detailed algorithm about permutation tests used in this study. The optimal peaks were analyzed by ROC (Receiving Operating Characteristics Curve) analysis. Principal component analysis was performed to visualize the separating power of the best peaks. All analyses were performed using the statistical package R and SPSS (SPSS, Inc., Chicago, IL).

Antibody-capture based confirmation of biomarker candidates' identities
The appropriate antibodies were coupled to Protein A Hyper D beads (BioSepra Inc., Marlboro, MA, USA) as follows: the antibody was diluted to 0.05 mg/ml in PBS. In a well of a 96 well 0.45 µm filter plate, a 50 µl aliquot of diluted antibody was mixed with 2 µl of Protein A Hyper D beads for 50 min at room temperature (RT). The beads were washed 3 times with 200 µl of PBS in the filter plate wells by means of a vacuum manifold. The antibody-coupled beads were then used to specifically capture proteins from samples: 10 µl of pleural effusion was diluted with 40 µl of PBS, added to the beads, and incubated for 30 min on a microtiter plate shaker (form 21, amplitude 7) (MicroMix5, Diagnostic Products Corporation, Gwynedd, UK). After the incubation step the beads were washed three times with 200 µl of PBS, two times with 200 µl (50 mM Tris, pH 7.5, 1 M urea, 0.2% CHAPS, 0.5 M NaCl), three times with 200 µl of PBS and finally once with 200 µl of 5 mM Hepes pH 7.4. Finally the proteins were eluted with 30 µl of 0.1 M acetic acid. Per sample a volume of 3 µl eluate fraction was profiled on NP20 ProteinChip arrays by incubation on-spot in a total volume of 10 ul (water added until final volume) for 30 min in a humid chamber, followed by two on-spot water washes, an air-drying step and two consecutive applications of 1 µl sinapinic acid (dissolved in a 400 µl volume of 50% acetonitrile, 0.5% TFA). To ascertain that the captured protein indeed is Apo CI the outcome of the eluate fraction analyses was compared to corresponding volumes of the depleted pleural effusion samples and of the non-depleted effusion samples when analyzed on the NP20 surface type. Five (out of fifty) µl volumes of the depleted fraction and two (out of ten) µl fractions of the non-depleted (original) effusion volume were analyzed in comparison to 3 out of 30 µl of the captured target.

Enzyme Immunoassays
Unless noted otherwise, all immunoassays were carried out following manufacturer's recommendations. Pleural fluid samples were thawed at RT and mixed by vortexing prior to use in the assays. SMRP levels in pleural effusions were determined using the MESOMARK assay from Fujirebio Diagnostics (Malvern, PA, USA). This sandwich ELISA uses monoclonal antibodies OV569 and 4H3 to quantitate SMRP in body fluid samples. The assay was carried out following manufacturers' instructions. Briefly, samples were serially diluted 1:2020 and applied to precoated microtiter plates. After incubation for 1 hr at RT, plates were washed and subsequently incubated with OV569-reactive antibody conjugated to horseradish peroxidase (HRP). Plates were developed using TMB substrate and the reaction was stopped by adding 1% hydrochloric acid followed by reading the absorbance values at 450 nm. Osteopontin (OPN) was measured using a sandwich ELISA kit from IBL (Japan) following manufacturer's instructions. Briefly, pleural fluid samples were serially diluted 1:11 to 1:264 in supplied buffer and applied onto the pre-coated microtiter plate for 1 hr at 37ºC. After 6x350 µl wash steps the detection antibody conjugated to HRP was applied for 30min followed by another wash step. The assay was developed with TMB substrate included in the kit and OD absorbance was measured at 450nm. HE4 was measured by sandwich ELISA using a RUO kit from Fujirebio Diagnostics. Plates were coated overnight at 4ºC followed by a block step at 4ºC overnight or until use. The coated plates were aspirated and samples (diluted 1:220) were applied for 1 hr at room temperature. After a 3x350 µl wash step, HRP-conjugated detection antibody was applied for 1 hr at roomtemperature. The assay was developed with TMB substrate included in the kit and OD absorbance was measured at 450nm. CYFRA 21-1 was measured using the RIA from Fujirebio Diagnostics following manufacturer's recommendation. Samples were applied undiluted or serially diluted to 1:50 if analyte concentrations were beyond the assay range. Antibody coated beads were added followed by radiolabeled detection antibody and the assay was incubated at 4ºC for 20 hrs. After a wash step, beads were measured in a gamma-counter and compared with a calibrator curve.

Statistical analysis
Data are expressed as mean±SD. Comparisons between groups were made using the one-way Anova and Mann-Whitney U-test for independent samples. Data were log-transformed before analysis. A two-tailed p-value<0.05 was considered significant. Statistical analysis were performed using the statistical package R and SPSS (SPSS, Inc., Chicago, IL, USA).

3. RESULTS

A total of 89 patients were recruited, of whom 41 with mesothelioma and 48 age-matched patients with effusions due to other causes (non-infective inflammatory exudates (n=3), malignancies with pleural involvement (metastasis of distant carcinoma [28], non-small cell lung carcinoma [6], sarcoma [3], lymphoma [2], and melanoma [1]), transudates (n=3), a still unknown cause (n=2) (Table 1). From some patients, effusions were taken at multiple time-points. Irrespective of the cause, all effusions were collected, processed, and stored at –80°C in the same way as described in Methods. Supernatants of pleural effusions were subjected to several immunoassays to determine their use in addition to the pathologist's findings. Immunoassays to measure osteopontin (OPN), human epididymes protein 4 (HE4), cytokeratin 19 fragment (CYFRA 21-1), and the soluble mesothelin-related protein (SMRP) were used to quantitate their levels in all pleural effusion supernatants. Results from these immunoassays were logarithmically transformed and compared using the independent t-test and presented in a box-and-whisker plot showing the median, quartiles and outliers ("o" = outlier [> 1.5x interquartile range (IQR), "∗" = extreme [≥ 3x IQR]) (Figure 1a). The differences in expression levels of OPN and HE4 in mesothelioma versus non-mesothelioma samples were not statistically significant. CYFRA 21-1 and SMRP assays were able to differentiate the group of mesothelioma samples from non-mesothelioma pleural effusions ($p < 0.001$, t-test). The outlier in the CYFRA 21-1 assay was obtained from a mesothelioma patient that had underwent 4 times a thoracentesis, all samples displayed the same high content in CYFRA 21-1 (outlier in HE4 is the same sample).

ROC curve analysis for MM versus non-MM showed an AUC of 0.736 (0.630-0.841 [95% confidence interval]) and 0.860 (0.782-0.938) for CYFRA 21-1 and SMRP, respectively. The sensitivities and specificities were dependent of the clinical cut-off values. For example, a sensitivity of 83% and specificity of 74% was obtained with a SMRP value of 7.05 nM/L but using 14 nM/L the sensitivity and specificity altered into 73% and 80% (Figure 1b).

Supernatants of pleural effusion samples were then subjected to anion exchange chromatography, generating six fractions containing subsets of the effusions' protein contents. Each fraction was applied to three ProteinChip array types (IMAC30, H50, and CM10), resulting in 18 fraction-array combinations. Peaks detected by the Expression Difference Mapping module in Ciphergen-Express were analyzed using the software package Prediction Analysis of Microarrays (PAM) to determine peaks with the greatest between-class variance while minimizing within-class variance. The five peaks with the best discriminating power identified by

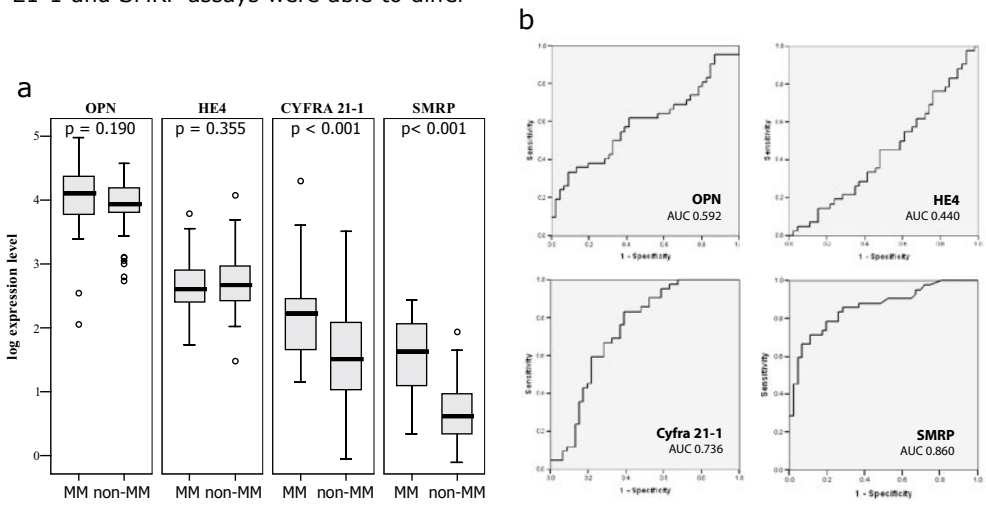


Figure 1: (a) Box-and-whisker plots showing the median and the quartiles of the four immunoassays for mesothelioma pleural effusions (MM) and other causes (non-MM). Using the independent samples t-test on logarithmic expression levels revealed a significant p value CYFRA 21-1 and SMRP. (b) Receiver operating characteristics (ROC) curves for OPN, HE4, CYFRA 21-1, and SMRP with areas under curve (AUC) values inside each graph.

Table 2: The best markers selected by PAM

m/z	Protein	Condition	AUC **	p value
6614	Apo CI	CM10 V1*	0.741	0.045
6626	Apo CI	H50 V1	0.755	0.001
6656	Apo CI (+48 Da)	CM10 V1	0.728	0.027
6821	Apo CI SPA adduct	CM10 V1	0.742	0.023
8799	Apo AII cysteinylated	H50 V6	0.688	0.001

* V = fraction, ** AUC = area under curve

Table 3: The statistics of the markers described in Table 2

m/z	Other causes		Mesothelioma	
	Mean	SD	Mean	SD
6614	165.5	84.5	111.7	75.1
6626	125.4	82.5	68.1	50.8
6656	77.3	40.3	51.4	31.9
6821	23.2	11.2	16.4	9.6
8799	15.4	7.0	10.9	8.4

PAM were at mass-to-charge ratios (m/z) 6614 (found on CM10, fraction 1), m/z 6626 (found on H50, fraction 1), m/z 6656 (found on CM10, fraction 1), m/z 6821 (found on CM10, fraction 1), and m/z 8799 (found on H50, fraction 6) (Table 2). Figure 2 displays the logarithmically transformed expression levels for each protein peak in box-and-whisker plots. Comparison between groups had P values < 0.005 for each protein. Table 3 shows for each of these peaks the mean and within-class variance in the mesothelioma subject group and the group suffering pleural effusions due to other causes.

ROC curve analysis showed an AUC of 0.741 (0.636 - 0.845 [95% confidence interval]) for protein peak 6614 Da and using a cut-off value of m/z 132, its sensitivity and specificity were found to be 76% and 69%, respectively (Figure 3).

Each of the five proteins found by SELDI analysis was down-regulated in the mesothelioma group. The proteins are highly correlated to each other, with correlation coefficients between 0.5 and 0.95. Based on previous experience with peaks at these m/z values eluting from these fractions and

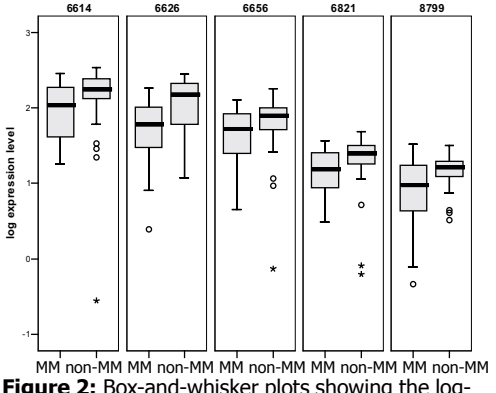


Figure 2: Box-and-whisker plots showing the logarithmically transformed expression levels of the SELDI detected protein peaks (p value of 0.045, 0.001, 0.027, 0.023 and 0.001 for m/z 6614, 6626, 6656, 6821, and 8799 respectively).

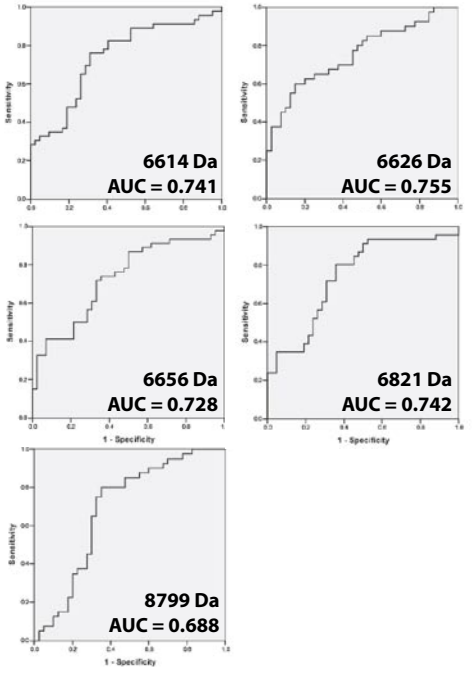


Figure 3: ROC curve for selected proteins.

binding to these arrays, we hypothesized that the first four peaks were Apo CI or adducts of Apo CI, and that the m/z 8799 peak was Apo AII.

To confirm the identity of the candidate markers, hypothesized as isoforms or adducts of Apo CI, we performed immunoprecipitation followed by analysis with mass spectrometry (immuno-MS). For this purpose negative control antibodies and anti-Apo CI antibodies were coupled to Protein A Hyper D beads and the antibodies' captured target antigens were eluted. Analyses of the eluates showing molecular weights (m/z values corresponding to the calibrator proteins' experimental molecular weights confirmed the observed markers' identities as Apo CI (Figure 4). The observed identity similarity between captured target and calibrator protein was further confirmed by the partial removal of the target antigen in the

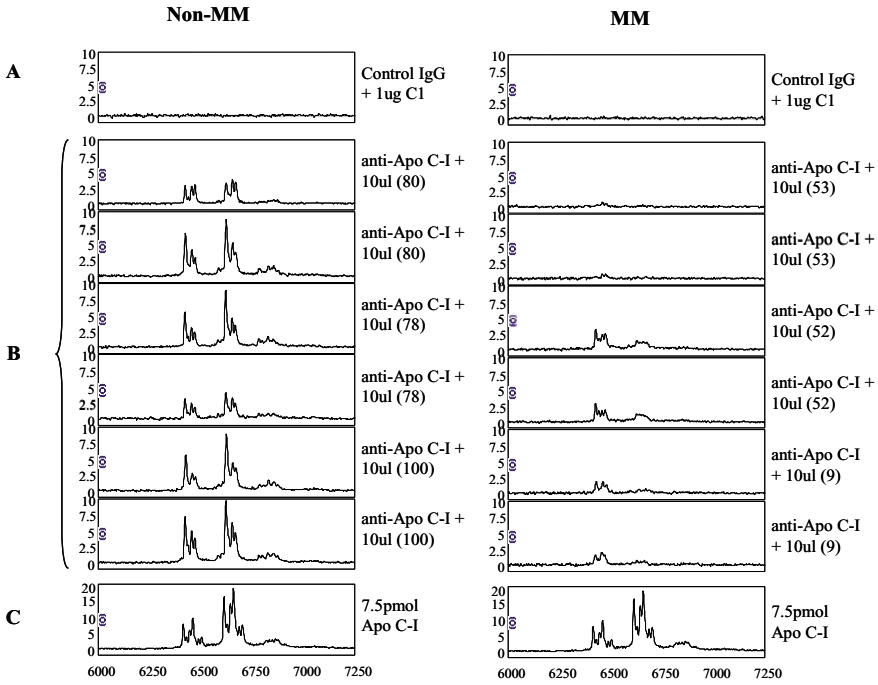


Figure 4: Apo CI isoforms expression levels in selected MM and non-MM pleural samples. (A) Negative control experiment in which 1 µg Apo CI was presented to Protein A Hyper D beads coupled with negative control antibody. (B) Eluate (3 out of 30 µl) after 10 µl of pleural effusion volumes for selected patient samples of MM (53, 52, 9) and non-MM (80, 78, 100) groups was incubated with Apo CI-coupled Protein A Hyper D beads. Replicate spectra represent independent technical duplicates of the whole process of capture, elution and analysis on NP20 surface type ProteinChip array. (C) Calibrator protein Apo CI (7.5 pmol) on NP20 ProteinChip array.

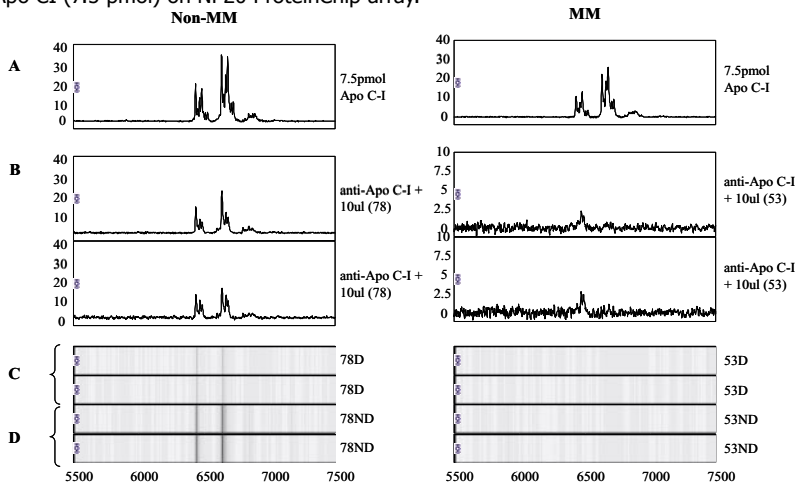
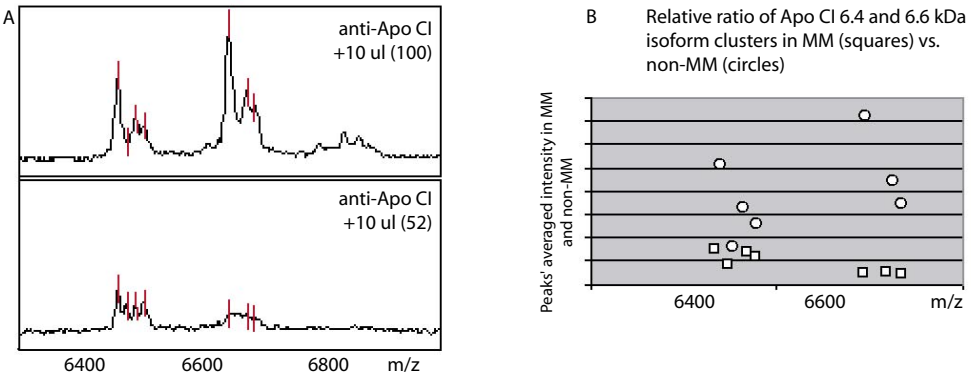


Figure 5: Captured Apo CI isoform molecular weights (m/z) correspond with decreased signal intensity after depleting incubations. In all spectra the same peaks as in the Apo CI calibrator protein sample are present, confirming the hypothesized identity of the 6.6 and 6.4 kDa peaks as Apo CI (A) SELDI-TOF spectrum for calibrator protein Apo CI (7.5 pmol) applied on NP20 ProteinChip array. (B) Representative SELDI-TOF spectra of eluates of MM patient (53) and non-MM patient (78) after bead based Apo CI antibody capture. (C) SELDI-TOF analysis of (Apo CI)-depleted samples (only 5 µl out of 50 µl final capture volume analyzed, corresponding with 3 µl out of 30 µl eluate analyzed) on CM10 ProteinChip array surface at pH 4.0. (D) SELDI-TOF analysis of non-depleted pleural effusion samples for MM and non-MM representative sample on CM10 ProteinChip array, pH 4.0.



C

m/z	6422.98	6437.54	6454.74	6470.62	6620.16	6651.93	6667.81
non-MM	5.07	1.61	3.25	2.61	7.13	4.41	3.47
MM	1.54	0.93	1.50	1.36	0.47	0.55	0.59
ratio	***3.30***	***1.73***	***2.17***	***1.92***	***15.17***	***8.02***	***5.88***

Figure 6: Relative abundance of different Apo CI isoforms in pleural effusions is dependent on sample being part of MM or non-MM groups. Relative ratio of the non-MM/MM average intensity values in the 6.4 kDa cluster peaks are smaller than in the 6.6 kDa cluster. (A) Representative images of the 6.4, 6.6 and barely detectable 6.8 kDa clusters in the spectra of a MM (52) and non-MM (100) patient pleural effusion sample. (B) Graph plotting the average intensity for the separate detectable peaks in the 6.4 and 6.6 kDa clusters of a selected MM and non-MM sample. (C) Table reflecting the selected MM and non-MM group samples' actual average values and the corresponding non-MM/MM ratios for the separate peaks.

depleted pleural effusions and the presence of the target antigen in the original non-depleted sample (Figure 5). In addition to the expected molecular weight for both the calibrator protein and the observed marker candidate, the calibrator protein and the capture experiments also revealed a common quadruplet peak cluster around 6.4 kDa for the Apo CI target. The 6.6 kDa and 6.4 kDa peak clusters captured do represent Apo CI and the postulated Apo CI form lacking the aminoterminal Thr-Pro sequence. The 6.8 kDa Apo CI SPA adduct was not visible in these immuno-MS spectra. In contrast to obvious expression level differences for the Apo CI isoforms in

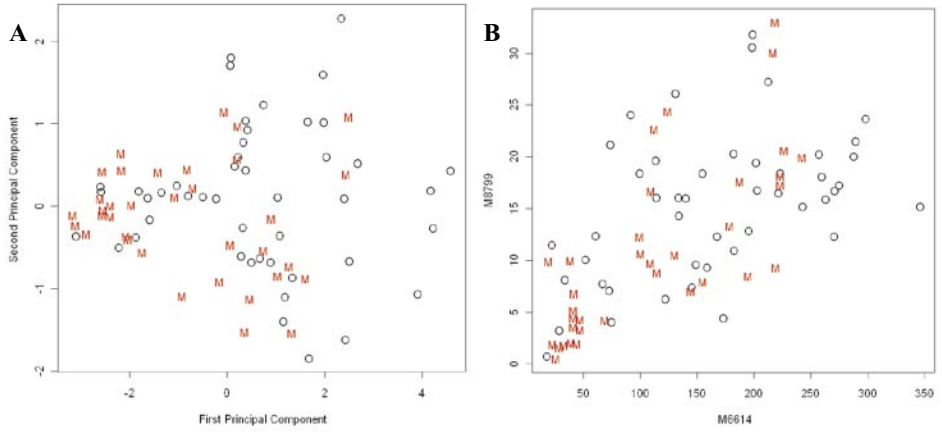


Figure 7: Multivariate and bivariate model performance. (A) Principal Component Analysis with all discovered expression level differences as listed in Table 4. Two-dimensional projection of the dominant vectors. (B) Scatter plot of samples' signal intensities of Apo CI (m/z 6614) vs Apo AII (m/z 8799).

the 6.6 kDa cluster between the MM and non-MM group the expression level difference for the peaks in the 6.4 kDa cluster are less pronounced considering selected patients with high (non-MM patients 80, 78 and 100) and low (MM patients 53, 52 and 9) extremes for the Apo CI 6.6 kDa cluster peaks (Figure 6).
Figure 7A shows the separating power of the two main contributing vectors in a principal component analysis and figure 7B shows scatter plots for the m/z 6614 peak and the m/z 8799 peak, of the Apo CI and Apo AII proteins respectively.
Patients with low levels of both the Apo CI and Apo AII protein signals were exceedingly likely to have MM versus other conditions (non-MM). However, because of the high correlation in amount of the two proteins, it was unfeasible to construct multivariable models encompassing both proteins or any other protein features in the generated SELDI-TOF spectra, that had significantly better classifying ability than either of the two proteins alone.

4. DISCUSSION

As the presence of pleural effusion may be indicative for MM, removal of fluid or a biopsy is normally investigated to confirm the presence and type of cancerous cells. However, conclusive diagnosis of MM as such or as discrepant from other cancer forms with similar histological characteristics has proven difficult in the past since exclusive immunostains have been lacking. Therefore, research into novel biomarkers for MM has been performed for several years to yield information that may contain potential to a correct and early diagnosis in the near future.
In addition to the proteins present in serum and pleural effusion of MM patients reported on in literature (25-27), we have pursued to investigate the presence of alternative proteins in pleural effusions collected during thoracenteses. Pleural fluid is present in large volumes in cases of pleural pathologies; in addition to alleviating the patient's discomfort, it can also provide a valuable matrix for measurements of molecules reflecting the subject's condition or a specific systemic response.
By means of SELDI-TOF MS we investigated pleural effusion samples (n=108) of both pleural mesothelioma diagnosed subjects (n=41, from which few had undergone more than 1 thoracentese, leading to a total of 54 samples) and of subjects with other malignancies, lymphatic abnormalities, and transudative pleural effusions correlated with diagnosed renal failures and a pulmonary embolism.
For several of the SELDI-TOF MS spectral features (6614 Da, 6656 Da and 6821 Da in pleural effusion fractions 1 applied to CM10 surface type; 6626 Da in effusion fraction 1 and the 8799 Da feature in effusion fraction 6 on the H50 surface type) the average intensities in the MM group showed to be decreased significantly in comparison to the pleural effusions correlated with other causes (Table 2 & 3).
Experience from earlier protein identification work for similar spectral features in serum (combinations of molecular weight labels and ProteinChip array surface type) suggested that the molecules were Apolipoprotein (Apo) CI and Apo AII isoforms. Confirmation of that hypothesized identity was obtained by means of SELDI-based antibody capture approaches using an anti-Apo CI antibody method. Apo CI was successfully captured from pleural effusions from selected subjects (n=6) of both the pleural mesothelioma (n=3) and other cause (n=3) exudative effusion groups. The additionally captured 6.4 kDa peak is postulated to be Apo CI lacking the amino-terminal Thr-Pro dipeptide. A similar Apo CI peptide was also observed and identified in serum samples by means of SELDI-TOF MS (33); Rossi et al postulated this form to be Des Thr-Val Apo CI, although the second amino acid of Apo CI (SwissProt # P02654) is Proline. We also know about data for a similar peptide with the same molecular weight from actual MS/MS sequencing experiments that has shown to contain Pro (personal communications) as the second amino acid rather than the Val residue suggested by Rossi et al (33). Further investigation would be required to understand the origin of the Des Thr-Pro Apo CI form: it could either be due to exoproteolytic digestion of the full-length 6.6 kDa Apo CI during the sample handling or alternatively could be caused by cleavage of the signal sequence prior polypeptide secretion. The latter explanation might be acceptable: this 6.4 kDa form is also present in the capture of the purified calibrator protein dilution in PBS, where no exoprotease activity is added as part of a lysate. In addition: there is also a significant difference of the 6.4 kDa to 6.6 kDa peak ratio in the MM vs. non-MM; where the MM effusions show mainly the 6.4 kDa Des Thr-Pro peak, from the non-MM pleural effusions a similar amount of both the 6.4 kDa N-terminally cut form and the intact 6.6 kDa peptide are captured in an equal ratio.
There are some limitations to our approach. First, experiments have been performed

on almost equal group sizes of MM and non-MM samples, but this way of clinical screening does not reflect the normal situation. Second, protein profiles were analyzed in a training set where classification was known ahead of time, and third, the reproducibility in different laboratories and standardization needs to be addressed. We did not intend to provide a complete coverage of the whole proteome in human pleural effusions. The sets of proteins we found in this study were not known as cancer involved, and expected proteins such as SMRP were not detected, indicating that this affinity-based biomarker discovery platform gives a bias towards certain proteins. Because variations in instrument and sample preparation were limited in our single center study makes the picked up apolipoproteins valid for separating MM from non-MM due to biology and not to artifacts of differential processing and study design. Validation of the apolipoprotein findings, developing an immunoassay and testing it in a randomized prospective study will have to demonstrate the use of apolipoproteins in the diagnosis of MM in the future.

Aiming at trying to understand the physiological relevance for the observed decreased Apo CI expression level in pleural effusions upon mesothelial malignancies two potential hypotheses could be postulated. First, one can consider Apo CI's described role in the lipid metabolism as an inhibitor of cholesteryl ester transfer protein (CETP (34)) and of phospholipase A2 (PLA2 (35). The observed decreased expression level of the Apo CI forms might reduce inhibition on the CETP and/or PLA2 enzymes, leading to an increased lipid metabolism as a response to disease-induced lipidemia in the pleural effusions (36). A second hypothesized effect for the Apo CI expression level change effect upon pleural MM differentiation could be recruitment of Apo CI to malignant cells (and thus decreased pleural effusion levels) inducing apoptosis of the malignant cells. Apo CI stimulates Neutral Sphingomyelinase, inducing increased synthesis of ceramide eventually leading to apoptosis initiation, as shown for aortic smooth muscle cells (ASMC) (37) and in breast cancer cells (38). In contrast, Apo B, AI, AII and E have been described not to alter apoptosis in ASMC (37, 39).

The Apo CI forms in pleural effusions perform reasonably as single biomarkers for separation of pleural mesothelioma subjects from subjects with other pleural affections: their performance is characterized by areas under ROC curves (AUC) between 0.688 and 0.755, with a sensitivity of 76% and specificity of 69%. As single markers Apo CI's isoforms may currently not provide improved prediction success for MM cases but might be considered as candidates in future multi-marker panels. Several publications have already reported on pathology situations where Apo CI was detected as significantly up or down regulated, thereby reflecting the disease status or the body's systemic responses against the aberrant disease processes. Apo CI has been reported as one of a set of five potential plasmatic markers to distinguish between ischemic and hemorrhagic stroke (40), and as one of the panel markers in postoperative serum samples with predictive power for metastatic relapse in high-risk primary breast cancer patients receiving adjuvant chemotherapy (41). Therefore, in the pleural mesothelioma context, it could be worthwhile to engage in a prospective follow-up study to determine the expression levels for selected Apo CI isoforms and other potential diagnostic markers in body material samples of subjects with pleural MM versus other effusion causes. Upon removal of the effusion fluid from the subject's pleural cavity multiple analyses could be performed to generate data in a complementary way.

An initial work plan for all pleural effusions could consist of immunocytological staining of the effusion's cellular components with a panel of immunohistochemical stains for MM (e.g. calretinin, cytokeratin 5/6, D2-40, epithelial membrane antigen (EMA), HBME-1, mesothelin, N-cadherin, thrombomodulin, vimentin, or Wilms' tumor 1 (WT1) protein, and immunostains for adenocarcinoma (e.g. B72.3, BerEP4, carcinoembryonic antigen (CEA), E-cadherin, CD15, CD138 (syndecan-1), MOC-31 and thyroid transcription factor-1 (TTF-1))(21, 42-46). DNA ploidy analysis on the cells in the pleural fluid may have special value in the differential diagnosis of benign reactive mesothelial cells versus MM (47-49). Given the fact these techniques are of limited value and that the obtained cell material is often inadequate for proper diagnosis (23, 50), it is important not to render a diagnosis of MM solely on the basis of cytology specimens. The pathologist could consider going down the route of further confirmation of potential mesothelioma diagnosis on determination of soluble forms of certain proteins in serum or pleural effusion fluids. The next steps towards positive diagnosis of pleural malignant mesothelioma could comprise the combined analyses of Apo CI isoforms and other commercially available immunoasays in the fluid part of the pleural effusion.

Therefore, we measured the expression of human epididymes protein 4 (HE4), human

osteopontin (OPN), soluble mesothelin-related proteins (SMRP), and the cytokeratin 19 fragment (CYFRA 21-1) in all pleural effusion samples. We and others have shown that OPN and HE4 (WFDC2) can not be used to differentiate MM from other cancers. Others have also described the overexpression of HE4 and OPN in mesothelioma compared to benign lung conditions (25, 51) but also in other malignancies (52-61). Initially OPN's mRNA level has been described as increased in tumorous tissue vs. non-tumorous tissue of a rat model system upon asbestos-induced carcinogenesis (62). The parallel increased mRNA transcription levels for the signaling phosphoprotein zyxin and integrin-linked protein kinase (62) suggest a link with disturbance of integrin β1 cell signaling systems (63), leading to anchorage-independent growth of the epithelial cells in the pleural cavity (64).

It is interesting to bring together the independent observations of the teams Sandhu et al. (62) and Huang et al. (36) to understand the biological relevance of the osteopontin upregulations in MM. The findings of Sandhu and co-workers on increased transcription levels could be related to the wounding by asbestos fibers and consecutive scarring in the lung and pleural tissues (62). In rats, wound healing consecutive to tissue damage involves cell signaling through integrins in general (65-67) and through integrin α9β1 in specific (68, 69). In a mouse model system the knock-out of the α9 chain of the integrin α9β1 did lead to mouse death between the 6th and 12th day after birth due to respiratory failure. Death was caused by accumulation of large volumes of pleural fluid (36), a phenotype also very common and characteristic for many of the pleural MM cases. A distant view at the interference results in these two animal model systems suggests involvement of the β1 integrin cell signaling routes upon (asbestos-induced) tissue damage in the pleural cavity and surroundings. Thus as a known interaction partner in this integrin pathway, OPN, could be a good candidate monitoring marker for aberrant signaling in this pathway. OPN so far has been described as a marker protein positively characterizing pleural MM in immunohistochemical stainings on the cellular components of pleural effusions and in a free form in the serum of mesothelioma patients (25). This study showed that OPN does not provide a useful parameter for the differential diagnosis between between MM and other malignancies in contrast to CYFRA 21-1 (AUC in the ROC curve of 0.736).

Mesothelin is a cell surface protein present on a restricted set of normal adult mesothelial tissue cells lining the body cavities, but is aberrantly expressed by several tumor types (ovarian epithelial, serous papillary ovarian cancers, pancreatic adenocarcinomas, endometrioid uterine adenocarcinomas, epithelioid MM, and squamous cell carcinomas of the esophagus, lung and cervix)(70-76). Mesothelin and the corresponding soluble form SMRP are released from a 69 kD precursor protein that also gives rise to megakaryocyte potentiating factor (MPF) (77-79). Soluble members of the mesothelin/megakaryocyte potentiating factor family are detectable in sera from patients with ovarian carcinoma (80). The biologic functions of mesothelin and SMRP remain speculative, but pleural SMRP levels are significantly higher in mesothelioma in comparison to benign lesions and pleural metastases (27). Recently, a diagnostic commercial kit is available for SMRP in the serum for the diagnosis of epithelioid MM (MESOMARK; Fujirebio Diagnostics, Inc., Malvern, PA, USA). However, the caveat that the serum levels measured might only partially mirror the SMRP levels in the pleural cavity is corroborated by Scherpereel and co-workers' results showing that the levels of SMRP are typically higher in the pleural fluids in comparison to the corresponding serum samples (29), and that therefore the discrepancy between the AUC for serum SMRP (AUC = 0.693) differentiating between mesothelioma and pleural metastasis is lower than based on the pleural SMRP values (AUC = 0.793).

In this study ROC curve analysis for MM versus non-MM showed an AUC of 0.860 (with a sensitivity of 83% and specificity of 74% for SMRP value of 7.05 nM/L) demonstrating the usefulness of SMRP as diagnostic marker. Serum SMRP levels have been described as tumor-size related and to decrease upon surgical cytoreduction interventions (26, 27, 81, 82). This leads to the suggestion that analysis of SMRP in the pleural effusion fluid might be preferable for positive primary diagnosis while the serum SMRP values might contain an opportunity as a treatment monitoring tool in a follow-up population.

We have found that the expression level of Apo CI is decreased significantly in pleural effusions of subjects diagnosed with malignant mesothelioma when comparing with subjects with other pleural cavity affections. As a future application we suggest the potential of Apo CI as a candidate biomarker for inclusion as one of the variables in a positive decision tool for pleural mesothelioma diagnosis in individual effusion cases, next to cytology and SMRP analysis for this pathology.

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Chapter 7

Proteomic analysis of exosomes isolated from human malignant pleural effusions

American Journal of Respiratory Cell and Molecular Biology 2004; 31:114-121

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Proteomic Analysis of Exosomes Isolated from Human Malignant Pleural Effusions

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Exosomes are membrane vesicles from endosomal origin secreted by various cells such as hematopoietic, epithelial, and tumor cells. Exosomes secreted by tumor cells contain specific antigens potentially useful for immunotherapeutic purposes. Our aim was to determine if exosomes are present in human cancerous pleural effusions and to identify their proteomic content. Exosomes were purified by sucrose gradient ultracentrifugation, and electron microscopy was used to check both concentration and purity of exosomes. Proteins were separated by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and protein bands were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and Western blotting. Exosomes were present in pleural fluid obtained from patients suffering from mesothelioma (n = 4), lung cancer (n = 2), breast cancer (n = 2), and ovarian cancer (n = 1). As previously reported by others, antigen-presenting molecules, cytoskeletal proteins, and signal transduction–involved proteins were present. Proteins not previously reported were identified (SNX25, BTG1, PEDF, thrombospondin 2). Different types of immunoglobulins and complement factors were abundantly present in the sucrose fractions containing exosomes. Exosome-directed specificity of these immunoglobulins was not observed. In conclusion, sucrose gradient ultracentrifugation allows isolation of exosomes from malignant pleural effusions. However, pleural fluid proteins and especially immunoglobulins are coisolated and may hamper the use of exosomes isolated from malignant effusion for immunotherapy programs.

Exosomes are membrane vesicles from endosomal origin that are secreted by various cells, e.g., hematopoietic, epithelial, and tumor cells. The function of exosomes is largely unknown, but some studies have shed some light on the effect of exosomes on immunity. Exosomes produced by intestinal epithelial cells have the ability to induce an antigen-specific tolerance (1). These "tolerosomes," which carry MHC class II molecules may be used by epithelial cells to induce tolerance to food antigens. In contrast, a tumor mice model study has demonstrated that exosomes secreted by tumor peptide-pulsed dendritic cells (DCs) have the ability to induce a specific anti-tumor response (2). The presence of tumor antigens on tumor-derived exosomes and their ability to be taken up by DCs has been demonstrated in an *in vitro* human study (3). These studies have demonstrated that exosomes from tumor cells and DCs can be implemented into cancer immunotherapy programs (4, 5). Little information is available on *in vivo* production and function of tumoral exosomes in humans. Recently, André and colleagues have reported the purification of exosomes from cancerous ascites fluid and demonstrated the induction of a specific anti-tumoral response by autologous exosome-loaded DCs (6). This study confirms that exosomes are secreted in human malignant ascites effusion and demonstrated that exosomes have the capacity to transfer specific tumoral antigens to DCs and to induce a specific anti-tumoral response.

The protein composition of *in vitro* produced exosomes has been studied using Western blotting (2, 7), flow cytometry of exosomes-coated beads (8), and mass spectrometry (9–12). These studies have demonstrated that exosomes from different cellular origins share common groups of proteins (13). These groups are (*i*) proteins involved in antigen binding and presentation such as heat-shock proteins, MHC class I, and II proteins; (*ii*) proteins involved in intracellular membrane fusion and transport such as annexins and rab proteins; (*iii*) proteins involved in targeting and cell adhesion such as tetraspanins and integrin proteins; (*iv*) cytoskeletal proteins such as actin and tubulin; and (*v*) metabolic enzymes.

These previous studies suggest that exosomes express a limited set of proteins and can be used as an antigen source for immunotherapeutic purpose especially in cancer therapy. The aim of our study was to determine if exosomes are present in pleural effusions of patients suffering from different cancer types involving the pleura and to analyze their protein composition.

Materials and Methods

Patients

Patients with a histologically proven malignancy were asked to participate in the study. All patients were more than 18 yr old and signed informed consent. Indication for pleural effusion evacuation was in most cases exertional dyspnea relief. Clinical characteristics of the patients (n = 9) are summarized in Table 1. Primary tumor sites were mesothelioma (n = 4), non–small cell lung carcinoma (n = 2), adenocarcinoma of the breast (n = 2), and ovarian adenocarcinoma (n = 1). All patients with mesothelioma proved to be nonoperable due to mediastinal involvement or low performance status at presentation. A pleural involvement was the presenting symptom of the two patients with lung cancer and the patient with ovarian cancer. The two patients with breast cancer were initially treated with surgery and locoregional radiotherapy, 6 and 9 yr after diagnosis multiple metastases appeared in these two patients.

Pleural Fluid

The pleural fluid evacuation procedure was standard. After a local anesthesia (Lidocaine 1%), a metallic needle was introduced in the pleural cavity through an intercostal space. Pleural fluid was gently aspirated and used immediately for exosome isolation. All fluids were nonhemorrhagic and proved to be exudates. Biochemical characteristics of the pleural fluids are summarized in Table 2. High level of albumin, immunoglobulin, and complement were observed in pleural effusion (Table 2). The microscopical analysis after May-Giemsa staining showed that lymphocytes and tumor cells were the most abundant present cells. Neutrophils, eosinophils, and mesothelial cells were occasionally

(*Received in original form June 20, 2003 and in revised form January 30, 2004*)

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Abbreviations: B-cell translocation gene, BTG; dendritic cell, DC; electron microscopy, EM; immunoglobulin, Ig; heat shock protein, HSP; matrix-assisted laser desorption ionisation time-of-flight, MALDI-TOF; major histocompatibility complex, MHC; polyacrylamide gel electrophoresis, PAGE; pigment epithelium-derived factor, PEDF; sodium dodecyl sulfate, SDS; sorting nexin, SNX.

Am. J. Respir. Cell Mol. Biol. Vol. 31, pp. 114–121, 2004
Originally Published in Press as DOI: 10.1165/rcmb.2003-0238OC on February 19, 2004
Internet address: www.atsjournals.org

TABLE 1. Clinical characteristics of nine patients presenting cancerous pleural effusions from which exosomes were extracted

Patient No.	Age/Sex	Primary Tumor Site	Histologic Diagnosis	Stage	Therapy
1	59/F	Breast	Adenocarcinoma	Stage IV	Mammectomy + radiotherapy tamoxifen 5-FU-adriamycin-cyclofosfamide
2	58/F	Lung	Adenocarcinoma	Stage IIIb	Cisplatin-gemcitabin docetaxel
3	67/M	Pleura	Mesothelioma	Stage III (IMIG)	Best supportive care
4	61/M	Pleura	Mesothelioma	Stage II (IMIG)	Best supportive care
5	59/F	Breast	Adenocarcinoma	Stage IV	Tumorectomy + radiotherapy arimidex
6	64/F	Ovary	Adenocarcinoma	Stage IV	Debulking surgery carboplatin-paclitaxel
7	64/M	Pleura	Mesothelioma	Stage II (IMIG)	Best supportive care
8	64/M	Pleura	Mesothelioma	Stage III (IMIG)	Cisplatin-raltitrexed
9	65/F	Lung	Adenocarcinoma	Stage IV	Best supportive care

Definition of abbreviation: IMIG, International Mesothelioma Interest Group.

observed. In all cases, no infectious agent was observed and bacterial cultures were negative.

Sucrose Gradient Ultracentrifugation

Exosome isolation was performed as previously described (6). In brief, 200–400 ml of pleural fluid was sequentially centrifuged at 300 × *g* for 10 min, 2,000 × *g* for 20 min, and 10,000 × *g* for 30 min to pellet cells and debris. Exosomes were then pelleted at 64,000 × *g* for 90 min. Pellet was resuspended in 2 ml 0.32M sucrose containing 10 μl 5 μg/ml phospholipid analog fluorescein-DHPE (N-(fluorescein-5-thiocarbamoyl) 1,2-dihexadecanoyl-sn-glycero-3-phosphaethanolamine, triethylammonium salt; F-362: Molecular Probes, Leiden, The Netherlands). Fluorescein-DHPE was used to label the phospholipid bi-layers of both cell and exosome membranes. The fluorescein labeled pellet was layered on a continuous sucrose density gradient (0.32–2.5 M sucrose, 20 mM HEPES, pH 7.2) and centrifuged overnight at 100,000 × *g* (SW41 rotor; Beckman Instruments [Fullerton, CA]). Two hundred–microliter fractions were collected from the top (low density) to the bottom of the tube (high density) of the sucrose gradient. For each fraction, fluorescence was measured in a fluorescence microplate reader (Bio-Rad Benchmark, Hemel Hempstead, UK; emission 519 nm, excitation at 495 nm). Fluorescent fractions from the gradient were harvested, diluted in phosphate-buffered saline, and centrifuged at 100,000 × *g* for 60 min (SW60 rotor; Beckman Instruments). Pellets were resuspended in phosphate-buffered saline, aliquoted and stored at −80°C. The final quantification of exosomal proteins was measured by CBQCA kit according to the manufacturer's recommendations (Molecular Probes).

Electron Microscopy

Fluorescent fractions were thawed and incubated on formar-coated grids for 15 min. After three washes with milli-Q (Millipore Corp, Etten-Leur, The Netherlands) water for 2 min each, samples were negatively stained with uranyl acetate and examined with a Philips CM 100 electron microscope (EM) at 80 kV (Philips Industries, Eindhoven, The Netherlands). Exosomes were defined as round shaped membrane vesicles rather homogenous in size not exceeding 100–150 nm in diameter (13). Membrane debris were defined as inhomogeneous membrane fragments variable in shape and size > 150 nm.

Indirect immunogold labeling of exosomes was performed with a goat anti-human immunoglobulin Fc antibody coupled to 10-nm gold particles (Aurion, Wageningen, The Netherlands).

One-Dimensional Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

One-dimensional electrophoresis of sucrose gradient fractions was performed under reducing conditions on 7.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) according to manufacturer's recommendations (PROTEAN II xi Cell; Bio-Rad Laboratories, Hemel Hempstead, UK). Samples were resuspended in 8 M urea (Sigma-Aldrich Chemie BV, Zurijndrecht, The Netherlands), 2% CHAPS (Amersham Pharmacia Biotech, Essex, UK) 20 mM dithiothreitol (DTT, Sigma-Aldrich Chemie BV), 0.01% bromophenol blue (Sigma-Aldrich Chemie BV), and transferred onto a 1.0-mm thick 7.5% SDS-PAGE gel. A constant voltage of 200 V at 10°C was applied. After 16 h, gels were stained with Novex Colloidal blue staining kit according to the manufacturers instructions (Invitrogen, Breda, The Netherlands).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Analysis

Colloidal-stained protein bands were excised manually with a plastic plunger and transferred to a 96-well low protein binding microtiter plate (Nunc, Life Technologies). Each excised spot was washed with 100 μl water for 5 min with shaking (650 rpm, Eppendorf shaker). Gel plugs were destained by incubating with shaking two times for 20 min with 0.4% (wt/vol) ammonium hydrogen carbonate, 30% acetonitrile in water at room temperature. After a short wash with water, gel spots were dried in a SpeedVac rotary evaporator (Savant, Farmingdale, NY) for 30 min. Digestion was performed by the addition of 4 μl trypsin

TABLE 2. Biological and cytologic characteristics of nine cancerous pleural effusions

Patient No.	Prot/Alb (*g/liter*)	LDH (*U/liter*)	pH	Gluc (*mmol/liter*)	IgG/IgM (*g/liter*)	C1q/C4 (*g/liter*)	Tum	Lym	Eos	Neu	Meso
1	52/23	ND	8.0	6.2	5.9/0.4	0.11/0.18	+	+	+	0	0
2	47/31	825	8.0	17	7.9/1.9	0.09/0.15	+	+	0	0	+
3	36/19	1,984	8.0	0.5	5.9/0.5	0.11/0.14	0	+	0	0	+
4	37/20	202	8.0	4.5	ND	ND	ND	ND	ND	ND	ND
5	40/20	175	7.7	8.7	5.1/0.3	0.07/0.09	+	+	0	0	+
6	57/31	443	7.4	5.0	8.9/0.3	0.12/0.15	+	+	0	+	0
7	36/22	1,008	7.4	5.3	4.0/0.3	0.09/0.12	+	+	0	+	+
8	49/26	403	7.4	6.5	5.1/0.8	0.08/0.12	+	+	0	0	+
9	45/24	2,396	ND	1.5	4.0/0.7	0.12/0.13	+	+	0	0	0

All fluids were exudates with high immunoglobulin and complement concentrations. Lymphocytes and tumor cells were abundantly present.
Definition of abbreviations: +, present; 0, absent; Eos, eosinophil; Lym, lymphocyte; Meso, mesothelial cell; ND, not done; Neu, neutrophil; Tum, tumor cell.

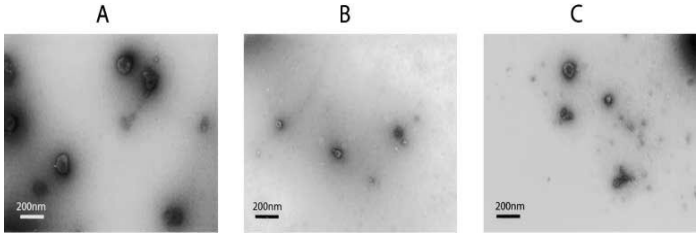

Figure 1. Electron micrograph of exosome containing gradient fractions isolated from pleural fluid in patients suffering from breast cancer (Patient 1, *A*), lung cancer (Patient 2, *B*), and mesothelioma (Patient 3, *C*), respectively. Exosomes were negatively stained with uranyl acetate and examined at 80 kV. Exosomes were defined as round shaped membrane vesicles rather homogenous in size not exceeding 100–150 nm in diameter.

(Promega, Madison, WI) to each gel piece. The plates were sealed with an adhesive film and incubated at room temperature overnight. After the tryptic hydrolysis of the different proteins, 7 μl (1:2) acetonitrile (0.1%) trifluroacetic acid was added to the gel plugs. After mixing, 0.5 μl of the tryptic digest was taken and mixed with 2.5 μl 2 mg/ml α-cyano-4-hydroxy-trans-cinnamic acid (ACCA; Bruker Daltonics, Billerica, MA) in acetonitrile. From this sample-matrix solution 0.5 μl was pipetted onto a 400-μm 384-well anchorchip matrix-assisted laser desorption ionization (MALDI) plate (Bruker Daltonics, Bremen, Germany) and air-dried for 5 min. Mass spectra were acquired on a Biflex III (Bruker Daltonics) MALDI–time-of-flight (TOF) mass spectrometer equipped with a 337-nm nitrogen laser. A mass list of peptides was obtained for each protein digest with X-tof software and submitted to Matrix Science Mascot (London, UK) software using the most recent MSDB databank of the NCBI to identify the proteins.

Western Blot Analysis

For Western blotting following one-dimensional SDS-PAGE, proteins were electroblotted onto Immobilon P membranes (Millipore Corp.) and incubated with specific antibodies, followed by horseradish peroxidase–conjugated secondary antibodies, and detected using SuperSignal West Pico chemiluminescent substrate (Pierce Perbio Science, Etten-Leur, The Netherlands). Antibodies used in this study to confirm the proteins detected by MALDI-TOF were: anti–HLA-DR/DP/DQ (clone 3/43; DAKO, Glostrup, Denmark), anti-HSP90 (clone AC88; Stressgen, Victoria, BC, Canada), and anti-immunoglobulin G, M, A, and E (IgG-HRP, IgM-HRP, IgA-HRP, IgE-HRP; all Zymed, San Francisco, CA).

Results

Sucrose Gradient Ultracentrifugation

After overnight centrifugation of fluorescent-labeled pleural sample, various yellow-white layers appeared in the sucrose gradient. In all patients, electron microscopic analysis of these fluorescent fractions confirmed the presence of round shaped homogeneous membrane vesicles which fulfilled the exosome definition. As illustrated in Figure 1, some variations in both shape and diameter of exosomes could be observed between the patients. For the same patient, the concentration of exosomes could vary between different fluorescent fractions and were occasionally mixed with cell membrane fragments (data not shown).

One-Dimensional Gel Separation

Proteins from fractions containing only exosomes (without membrane fragments) were separated under reducing condition on 7.5% SDS-PAGE. For all patients, it appeared that the patterns of bands on the SDS gel were grossly similar (Figure 2).

In three representative patients (Patients 1, 2, and 3)—suffering from breast cancer, lung cancer, and mesothelioma, respectively—one fraction containing no exosomes (no), one fraction containing a mix of exosomes and cell membrane fragments (mix), and one fraction containing only exosomes (ex) were separated on 7.5% SDS-PAGE and stained by colloidal blue (Figure 3). For each three patients, common bands were observed in fractions with or without exosomes.

MALDI-TOF Analysis

According to the common band pattern observed on the 1D gel for all patients, the MALDI-TOF analysis was performed in three representative patients (Patients 1, 2, and 3) suffering from breast cancer, lung cancer, and mesothelioma, respectively. This MALDI-TOF analysis was performed for fractions of the sucrose gradient containing only exosomes without membrane fragments (Figure 4). The results of this analysis are summarized in Tables 3, 4, and 5 for Patients 1, 2, and 3, respectively. Both protein names and accession numbers are related to MSDB databank. The calculated molecular mass was deduced from the mass fingerprint analysis, the apparent molecular mass was measured on the SDS-PAGE gels using molecular weight standard proteins. A facultative modification during the peptide mass fingerprint analysis was the presence of oxidative methionine. The number of peptides matched and the coverage percentage were deduced from the comparison between the identified peptides and the whole primary protein structure. The last column corresponded to the score given by Matrix Science Mascot UK software analysis, which was significant ($P < 0.05$) when higher than 61. For each protein digest, all significant results were given, and in case of a not significant score only the top-scored protein was indicated.

Surprisingly, an important group of identified proteins was composed of peptides originating from immunoglobulin (Ig) light and heavy chains (IgM, IgG1, IgG3 heavy chains, and Ig kappa light chain) and from various complement factors (C1q, C1r, C4a, H). These proteins corresponded to the large and most intense bands and could be detected in all patients. These

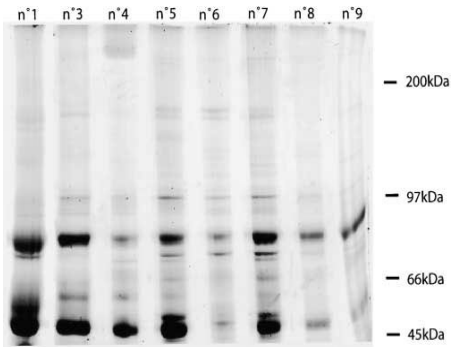

Figure 2. Exosomes were isolated from pleural fluid of nine patients suffering from various types of cancer (Table 1). After sucrose gradient ultracentrifugation and EM examination, gradient fractions containing only exosome (without membrane fragment) were separated on 7.5% SDS-PAGE and stained by colloidal blue. For all patients the same protein pattern was observed.

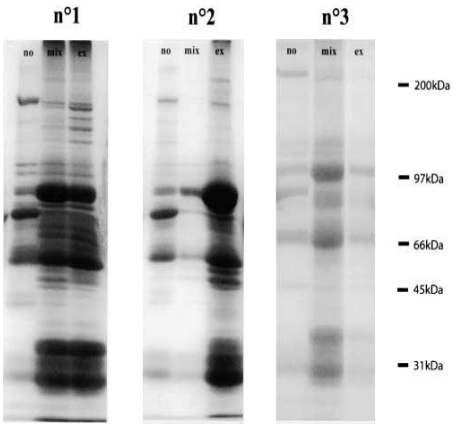

Figure 3. Sucrose gradient separation of exosome from pleural fluid in patients suffering respectively from breast cancer (Patient 1), lung cancer (Patient 2), and mesothelioma (Patient 3). After sucrose gradient ultracentrifugation and EM examination one fraction containing no exosome (no), one fraction containing a mix of exosomes and cell membrane fragments (mix), and one fraction containing only exosomes (ex) were separated on 7.5% SDS-PAGE and stained by colloidal blue. Some common bands were observed between fractions with or without exosomes. These common bands corresponded to immunoglobulin and complement proteins.

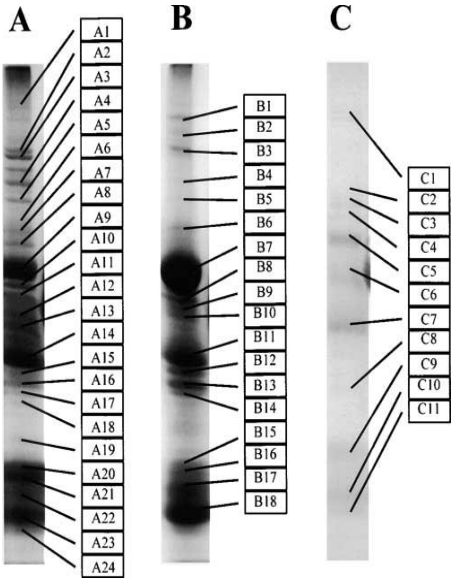

Figure 4. A proteomic analysis of exosome containing fractions was performed in patients suffering from breast cancer (Patient 1, *A*), lung cancer (Patient 2, *B*), and mesothelioma (Patient 3, *C*). After sucrose gradient ultracentrifugation and EM examination, gradient fractions containing only exosome (without membrane fragment) were separated on 7.5% SDS-PAGE and stained by colloidal blue (*A*, *B*, and *C*). The fractions that were subsequently analyzed by MALDI-TOF are indicated by the *numbered boxes* (corresponding to Tables 3, 4, and 5).

immunoglobulins and complement proteins corresponded also to the common bands observed in the sucrose fractions containing no exosomes (Figure 3). Moreover, immunogold-labeled electron microscopy analysis showed that the exosomes were not labeled with the monoclonal antibody to human immunoglobulin Fc component in any patient (data not shown).

We identified proteins already reported to be present in exosomes derived from tumor cells or antigen-presenting cells. MHC class I molecules were identified in exosomes from Patient 1 with a peptide coverage of 15% but a nonsignificant Matrix Science Mascot UK score. The cytoskeletal protein actin was identified in Patients 1 and 2. Some proteins involved in signal transduction as G protein and protein kinase were identified in Patients 1 and 2, respectively.

In addition, we identified proteins that have not been previously described. Some were related to intracellular membrane trafficking proteins such as sorting nexin (SNX25) protein. Some were related to cell growth and differentiation such as B-cell translocation gene 1 (BTG1) protein and pigment epithelium-derived factor (PEDF), both overexpressed in malignant processes, suggesting a role in tumoral exosome biogenesis. Others were related to extracellular matrix organization and cell–matrix interaction such as bamacan (basement membrane-chondroitin sulfate proteoglycan) protein and thrombospondin-2.

Western Blot Analysis

Western blotting of the exosomes isolated from the nine patients showed the presence of MHC class II molecules and HSP90 (Figure 5). Presence of immunoglobulin G and M were confirmed by Western blot in the gradient fraction containing exosomes (Figure 6). No antibodies against immunoglobulin A and E could be visualized (data not shown).

Discussion

We isolated exosomes from nine malignant pleural effusions by sucrose gradient ultracentrifugation and used MALDI-TOF mass spectrometry technology and Western blotting to identify their protein composition. We identified MHC class I and II proteins, heat shock proteins, cytoskeletal proteins, and signal transduction–involved proteins, all proteins that have already been reported as constituent of exosomes from other origin. We are the first to report the presence on exosomes of sorting nexin, BTG1, PEDF, bamacan, and thrombospondin 2. Surprisingly, the most abundantly present proteins were immunoglobulins and complement components.

Exosomes present in cancerous pleural fluid may have various cellular origins. Cancerous pleural effusion is accompanied by a strong inflammatory response involving both cellular and humoral immunity. The most abundantly reported cells in the pleural effusion of our nine patients were lymphocytes and tumor cells. B-lymphocytes, T-lymphocytes, and antigen-presenting cells such as dendritic cells are important participants of the anti-tumoral immune response, and are all known to produce exosomes (7, 9, 11). Moreover, tumor cells are also able to produce exosomes (3, 6, 12). Although a lot of proteins are commonly present in exosomes secreted by various cell types (13), particular proteins are specifically enriched in exosomes secreted by specialized cells. For example, exosomes secreted by dendritic cells are rich in MHC class II and CD86 protein, both proteins involved in antigen presentation and stimulation of T cells (2). T cell receptors are abundantly present in exosomes secreted by T lymphocytes, suggesting a role of exosomes in intercellular

TABLE 3. MALDI-TOF analysis of exosome containing gradient fraction isolated from the pleural fluid of a patient with breast cancer (Patient 1)

Band (Figure 4A)	Protein	Accession No.	Calculated Mol Mass (*D*)	Apparent Mol Mass (*D*)	Variable Modification	Peptides Matched	Coverage	Score
A1	Myosin heavy chain	I38055	222,732	230,570	Oxidation	11	6%	62
A2	Complement factor H precursor	NBHUH	139,034	175,570	No	12	8%	87
A3	MSTP043 (sorting nexin snx25)	Q9H3E2	62,630	164,670	No	8	15%	65
A4	Yotiao protein	AAC60380	188,676	133,690	No	12	9%	64
A5	Bamacan protein	O60464	141,454	118,550	No	11	7%	60
A6	Sequence 3 from patent WO0114564	CAC34689	202,399	107,280	Oxidation	12	7%	66
	TNF-inducible (TSG14) protein	HUMTSG14A	41,975	107,280	Oxidation	7	17%	63
A7	Fibrinogen fragment d	1FZAC	35,144	103,530	No	9	33%	89
A8	KIAA0622 protein	T00387	141,517	93,500	Oxidation	11	10%	63
A9	μ Ig heavy chain constant region	CAC20458	49,433	85,780	No	12	26%	110
A10	Complement C4A precursor	C4HU	192,741	71,160	No	17	11%	88
A11	Albumin	1UOR	65,936	68,650	No	13	20%	111
A12	Nebulin (fragment)	Q14215	348,750	63,230	Oxidation	17	6%	70
	Hypothetical 51.5 Kda protein	Q9H0X1	51,502	63,230	Oxidation	8	16%	63
A13	γ3 Ig constant heavy chain	CAA67886	39,637	58,730	No	6	19%	64
A14	γ1 Ig constant heavy chain	CAC20454	36,129	50,670	No	5	18%	64
A15	PEDF	A47E281	46,300	49,060	No	6	14%	69
A16	μ Ig chain C region	MMHU	49,310	46,410	No	6	15%	56
A17	Actin β (fragment)	Q96HG5	40,978	44,940	No	6	20%	71
A18	HLA-A30.3 precursor	I56039	40,939	44,210	No	4	15%	47
A19	Guanine nucleotide binding protein	Q96F32	14,403	37,970	Oxidation	4	34%	50
A20	Acidic ribosomal protein P0	R5HUP0	34,252	35,110	No	6	19%	62
A21	Acidic ribosomal protein P0	R5HUP0	34,252	33,980	No	5	13%	57
A22	BTG1 protein	I48272	19,197	31,940	No	7	27%	65
	HSPC059	Q9NZX7	70,081	31,940	No	10	22%	61
A23	κ Ig light chain (fragment)	AAC17968	23,035	26,600	No	6	41%	65
A24	Complement C1q chain C precursor	C1HUQC	25,731	24,780	No	4	21%	58

The first column corresponds to the SDS-PAGE gel codes (Figure 4A).
Definition of abbreviations: BTG1, B cell translocation gene 1; HSPC, hematopoietic stem/progenitor cells; PEDF, pigment epithelial derived factor.

communication (14). Tumor cells express MHC class I molecules and tumor markers (6, 12). Our results suggest that cancerous pleural fluid contains a combination of exosomes from various origins (mostly from lymphocytes and tumor cells). However, we are not able to determine the cellular source of these exosomes with currently available technology. As direct proof that mesothelioma cells can produce exosomes, we have found that cell lines derived from pleural fluid indeed produce exosomes (12).

Immunoglobulin peptides were the most common proteins identified in the exosome-containing fractions from the malignant pleural effusions, corresponding to the most intense bands on the SDS-PAGE gel. IgM is the first antibody to be produced in a humoral immune response. IgG is the principal isotype found in blood and extracellular fluid. Both IgG1 and IgG3 can efficiently opsonize pathogens for engulfment by phagocytes and activate the complement system. Complement (C1q, C1r, C4a,

TABLE 4. MALDI-TOF analysis of exosome containing gradient fraction isolated from the pleural fluid of a patient with lung cancer (Patient 2)

Band (Figure 4B)	Protein	Accession No.	Calculated Mol Mass (*D*)	Apparent Mol Mass (*D*)	Variable Modification	Peptides Matched	Coverage	Score
b1	μ Ig chain C region	MHMU	49,310	222,600	No	7	12%	66
b2	Thrombospondin 2 precursor	TSHUP2	129,872	198,770	No	16	16%	80
	Protein kinase	S67527	111,964	198,770	No	15	14%	80
b3	μ Ig chain	AAB59418	8,815	169,080	No	2	19%	33
b4	Bamacan protein	O60464	14,1454	134,820	No	20	13%	97
b5	μ Ig heavy chain constant region	CAC20458	49,433	120,370	No	7	15%	62
b6	Hypothetical protein (fragment)	Q9HAJ5	71,444	104,600	No	16	17%	95
	Hypothetical protein DKFZp434A2017.1	T43445	115,884	104,600	No	19	14%	93
	Hypothetical protein KIAA0542	T00322	117,525	104,600	No	19	14%	93
	KIAA0291	Q9UDT6	115,767	104,600	No	18	11%	80
b7	μ Ig chain C region	S37768	49,439	87,320	No	14	26%	124
b8	Serum albumin	1BKE	65,993	66,780	No	12	18%	111
b9	μ Ig chain C region	S37768	49,439	63,840	No	10	22%	73
b10	μ Ig chain C region	MHHU	49,310	60,930	No	7	17%	77
B11	γ 1 Ig FC fragment	AAD38158	25,061	52,740	No	8	43%	116
B12	μ Ig chain C region	S37768	49,439	49,270	No	8	21%	98
B13	SP α	43866	38,063	47,160	No	13	26%	141
B14	Actin β (fragment)	Q96HG5	40,978	44,460	No	4	14%	61
B15	C1qB-chain precursor	CAA26880	23,926	34,800	No	6	25%	71
B16	C1qB-chain precursor	CAA26880	23,926	33,110	No	5	25%	54
B17	btg1 protein	I48272	19,197	31,760	No	5	17%	39
B18	κ Ig chain fragment	BAA37169	23,404	28,640	No	8	30%	66

The first column corresponds to the SDS-PAGE gel codes (Figure 4B).
Definition of abbreviation: BTG1, B cell translocation gene 1.

TABLE 5. MALDI-TOF analysis of exosome containing gradient fraction isolated from the pleural fluid of a patient with mesothelioma (Patient 3)

Band (Figure 4c)	Protein	Accession No.	Calculated Mol Mass (*D*)	Apparent Mol Mass (*D*)	Variable Modification	Peptides Matched	Coverage	Score
C1	Complement factor H precursor	NBHUH	139,034	178,064	No	8	6%	57
C2	KIAA0291	Q9UDT6	115,767	110,984	No	7	7%	44
C3	Fibrinogen fragment d, chain F	1FZEF	34,343	106,255	No	13	44%	157
C4	Fibrinogen fragment d, chain F	1FZEF	34,343	98,612	No	8	30%	93
C5	μ Ig chain C region	S37768	49,439	86,000	No	10	20%	70
C6	γ3 Ig heavy chain	CAA67886	37,974	67,895	No	7	21%	49
C7	Ig heavy chain variable region	CAC12584	8,086	50,801	Oxidation	7	44%	49
C8	β-Fibrinogen precursor	AAA52429	54,861	40,355	No	7	17%	79
C9	Complement C1q sub component	CAA26880	23,926	31,807	No	5	25%	45
C10	κ Ig chain (fragment)	BAA37169	23,404	27,105	No	7	41%	66
C11	Complement C1q precursor	C1HUQC	25,731	25,465	No	6	32%	63

The first column corresponds to the SDS-PAGE gel codes (Figure 4C).

and H factors) components were also frequently identified in exosomes from malignant pleural effusion. Both C1 and C4 complement factors are involved in the so-called classical complement pathway. Additionally tumor necrosis factor–stimulated gene 14 (TSG-14) protein is structurally related to C reactive protein and serum amyloid C component both involved in the first response to infections or tumor with the ability to activate complement (15).

The importance of the high Ig and complement levels in our exosome preparations is threefold. First, Ig and complement determine and modify the anti-tumoral immune response. Presence of Ig, complement factors, and TSG-14 protein in pleural effusions could be related to the strong immune response occurring in the pleural space of patients with cancer. High concentration of Ig and complement has already been reported in malignant effusions (16–18) and was measured in the pleural fluid of our nine patients. Pleural Ig diffuse mostly from the bloodstream but can also originate from locally stimulated B-lymphocytes (19). Second, ultracentrifugation in sucrose gradient has been shown to be a reliable method for isolation of exosomes from cell culture supernatant (2, 13). This methodology has been recently used with success to isolate exosomes from malignant ascities (6). However, ultracentrifugation in sucrose gradient coisolates Ig and complement proteins. The presence of Ig in the sucrose gradient fractions that contain no exosomes and the absence of exosome labeling with an antibody targeted against Fc component of human Ig argues for the presence of free Ig and not for Ig bound to exosomes. Third, the potential presence of proteins not binding to exosomes in the exosome preparation had no functional consequences for an *in vitro* autologous cytolytic test as observed by André and colleagues. However, the use of exosome samples containing nonexosomal proteins may have deleterious consequences, especially in the case of heterologous cross-utilization. Induction of polyantigenic immune responses targeted to noncancer proteins could be not only responsible for a decrease of the anti-tumoral effect of the vaccine but could also induce an autoimmune response potentially dangerous to the patient. Use of exosomes as antigen source for cancer immunotherapy is promising. However, risk of presence of contaminating proteins, especially when exosomes are isolated from malignant effusion, must be taken into account before their *in vivo* use can be generalized.

Antigen-presenting molecules such as MHC molecules (class I and II) and heat shock proteins were identified in exosomes isolated from cancerous pleural fluid. These molecules have been commonly identified in exosomes originating from various cells such as B cells, T cells, dendritic cells, and tumor cells (2, 3, 14). Moreover, in a recent report, André and colleagues have confirmed the presence of MHC class I molecules in malignant ascites-derived exosomes by electron microscopic immunostaining and Western blotting (6). Functions of these proteins in exosomes have been related to their capacity to transfer antigens to antigen presenting cells and to induce a specific immune response. Other proteins that we identified have already been reported as constituents of exosomes from other origins (e.g., actin, myosin, G protein, or protein kinase).

We described several new proteins not previously described in exosomes by examining exosomes from malignant effusions. Sorting-nexin (SNX) family is a group of hydrophilic proteins implicated in the intracellular trafficking of proteins to various organelles. SNX1 has the capacity to bind membrane receptors such as epidermal growth factor receptor (EGRF), platelet derived growth factor or insulin (20). Moreover, overexpression of SNX1 induced an EGFR decrease on human cell surface suggesting that SNX1 plays a role in sorting EGFR to lysosomes for degradation (21). The presence of an endosomal trafficking protein such as SNX1 in exosomes, which are known to originate from the multivesicular late endosomal compartment, seems a quite likely possibility. Moreover, proteins involved in membrane intracellular transport such as annexins or rab proteins have been previously observed in exosomes secreted by dendritic

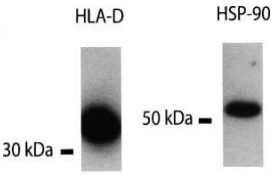

Figure 5. The presence of MHC class II molecules and heat shock protein 90 was confirmed by Western blotting in the exosome containing fraction isolated from cancerous pleural fluid.

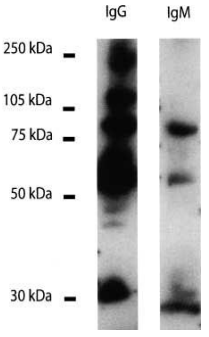

Figure 6. The presence of immunoglobulins G and M was confirmed by Western blotting in the exosome-containing fraction isolated from cancerous pleural fluid.

cells (10). It could then be hypothesized that SNX1 plays a role in the intracellular membrane trafficking to the endosomal and subsequently to the exosomal compartment.

Acidic ribosomal phosphoproteins from the 60S subunit of ribosomes, interact with elongation factors EF-1 and EF-2 and play an important role in the elongation step of protein synthesis. Elongation factor EF-1 has already been identified in B cell– and DC-derived exosomes (10, 11). However, various tumor cells like colon or hepatocellular carcinoma have an increased expression of acidic ribosomal phosphoprotein (22). The presence of translation-related proteins in exosomes may therefore also be explained by their high concentration in the cytosol of cancer cells.

Identification of extracellular matrix organization– and cell matrix interaction–related proteins as bamacan (basement membrane-chondroitin sulfate proteoglycan) protein or thrombospondin-2 may be related to their high concentration in malignant pleural fluid. Indeed, an increased level of proteoglycans is a known event in epithelial tumors such as breast or lung carcinoma and overexpression of bamacan protein has been reported in cancer cells (23). Thrombospondin 2 is involved in the regulation of proliferation, adhesion, and migration of various cells, and has an inhibitory function of both angiogenesis and tumor growth (24, 25). Thrombospondin 2 interacts with cell surface receptors such as integrin or heparan sulfate proteoglycan (26, 27). A link between thrombospondin 2 expression and tumor progression has been reported in melanoma and breast carcinoma (28, 29). The binding of these extracellular matrix proteins on exosomes may be explained by the presence of matrix-binding proteins like integrins on the surface of exosomes, which have been reported previously (13). However the function of these extracellular matrix molecules in exosomes remains unknown.

PEDF is a secreted protein that is expressed by various healthy and tumoral tissues. PEDF belongs to the serine protease inhibitor (SERPIN) gene family and is known to possess anti-angiogenic activities (30). Secretion of PEDF by Schwann cells induced differentiation of neuroblastoma tumor cells, which consecutively secrete PEDF, suggesting an anti-tumor feedback loop with the potential to limit tumor growth (31). The function of BTG1 (B-cell translocation gene 1) protein is incompletely known, but it may be involved in cell growth and differentiation and control of the cell cycle at a transcriptional level (32). Both PEDF and BTG1 proteins are then related to cell growth control, and their expression can be modified in cancerous cells, but their putative role in exosomes remains unclear.

We could not identify any integrins, tetraspanins, or annexins in our exosome preparations by the technology used. These proteins have been isolated from exosomes derived from DC and tumor cells. The fact that we do not detect these proteins is probably caused by the relatively poor ability of the 1D SDS-PAGE gel to separate the different proteins. An extra initial step to separate the Ig and complement factors from the exosomes and the use of two-dimensional gel electrophoresis separation or other high resolving techniques may be a way to identify more proteins that are less profusely present.

In conclusion, cancerous pleural effusion contain exosomes from various cellular origin. The proteomic analysis of pleural effusion-derived exosomes was marked by the identification of proteins that had not been identified in exosomes before and by a high concentration of antigen-antibody humoral immunity components. The latter might have important consequences both for *in vitro* and *in vivo* applications of exosomes that are isolated directly from body fluids.

Acknowledgments: M.P.L.B. received a research fellowship from the European Society of Medical Oncology.

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Chapter 8

8

Proteomic analysis of exosomes secreted by human mesothelioma cells

American Journal of Pathology 2004; 164:1807-1815

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Proteomic Analysis of Exosomes Secreted by Human Mesothelioma Cells

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Exosomes are small membrane vesicles secreted into the extracellular compartment by exocytosis. Tumor exosomes may be involved in the sampling of antigens to antigen presenting cells or as decoys allowing the tumor to escape immune-directed destruction. The proteins present in exosomes secreted by tumor cells have been poorly defined. This study describes the protein composition of mesothelioma cell-derived exosomes in more detail. After electrophoresis of exosome preparations, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) was used to characterize the protein spots. MHC class I was found to be present together with the heat shock proteins HSC70 and HSP90. In addition, we found annexins and PV-1, proteins involved in membrane transport and function. Cytoskeleton proteins and their associated proteins ezrin, moesin, actinin-4, desmoplakin, and fascin were also detected. Besides the molecular motor kinesin-like protein, many enzymes were detected revealing the cytoplasmic orientation of exosomes. Most interesting was the detection of developmental endothelial locus-1 (DEL-1), which can act as a strong angiogenic factor and can increase the vascular development in the neighborhood of the tumor. In conclusion, mesothelioma cells release exosomes that express a discrete set of proteins involved in antigen presentation, signal transduction, migration, and adhesion. Exosomes may play an important role in the interaction between tumor cells and their environment. *(Am J Pathol 2004, 164:1807–1815)*

Like most cells of hematopoietic origin, tumor cells secrete exosome-like vesicles. These subcellular membrane vesicles from endosomal origin are secreted on fusion of multi-vesicular bodies with the plasma membrane.[1,2] As a consequence, exosomes have a "cellular" membrane orientation with a limited range of proteins derived from the cytosol, endocytic compartment membranes, and plasma membranes.[3] They are 60 to 110 nm in diameter, and may be involved in the communication between cells. Exosomes from a murine dendritic cell (DC) line D1 are best characterized for protein composition.[4,5] Proteins expressed on these DC-derived exosomes are involved in the regulation of basic processes like signal transduction, adhesion, activation, and migration. In addition, MHC-I and MHC-II, proteins normally involved in antigen presentation, are expressed on DC-derived exosomes.

Although DC-derived exosomes are able to activate cytotoxic T cells and to elicit potent anti-tumor immune responses,[4] the function of tumor cell-derived exosomes is unknown. They may serve as decoys by allowing the tumor to escape immune-directed destruction or for sampling antigens to DC. Wolfers et al[6] demonstrated that tumor-derived exosomes are capable of transferring tumor antigens to DC, inducing a $CD8^+$ T-cell-dependent cross-immunization of tumor-bearing mice. These exosomes seem to concentrate a set of whole native shared tumor antigens opening the possibility that exosomes could be used as a source of antigen in vaccination protocols.[6,7] Proteomics offers the possibility to understand more about human tumor-derived exosomes and these organelles may, like DC-derived exosomes, give new perspectives to improve the diagnosis and therapy of cancer patients.[8–10]

Malignant mesothelioma (MM) is a tumor of mesodermally derived tissue lining the coelomic cavities with no satisfactory curative treatment.[11] This tumor was chosen as a model system to study the characteristics of tumor-derived exosomes because only a small amount of data are available on tumor antigens in this tumor.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was used for the proteomic analysis of exosomes derived from well-characterized mesothelioma cell lines. The focus of this article will be on the proteins present in tumor exosomes.

Supported by "Stichting Asbestkanker Rotterdam."

Accepted for publication January 6, 2004.

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Materials and Methods

Establishment of Human Mesothelioma Cell Lines

Mesothelioma cell lines have been derived from pleural effusions or primary solid tumor biopsy material. After informed consent, patient material was collected under sterile conditions and transported immediately to the laboratory. Solid tissue was minced into small pieces with sterile scissors and gently pressed through a 100-μm mesh cell strainer (Falcon/Becton Dickinson Labware, Franklin Lakes, NJ) with a syringe piston. Dispersed cells and clumps were washed through gauze with HBBS (GIBCO/Invitrogen, Breda, The Netherlands), and the suspension was transferred to a second finer (40-μm mesh) gauze (Falcon/Becton Dickinson Labware). Suspension was centrifuged at 400 $\times$ *g* for 15 minutes at room temperature (RT) and cells placed into culture flasks (Falcon/Becton Dickinson Labware). Pleural effusions were centrifuged 400 $\times$ *g* for 15 minutes and cells were placed into culture flasks. Cells were cultured at 37°C in RPMI 1640 medium containing 25 mmol/L HEPES, Glutamax, 50 μg/ml gentamicin, and 10% (v/v) fetal bovine serum (FBS) (all obtained from GIBCO/Invitrogen) in a humidified atmosphere of 5% CO_2, in air. Media were changed once or twice a week and when flasks were confluent, then cells were passaged to a new flask by treatment with 0.05% trypsin and 0.53 mmol/L EDTA in phosphate-buffered saline (PBS, all from GIBCO/Invitrogen). Two cell lines (PMR-MM7 and PMR-MM8) were extensively characterized and kept in long-term cell culture (>50 passages, 6 months of culturing) while using for exosome isolation.

Characterization of Cell Lines

Cellular DNA Content

Cell lines were characterized for cellular DNA content by propidium iodide. In short, cells were trypsinized and washed twice in 0.1% (w/v) glucose (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) in PBS. A pellet containing 1 $\times$ 10^6 cells was resuspended by slowly adding 1 ml of ice-cold 70% ethanol under vigorous vortexing. The suspension was fixed overnight at 4°C and the next day the pellet was resuspended in 1 ml 0.1% (w/v) glucose in PBS supplemented with 50 μg/ml propidium iodide (Sigma-Aldrich Chemie BV) and 100 Kunitz units RNAseA (Amersham Pharmacia Biotech, Essex, UK) and incubated for 60 minutes at RT. Flow cytometric analysis of nuclear DNA content was performed on a FACSCalibur (BD Immunocytometry Systems, Erembodegem, Belgium).

Immunohistochemical Studies

Cytocentrifuge preparations were stained using the rabbit-anti-mouse (RαM) and APAAP method for the following mouse antibody clones: 5B5 (anti-prolyl 4-hydroxylase (collagen synthesis)), E29 (anti-epithelial membrane antigen), II-7 (anti-carcinoembryonic antigen), HBME-1 (anti-mesothelial cell), Ber-EP4 (epithelial antigen), RCK108 (anti-cytokeratin 19) (all antibodies were obtained from DAKO, Glostrup, Denmark). Appropriate positive controls were used in each case. Specificity of the primary and secondary antibodies was checked by using protein concentration and isotype-matched non-relevant monoclonal antibodies and PBS. Naphtol-AS-MX-phosphate (0.30 mg/ml, Sigma-Aldrich Chemie BV) and new fuchsine (160 mg/ml in 2 mol/L HCl, Chroma-Gesellschaft, Kongen, Germany) were used as substrate for alkaline phosphatase (AP). Levamisol (0.25 mg/ml, Sigma-Aldrich Chemie BV) was added to block endogenous AP activity. Finally, sections were counter-stained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and mounted in Kaiser's glycerol-gelatin (Merck).

Tumorigenicity in Vitro *and in Immune-Deficient Mice*

The tumorigenicity of cell lines was determined by their capacity of forming colonies in semi-solid media.[12] An agarose underlay was prepared by adding 1 ml autoclaved 0.8% (w/v) agarose (GIBCO/Invitrogen) in PBS to a 6-well plate per well and allowed to gel for 30 minutes. Cells were collected by trypsinization and adjusted to a concentration of 5 $\times$ 10^4 cells per 3 ml RPMI 1640 medium containing HEPES, Glutamax, gentamicin, and 10% FBS. The cells were diluted in 3 ml StemPro 2.3% methylcellulose (GIBCO/Invitrogen), and the tube was vigorously vortexed until the cells were uniformly suspended. After 10 minutes of allowing the air bubbles to rise, the suspension was added to the agarose underlay and incubated in a humidified incubator at 37°C for 14 days or until colonies were formed. The tumor-forming capacity of the cell lines was also tested in athymic *nude* mice. Monolayer cells were harvested by trypsinization, and 2 $\times$ 10^6 cells suspended in 0.2 ml of PBS were injected subcutaneously into 4- to 6-week-old BALB/c athymic *nu/nu* mice (Jackson Laboratory, Bar Harbor, ME). Mice were maintained in sterile-air laminar flow cage racks and examined regularly for tumor development for at least 2 months following the injection.

Virus Contamination, HLA Typing, and Karyotyping

Contamination of the cell lines with HCV, HBV, and HIV viruses was analyzed with (quantitative) polymerase chain reaction at the virology laboratory of the Erasmus MC-Dijkzigt according to World Health Organization references. The Department of Immunohematology and Blood Transfusion of the Leiden University Medical Center performed HLA typing. Karyotyping was carried out in the Department of Clinical Genetics of the Erasmus MC.

Isolation of Mesothelioma-Derived Exosomes

Mesothelioma cell lines at 80% confluency were washed twice with PBS and incubated in RPMI medium (containing HEPES, Glutamax, and gentamicin) and the serum replacer TCH (1X working strength [ICN, Irvine, CA]) for 48 hours in a humidified atmosphere of 5% CO_2, 95% air.

Cell culture supernatants were subjected to three successive centrifugations to remove cells and debris: 300 × *g* for 10 minutes, 2000 × *g* for 20 minutes, and finally at 10,000 × *g* for 30 minutes, all at 4°C. Exosomes were then pelleted at 64,000 × *g* for 100 minutes using a SW28 rotor (Beckman Coulter Instruments, Fullerton, CA). Pellets were resuspended and washed in PBS and centrifuged at 100,000 × *g* for 1 hour (SW60 rotor, Beckman Coulter Instruments). Exosomes were resuspended in PBS, aliquoted, and stored at −80°C. The quantification of exosomal proteins recovered was measured by CBQCA kit according to the manufacturer's recommendations (Molecular Probes, Leiden, The Netherlands). In the presence of cyanide, the ATTO-TAG CBQCA reagent reacts with the primary amides found on proteins and functions well in the presence of lipids and detergents. The fluorescence emission was measured at ~550 nm (filter 530 ± 30 nm) with excitation at ~465 nm (filter 485 ± 20 nm) in a CytoFluor 4000 fluorescence microplate reader (gain 40) (PerSeptive Biosystems, Foster City, CA).

Electron Microscopy

Exosomes obtained after centrifugation of cell-culture supernatants were loaded onto Formvar carbon-coated grids. Adsorbed exosomes were fixed in 2% paraformaldehyde and immunolabeled with CLB-gran1/2, 435 (anti-CD63; CLB, Amsterdam, The Netherlands) and 10 nm protein A gold particles.

Protein Electrophoresis

One-dimensional electrophoresis of mesothelioma-derived exosomes onto 10% SDS-PAGE gels was performed according to manufacturer's recommendations (PROTEAN II xi Cell, BioRad Laboratories, Hemel Hempstead, UK). Samples were taken-up in 8 mol/L urea (Sigma-Aldrich Chemie BV), 2% CHAPS (Amersham Pharmacia Biotech), 20 mmol/L dithiothreitol (DTT, Sigma-Aldrich Chemie BV), 0.01% bromophenol blue (Sigma-Aldrich Chemie BV), and transferred onto a 1.0-mm thick 10% SDS-PAGE gel. A constant current of 7 mA per gel at 10°C was applied. After 16 hours, gels were stained with Novex Colloidal blue staining kit according to the manufacturer's instructions (Invitrogen).

Enzymatic Digestion of Protein Spots

Colloidal blue stained protein spots were excised manually with a plastic plunger and transferred onto a 96-well low protein binding microtiter plate (Nunc A/S, Roskide, Denmark). Each excised plug was washed with 100 μl milli-Q for 5 minutes with shaking (650 rpm, Eppendorf Geratebau GmbH, Hamburg, Germany). Gel plugs were de-stained with 0.4% (w/v) ammonium hydrogen carbonate (Sigma-Aldrich Chemie BV), 30% acetonitrile in water by incubating two times for 20 minutes at RT. After a short wash with Milli-Q, gel spots were dried in a rotary evaporator (Savant, Farmingdale, NY) for 30 minutes. Protein digestion was performed with the addition of 4 μl of 100 μg/ml sequencing grade-modified trypsin (Promega, Madison, WI) to each gel piece. The plate was sealed with an adhesive aluminum foil and incubated overnight at RT.

MALDI-TOF Analysis of Peptides

After the specific hydrolysis at the carboxylic sides of lysine and arginine residues by trypsin, 7 μl (1:2) acetonitrile:0.1% trifluoroacetic acid was added to the gel plugs. After mixing, 1 μl of the tryptic digest was taken and co-crystallized with 2.5 μl 2 mg/ml of the photoactive compound α-cyano-4-hydroxy-*trans*-cinnamic acid (α-HCCA, Bruker Daltonics, Billerica, MA) in acetonitrile. This sample-matrix solution (0.5 μl) was pipetted onto a 400-μm 384-well anchor chip MALDI-TOF plate and air-dried for 5 minutes. Peptide mass spectra were acquired on a Biflex III MALDI-TOF mass spectrometer equipped with a 337-nm nitrogen laser (Bruker Daltonics, Bremen, Germany). The instrument was calibrated with a peptide calibration standard (Bruker Daltonics). Spectra were compared using autolytic fragments from trypsin. A mass list of peptides was obtained from each digest and submitted to Matrix Science Mascot UK software to identify the proteins in the MSDB database of the NCBI. The criteria for identification of proteins were determined as follows: maximum allowed peptide mass error of 200 ppm, at least five matching peptide masses, molecular weight of identified protein should match estimated values by comparing with marker proteins, and top scores given by software higher than 61 ($P < 0.05$).

Western Blotting

For Western blotting following one-dimensional SDS-PAGE, proteins were electroblotted onto Immobilon P membranes (Millipore Corp, Etten-Leur, The Netherlands) and incubated with specific antibodies, followed by horseradish peroxidase-conjugated secondary antibodies, and detected using SuperSignal West Pico chemiluminescent substrate (Pierce Perbio Science, Etten-Leur, The Netherlands). Antibodies used in this study to confirm the proteins detected by MALDI-TOF were: anti-HSC70 (clone 13D3; Affinity BioReagents, Golden, CO), anti-HSP90 (clone AC88; Stressgen, Victoria, Canada), anti-fascin (clone FCN01, Abcam Ltd, Cambridge, UK), and anti-β-tubulin (clone E7, Developmental Studies Hybridoma Bank, Iowa City, IA).

Results

Establishment and Characterization of Human Mesothelioma Cell Lines

Since 1997, the Department of Pulmonary Medicine Rotterdam (PMR) has established 10 continuously growing cell lines originally initiated from pleural effusions from patients diagnosed as malignant mesothelioma (MM). One cell line was derived from a postmortem pleural biopsy. The

Table 1. Characteristics of the Mesothelioma Cell Lines PMR-MM7 and PMR-MM8

	PMR-MM7	PMR-MM8
Patient		
Gender	Male (Caucasian)	Male (Caucasian)
Age	63	57
Cellular DNA content	Aneuploid	Diploid
Immunohistochemistry		
5B5	Positive	Positive
II-7 (CEA)	Negative	Negative
HBME-1	Negative	Negative
Ber-EP4	Negative	Negative
RCK108	Negative	Positive
Tumorigenicity	Yes	Yes
Virus contamination	HCV, HBV, HIV negative	HCV, HBV, HIV negative
Bacterial contamination	No	No
Karyotyping	71~78<3>,XY,X,+Y,del(1p),add (2p),add(2p),?add(3q),del(5q),+del(5q),+del(5q),der(6)t(6;7),der(6)t(6;7),del(7p),−7,−7,add(8q),add(9p),add(9p),del(9p),del(10p),+11,+11,+der(12),t(2;12),add(13p),−14,−15,add(17q),add(19q),+20,+add(20q),+21?,22?,+mar1,+mar2,+mar3	40~41,add(Xq),−Y,add(1p),−1,add(2q),der(3)t(2;3),−4,add(5q),?5,del(6p),del(6q),inv(7),−8,add(10p),del(10q),del(11q),+add(12q),−13,−13,der(14p)t(14;15),der(15)t(8;15),der(16)t(14;16),−19,22?
HLA typing	A*02,A*68,A*28,B*27,B*40,Bw*04,Bw*06,Cw*0304,Cw*03Cw*0704,Cw*07	A*01,B*39,B*16,Bw*06,Cw*0501,Cw*05

10 patients from whom cell lines were derived were all males ranging in age from 45 to 79 years (mean, 61 years). Two mesothelioma cell lines, PMR-MM7 and PMR-MM8, were characterized as summarized in Table 1.

Based on these characteristics and by judgment of the Dutch mesothelioma expert panel these cell lines were regarded as true mesothelioma and were differentiated from pleural metastasis of adenocarcinoma. Furthermore, the cells were free from bacterial and viral contaminants, excluding the possibility of viral and bacterial proteins in the exosome preparation.

Isolation and Characterization of Mesothelioma-Derived Exosomes

Exosomes from seven mesothelioma cell lines were collected from 80% confluent cultures after culturing for 48 hours in medium supplemented with a serum replacer. Exosomes were purified by successive (ultra)-centrifugation steps. Initial experiments with medium containing fetal bovine serum deprived of cells pelleted also protein components from the serum. Medium containing the serum replacer TCH gave no protein contamination in the exosome preparation (data not shown). Typically, 15 to 50 μg proteins were isolated from 25-ml culture medium after 48 hours of incubation with an 80% confluent layer of mesothelioma cells (175-cm^2 flask). Protein content was based on the CBQCA quantitation kit because it functions well in the presence of lipids and can be used directly to determine the amount of proteins in lipid-protein samples. Electron microscopically, the extracellular particles isolated from the culture supernatant after removal of cells by centrifugation consisted of membrane vesicles as shown in Figure 1. Cellular debris was rarely found.

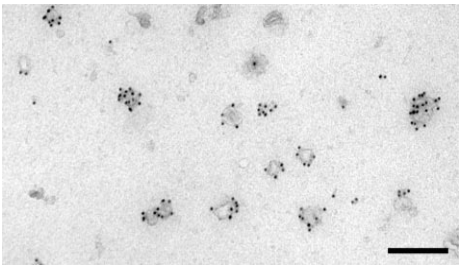

Figure 1. Electron micrograph of mesothelioma cell line PMR-MM7-derived exosomes, showing cup-shaped membrane vesicles. Exosomes were fixed in 2% formaldehyde and immunolabeled for CD63 as described in the Materials and Methods section (**bar**, 200 nm). Similar results were obtained with the PMR-MM8-derived exosome preparation (not shown).

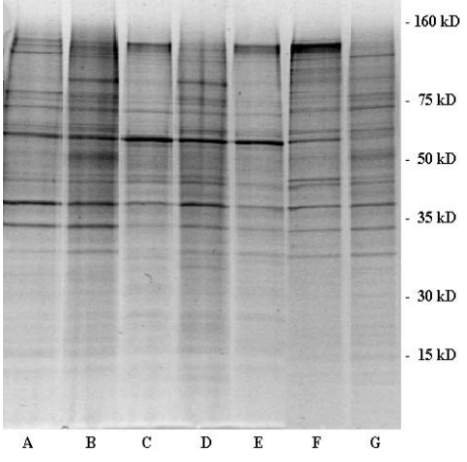

Figure 2. Separation of mesothelioma cell-derived exosomal proteins on 10% SDS-PAGE and stained by colloidal blue. **Lanes A** to **G** represent the different mesothelioma cell lines, PMR-MM1, PMR-MM3, PMR-MM5, PMR-MM7, PMR-MM8, PMR-MM9, and PMR-MM10, respectively.

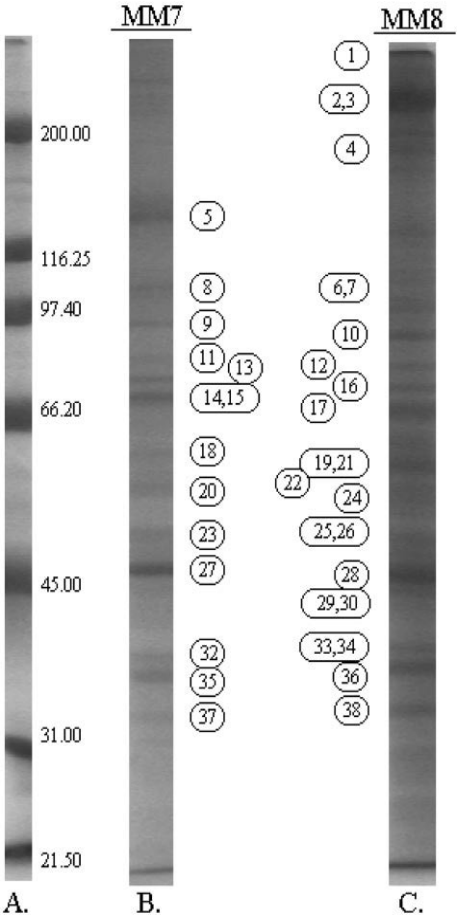

Figure 3. Exosomes derived from the mesothelioma cell lines PMR-MM7 (**B**) and PMR-MM8 (**C**) after electrophoresis in a denaturing polyacrylamide gel (**A**, broad range marker in kilodaltons (kd)). Numbers correspond to the excised protein bands (see Table 2)

Proteomic Analysis

The protein composition of exosomes isolated from seven different mesothelioma cell lines was determined by electrophoretic separation onto a 10% SDS-PAGE gel (Figure 2). Because protein bands showed similar patterns between the different exosome preparations, two cell lines were depicted to characterize all distinct bands by MALDI-TOF mass spectrometry. Therefore, 50 μg of exosomes derived from PMR-MM7 cells and PMR-MM8 cells were loaded onto a 10% SDS-PAGE gel (Figure 3). All distinct bands were subjected to MALDI-TOF analysis. As mentioned in the Materials and Methods section, criteria for positive protein identification were set as follows: maximum allowed peptide mass error of 200 ppm, at least five matching peptide masses, molecular weight of identified protein should match estimated values, and top score given by software. Results are presented in Table 2. The first column corresponds to the numbers on the SDS-PAGE gel followed by a description from which cell lines the exosomes were derived. Protein names, accession numbers, and calculated molecular weights were deduced from the mass fingerprint analysis in the MSDB database of the NCBI. Observed molecular weights were measured by interpolation by image analysis software with the molecular weight curve obtained from the molecular weight marker proteins run as a separate track on the gel. SDS-PAGE allows only an estimation of the mass of a protein. Differences between the calculated molecular weight and observed molecular weight can be caused by excessive post-translational modifications, which were not predicted in the theoretical digestion of the proteins in the database as well as precluding peptides from the fingerprint. The last column corresponds to the score given by the Matrix Science Mascot UK software analysis, which was significant ($P < 0.05$) when higher than 61.

Analysis of the exosomes by Western blot (Figure 4) confirmed the presence of the proteins detected by MALDI-TOF mass spectrometry. Antibodies against fascin, β-tubulin, HSC70, and HSP90 could be visualized by Western blot. As an illustration that some proteins were still present in both cell lines, even when only one gave a statistically significant result using our strict criteria for the MALDI-TOF technique, HSP70, fascin, and β-tubulin proteins were detected in one cell line by MALDI-TOF, whereas they were demonstrated in both cell lines using Western blotting.

Discussion

Tumor exosomes are poorly defined. In contrast to dendritic cell-derived exosomes, no studies have described an extensive protein characterization of tumor exosomes. Initial exosome isolations from pleural effusion turned out to be troublesome, caused by high amounts of immunoglobulins and complement factors present in the fluid.[13] Exosomes in pleural effusions will not only be secreted by mesothelioma cells, but also by mesothelial cells or from cells of hematopoietic origin present in the fluid. Therefore, mesothelioma cell line-derived exosomes were studied for their protein content. The seven mesothelioma cell lines described in this study secrete exosomes into their environment. Exosome-like vesicles were isolated from the culture supernatant after 48 hours of secretion by the cell lines through successive centrifugation steps. Electron microscopy showed 60 to 150 nm diameter vesicles. After one-dimensional electrophoresis of these exosome preparations, protein spots from two mesothelioma cell lines were analyzed by MALDI-TOF mass spectrometry.

Earlier studies showed that exosomes derived from mouse tumors concentrate tumor antigens and contain MHC class I molecules loaded with tumor peptides.[6] Similarly, MHC class I was found to be present on human tumor-derived exosomes that may be involved in the presentation of polypeptide fragments of antigens to T cells. Heat shock proteins (HSP) are a group of common proteins that play a role in the cell's response to elevated

Table 2. Identified Exosomal Proteins Secreted by Mesothelioma Cells (Ordered by Observed Molecular Weight)

No.	PMR MM	Protein	Accession number	Calculated mol wt	Observed mr	Peptides	Coverage	Top score*
1	8	desmoplakin I	A38194	309.797	>272	36	11%	116
2	8	fibronectin precursor	CAA26536	256.529	228	31	15%	148
3	8	myosin	CAB05105	226.392	205	29	13%	97
4	8	putative P150	O00378	148.786	174	15	10%	72
5	7	integrin alpha-3 chain precursor	A40021	116.538	136	15	17%	69
6	8	hypothetical protein fragment	Q9HAj5	71.444	115	17	15%	87
7	8	epithelial microtubule-associated protein	I37356	84.002	115	16	13%	68
8	7	actinin-4	BAA24447	102.204	106	12	11%	67
9	7	heat shock protein 90-alpha	HS9A_HUMAN	84.490	95	8	21%	112
10	8	heat shock protein 90	AAA36026	83.212	93	24	27%	197
11	7	ezrin	Q96CU8	69.370	84	16	25%	134
12	8	ezrin	EZRI_HUMAN	69.225	81	14	20%	125
13	7	moesin	MOES_HUMAN	67.647	79	9	13%	75
14	7	albumin	1A06A	65.695	74	15	14%	107
15	7	annexin VI	ANX6_HUMAN	75.695	74	14	16%	75
16	8	moesin	MOES_HUMAN	67.647	73	17	24%	161
17	8	heat shock cognate protein 70	A27077	70.854	70	12	17%	85
18	7	pyruvate kinase	KPY1_HUMAN	57.710	63	7	9%	87
19	8	integrin-binding protein DEL1 precursor	O43854	53.730	60	12	20%	102
20	7	beta-tubulin	I38369	48.848	60	7	13%	71
21	8	fascin	FSC1_HUMAN	54.365	60	8	15%	72
22	8	kinesin-like protein 2	Q9NS87	160.061	57	12	7%	70
23	7	enolase alpha	ENOA_HUMAN	47.008	52	8	17%	65
24	8	PV1 protein	Q9BX97	50.562	50	8	16%	65
25	8	protein kinase	A38643	53.676	50	11	21%	62
26	8	translation initiation factor	FIMS4A	46.125	50	12	31%	77
27	7	actin	AAH08633	40.978	49	12	29%	111
28	8	actin	AAH08633	40.978	47	12	36%	126
29	8	2′3′-cyclic-nucleotide 3′-phosphodiesterase	BAA02435	45.070	45	11	28%	115
30	8	MHC class I HLA-B	I68774	31.669	45	9	39%	103
31	8	MHC class I antigen (fragment)	Q9TP25	21.011	45	8	44%	67
32	7	glyceraldehyde 3-phosphate dehydrogenase	CAA25833	36.031	41	8	23%	89
33	8	glyceraldehyde 3-phosphate dehydrogenase	G3P2_HUMAN	35.899	39	7	14%	75
34	8	annexin I	1AIN	35.018	39	8	24%	92
35	7	annexin II	ANX2_HUMAN	38.449	39	21	52%	251
36	8	annexin II	ANX2_HUMAN	38.449	37	13	31%	139
37	7	annexin V	1HVE	35.068	36	9	26%	88
38	8	annexin V	1HVE	35.068	35	7	22%	64

*Top scores higher than 61 were significant ($p < 0.05$).

temperature, infection, cytokine stimulation, metabolic starvation, and other environmental stresses. Indicated by intense bands on the PAGE-gel, high amounts of HSP90 and heat shock cognate protein (HSC) 70 were present in mesothelioma-derived exosomes. HSP are normally present in small amounts within the cytoplasm of all cells in all life forms but can also be released into the extracellular environment in the absence of cellular necrosis.[14] The precise mechanisms by which HSP are actively released by viable cells have not yet been elucidated but we propose that exosomes may play a role in releasing HSP from cells. Inside cells, HSP play a role in protein trafficking, whereby they fold other proteins properly, keep them in correct and functional shape, or transport them from one location to another.[15] They act thus as chaperones, bringing along with them small fragments, or peptides, derived from other proteins expressed in that cell, providing a "fingerprint" of the cell's content.[16] Therefore, exosomes carrying high amounts of HSP from a patient's tumor may be good candidates for a cancer immune therapy without the need to identify what those antigens are. HSP in exosomes can be taken up by dendritic cells and macrophages (perhaps by CD91 receptor-mediated endocytosis[17–19]), and processed for presentation to the immune system in the lymph nodes. The tumor-specific antigens are released from the HSP inside the cell and presented to cytotoxic T cells (CTL), or "killer cells," which are then activated. Different studies showed that immune cells stimulated with heat shock proteins can eliminate different kinds of cancers,[20–26]

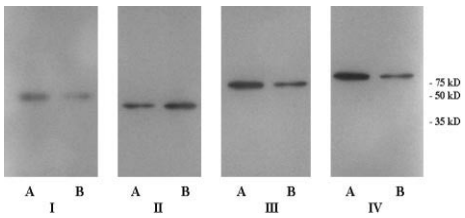

Figure 4. The presence of fascin (**I**), β-tubulin (**II**), HSC70 (**III**), and HSP90 (**IV**) were confirmed by Western blots of PMR-MM7-derived exosomes (A) and PMR-MM8-derived exosomes (B). The primary antibodies anti-fascin (1:1000), anti-β-tubulin (1:10.000), and anti-HSP90 (1:2000) were followed by horseradish peroxidase-conjugated goat anti-mouse IgG1. Anti-HSC70 (1:1000) was followed by Envision (DAKO). Blots were incubated with SuperSignal West Pico chemiluminescent substrate and exposed to Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, England).

and phase III trails are underway in renal cancer and metastatic melanoma. HSC70 has furthermore been described as an important factor in the release of exosomes during reticulocyte maturation[27] and HSP73, present in dendritic cell-derived exosomes, induced antitumor immune responses *in vivo*.[4]

Annexins comprise a structurally conserved family of proteins capable of binding in a Ca^{2+}-dependent manner to phospholipids.[28] Annexins participate in the regulation of membrane organization, membrane traffic, and the regulation of calcium currents across cell membranes or within cells.[29] After being exported outside of cells, some annexins have been shown to function as receptors for extracellular proteins and proteases and can interact with glycoconjugates.[30] Annexin A2 and annexin A6 participate in disconnecting the clathrin lattice from the spectrin membrane cytoskeleton during the final stages of coated pit budding.[31–33]

The annexins found in this study (annexin A1 (synonyms: annexin I/lipocortin 1)), annexin A2 (annexin II/calpactin 1), annexin A5 (annexin V), and annexin A6 (annexin VI)) may regulate membrane-cytoskeleton dynamics and besides being involved in membrane-fusion events between intracellular compartments, they play a role in the inward vesiculation process.[34]

We and others could not detect tetraspanin molecules by mass spectrometry techniques.[35,36] However, immunogold electron microscopy using an anti-CD63 (specific tetraspanin marker of late endosomes) antibody showed the presence of this protein at the exosome surface suggesting it was under the threshold of detection of the SDS-PAGE/MALDI-TOF technique.

Plasmalemma vesicle-associated protein (PLVAP) or PV-1 is a caveolae-specific glycoprotein associated with stomatal diaphragms of caveolae, transendothelial channels, and fenestrae and is highly conserved across species.[37] While for the diaphragms of fenestrae a "sieving" function of the blood plasma components has been documented,[38] there is no data documenting the function of diaphragms of caveolae. PV-1 is anchored in the membrane and could participate in protein-protein interactions via the proline-rich region at the C terminus.[38] Results of strong affinity between PV-1 and heparin suggests that PV-1 may interact with heparan sulfate proteoglycans located on cell surfaces or in the extracellular matrix.[39] Although data of PV-1 expression on mesothelial cells are lacking, PV-1 is expressed in many types of multiple endocrine and endothelial cell types.[39] Our data provide evidence for the presence of PV-1 protein in exosomes from mesothelioma cell lines but the role of this protein both in exosomes and normal mesothelial cells is a matter under current investigation.

Cytoskeleton proteins as actin and tubulin give structure to the exosome together with the associated proteins ezrin, moesin, actinin-4, desmoplakin I, and fascin. Ezrin and moesin belong to the ERM family that attach actin filaments to transmembrane glycoproteins and thereby stabilize cell-surface protrusions.[40–42] α-actinin is a microfilament bundling and cross-linking protein that is ubiquitously expressed in numerous actin structures of virtually all cells. Four different isoforms encoding α-actinin have been identified in humans. α-actinin-1 exists as a non-muscle or a smooth muscle isoform, α-actinin-2 and -3 are skeleton muscle isoforms, and α-actinin-4 is a non-muscle isoform.[43–46] Although both are non-muscle isoforms, α-actinin-4 exerts contradictory functions from those of α-actinin-1 with respect to their involvement in cell movement. α-actinin-4 is described to be associated with enhanced cell motility and cancer invasion, especially in patients with a cytoplasmic localization of this protein.[46] After disassembly of the actin skeleton, α-actinin-4 may reorganize the cytoskeleton by cross-linking actin filaments, a process supposedly requisite for exosome formation. Recently, mutations in the ACTN4 gene, which codes for α-actinin-4, were reported to cause familial focal segmental glomerulosclerosis.[47] Although rarely detected in lung tumors, a point-mutation (adenine $\rightarrow$ thymine) in the ACTN4 gene of a non-small cell lung cancer (NSCLC) cell line resulting in an asparagine instead of lysine in actinin-4, leading to the expression of a tumor-specific antigen recognized by autologous cytotoxic T lymphocytes (CTL).[48–50] Preliminary data suggest that the mesothelioma cell line (PMR-MM7, HLA-A02, A68) is not recognized by the mutated actinin-4-specific CTL clone (HLA-A02, A68, under current investigation (F. Mami-Chouaib, Institut Gustave Roussy, Villejuif, France)).

Like actinin-4, fascin organizes actin filaments into bundles and is predominantly present in dendrites, microspikes, microvilli, filopodia, and pseudopodia (or called lamellipodia depending on the morphology) at the cell periphery and in stress fibers in some cells. The expression of fascin is described in cells that have the morphological characteristic of membrane protrusions in common, like glial and neuronal cells, microcapillary endothelial cells, and antigen-presenting dendritic cells. Therefore, it is suggested that fascin plays a role in extending the membrane either for cell motility or for interactions with other cell types. Fascin expression is dramatically increased during maturation of DC.[51] On maturation of DC and their travel to lymph nodes to present the antigen to T cells, numerous fascin-containing membrane extensions appear. At this stage, the secretion of exosomes by DC is increased.[9] High levels of fascin is also observed in many cancer cells and appears to be correlated with aggressive cell behavior.[52,53] We suggest that fascin may also be involved in the inward budding of multi-vesicular bodies, which creates internal vesicles that after fusion with the plasma membrane leads to release of exosomes into the extracellular milieu.

Desmoplakin is required for assembly of functional desmosomes (a type of junction that attaches one cell to its neighbor) during epithelial sheet formation, maintaining cytoskeletal architecture, and reinforcing membrane attachments essential for stable intercellular adhesion.[54] This intracellular anchor protein is responsible for connecting the cytoskeleton to transmembrane adhesion proteins.

Molecular motor proteins mediate the intracellular transport of membrane-enclosed organelles. Kinesin-like protein, a microtubule-based motor protein, was found.

The membrane orientation of exosomes is identical to that of cells, during their formation cytoplasm is included

into the vesicle. The metabolic enzymes, glyceraldehyde 3-phosphate dehydrogenase, enolase-1, and pyruvate kinase, are involved in the glycolysis. This process in which glucose is converted into pyruvate with the concomitant production of ATP occurs in the cytosol. Regulation of the production of cyclic AMP is done by 2′3′-cyclic-nucleotide 3′ phosphodiesterase. The phenomenon of increased expression of glucose transporters and glycolytic enzymes in tumor cells was described as the Warburg effect[55] and is one of the most universal characteristics of solid tumors.[56–58] The genes of these products are also found to be up-regulated in expression mapping in mesothelioma oncogenesis (Singhal S, personal communication). High amounts of glycolytic enzymes in the cytoplasm of cells will thus be reflected by the presence of these enzymes in exosomes. There are no data published on the distribution and function of the putative P150 protein (code O00378).

Some of the proteins present in mesothelioma-derived exosomes like MHC class I, HSC70, HSP90α, annexins (A1, A2, A5, A6), actin, and tubulin were also described to be present in B cell-derived exosomes[35] and dendritic cell-derived exosomes.[5] Furthermore, proteomic analysis of B cell-derived exosomes revealed the presence of moesin, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, and enolase in common with our results on mesothelioma-derived exosomes.[35] Common proteins with intestinal epithelial cell exosomes are MHC class I, actin, tubulin, and enolase-1 and with other tumor-derived exosomes are MHC class I and HSC70.[59]

Most interesting was the detection of a protein, not described previously on exosomes, a precursor of the developmental endothelial locus-1 (DEL-1) protein. DEL-1 has structural homology to lactadherin and has a regulatory function in vascular remodeling during embryo genesis.[60] It is described as an extracellular matrix protein that promotes adhesion of endothelial cells via the $\alpha v\beta 3$ integrin receptor present on endothelial cells. The $\alpha v\beta 3$ integrin receptor is also present on dendritic cells and mediates the uptake of apoptotic vesicles, important for cross priming of tumor antigens. It is speculative that DEL-1 on tumor-exosomes is important for targeting exosomes to DC for cross-presentation. Recently, Aoka et al[61,62] suggested that DEL-1 acts as an angiogenic factor in the context of solid tumor formation and that the increase in vascular development accelerates tumor growth through decreased apoptosis. The role of DEL-1 in tumor exosomes can be bilateral first, attachment to dendritic cells and secondly, to increase the vascular development in the neighborhood of the tumor. Exosomes may also bind to extracellular matrix components by fibronectin, a ligand for integrins.

Proteomic analysis using MALDI-TOF mass spectrometry revealed several new proteins not previously described on (tumor) exosomes or for mesothelioma cell lines. In conclusion, mesothelioma cell line-derived exosomes express a discrete set of proteins involved in antigen presentation, signal transduction, migration, and adhesion and thereby may be an important pathway in the communication between cells.

Acknowledgments

We thank Hans Dalebout, Lies-Anne Severijnen, and Janice Griffith for their assistance in the MALDI-TOF mass spectrometry studies and electron microscopy studies, respectively. We also thank the Department of Clinical Genetics of the Erasmus MC for karyotyping the mesothelioma cell lines.

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Chapter 9

9

Mesothelioma environment comprises cytokines and T-regulatory cells that suppress immune responses

European Respiratory Journal 2006; jun 27(6): 1086-1095

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Mesothelioma environment comprises cytokines and T-regulatory cells that suppress immune responses

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ABSTRACT: Malignant mesothelioma is a cancer with dismal prognosis. The objective of the present study was to address the role of the immune system, tumour micro-environment and potential immunosuppression in mesothelioma.

Expression profiles of 80 cytokines were determined in the supernatant of mesothelioma cell lines and the original patient's pleural effusion. Influx of immune effector cells was detected by immunohistochemistry.

Angiogenin, vascular endothelial growth factor, transforming growth factor-β, epithelial neutrophil-activating protein-78 and several other proteins involved in immune suppression, angiogenesis and plasma extravasation could be detected in both supernatant and pleural effusion. Surrounding stroma and/or infiltrating cells were the most likely source of hepatocyte growth factor, macrophage inflammatory protein (MIP)-1δ, MIP-3α, neutrophil-activating peptide-2, and pulmonary and activation-regulated chemokine that can cause leukocyte infiltration and activation. There was a massive influx of CD4+ and CD8+ T-lymphocytes and macrophages, but not of dendritic cells, in human mesothelioma biopsies. It was further demonstrated that human mesothelioma tissue contained significant amounts of Foxp3+CD4+CD25+ regulatory T-cells. When these CD25+ regulatory T-cells were depleted in an *in vivo* mouse model, survival increased.

Mesothelioma is infiltrated by immune effector cells but also contains cytokines and regulatory T-cells that suppress an efficient immune response. Immunotherapy of mesothelioma might be more effective when combined with drugs that eliminate or control regulatory T-cells.

KEYWORDS: Cancer, Foxp3, immunosuppression, immunotherapy, micro-environment

Malignant mesothelioma (MM) is a highly aggressive neoplasm most often seen in patients with a history of asbestos exposure. There is a latency period of 20–40 yrs between the exposure to asbestos fibres and the first symptoms of disease. With median survival durations of 9–12 months from onset of symptoms, the prognosis is poor. To date, there is no standard curative therapy for MM. Combined modality approaches, such as extrapleural pneumonectomy followed by radiochemotherapy, result in high local recurrence rates and questionable survival benefit [1]. As MM is a weak immunogenic tumour, various groups have attempted to perform immunotherapy using cytokines or adjuvants to boost tumour immunity, with varying success [1]. In a previous study, the present authors evaluated the therapeutic efficacy of tumour lysate-loaded antigen-presenting dendritic cells (DCs) given before and/or after an *i.p.* tumour challenge with the mouse mesothelioma cell line AB1. DCs pulsed with tumour lysate or exosomes were effective in inducing protective cytotoxic CD8 T-cell responses and increasing survival, even when given after tumour implantation [2]. In these studies, DC treatment had a better outcome when DCs were injected early in tumour development, indicating that tumour load played an important role in survival. Although the exact sequence of events in mesothelioma induction and progression is still unknown, a range of defects that develop both inside and outside the mesothelial cell could be involved in the escape of the tumour from immune destruction. According to the immune surveillance theory, large tumours escape immune recognition by downregulating major histocompatibility complex (MHC) class I or by altering expression of tumour antigens, thus leading to an escape from cytotoxic killing

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Received:
November 16 2005
Accepted after revision:
February 22 2006

SUPPORT STATEMENT
This study was financially supported by Stichting Asbestkanker (Rotterdam, the Netherlands) and Mesothelioma Applied Research Foundation (MARF; Santa Barbara, CA, USA).

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European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

by CD8 cells [3]. This theory has recently been challenged, as spontaneously arising tumours in mice remain immunogenic and, instead, escape immune recognition by inducing anergy in tumour-infiltrating lymphocytes [4] or by attracting regulatory T-cells (Treg) that suppress anti-tumoural responses. It is now well established that certain tumours and the surrounding stroma generate an immunosuppressive microenvironment to suppress the effector arm of the anti-tumoural immune response (cytotoxic T-lymphocyte response inside the tumour) and the inductive arm of the immune response, *i.e.* the potential of antigen-presenting DCs to induce cytotoxic T-lymphocyte responses.

The present study takes an unbiased approach, using a proteomics platform and determining the presence of an array of 80 cytokines and chemokines in mesothelioma cell lines and pleural fluids of the original patients from whom the cell lines were generated. This allowed the authors to study which factors were tumour derived and which were derived from infiltrating immune cells or surrounding stroma. Tumours were heavily infiltrated with CD4+ and CD8+ T-cells and macrophages, but DCs were strikingly absent. Surprisingly, Foxp3+ CD25+ Treg, which were previously shown to promote tumour progression in other cancer models, were found. Removal of these cells led to increased survival in a transplantable mouse model of mesothelioma. These findings suggest there are multiple levels by which MM escapes immune recognition.

METHODS

Patient material and preparation of cell lines for analysis

After obtaining informed consent, pleural fluid was collected from histologically proven mesothelioma patients (n=6) who presented with large pleural effusions. In most cases, indication for pleural fluid evacuation was exertional dyspnoea relief. All patients were 67–88-yr-old males who were treated with best supportive care. Thoracocentesis was performed using fine-needle aspiration inserted into the pleural cavity and collected in sterile tubes without anticoagulant. Pleural cells were removed from pleural effusions using centrifugation at 3,000×*g* for 20 min at 4°C, and the supernatant was stored in aliquots at -80°C. Four long-term mesothelioma cell lines were generated from these pleural effusions and extensively characterised as described earlier [5]. This included determining the cellular DNA content, immunohistochemistry, tumourigenicity *in vivo*, virus and/or bacterial contamination, karyotyping, and human leukocyte antigen typing. Cell lines were kept in long-term cell culture (>50 passages) before using for supernatant testing. Pleural fluid from these patients was still available for analysis.

In a separate group of patients (n=4), tumour biopsies were obtained through medical thoracoscopy and processed for immunohistology.

Proteomics platform to analyse cytokines and chemokines

An antibody-based cytokine array system (RayBiotech, Inc., Norcross, GA, USA) was used to determine cytokine expression profiles in supernatant of mesothelioma cell lines (n=4) and the corresponding patient's pleural effusions (n=6). Cell lines were grown in T175 culture flasks to 80% confluence. Medium was then replaced by 12-mL RPMI containing 1% foetal calf serum and incubated at 37°C. The following day, supernatant was collected and centrifuged for 20 min at 3,000×*g* to remove the cells. Samples were concentrated using Centricon YM3 columns (Millipore, Billerica, MA, USA). As a negative control, 12 mL of the previously mentioned medium was prepared in the same way as the cell supernatant. Of these concentrated samples, 1 mL were applied to the RayBio membranes (human cytokine array V; table 1). The detection was performed according the manufacturer's protocol. Quantification of cytokine expression was performed in duplicate by two independent observers (J.P.J.J. Hegmans and A. Hemmes) using a standard scale of six spots with increasing density (score 0 to 5; landing lights (positive controls) were scored as 4).

Immunohistology on tumour biopsies

Tumour biopsies were taken using medical thoracoscopy and embedded in Tissue-Tek II optimum cutting temperature medium (Miles, Naperville, IL, USA), snap-frozen and stored at -80°C. Tissue sections (6 μm) were cut on an HM-560 cryostat (Microm, Heidelberg, Germany) and immunostaining was carried out using antibodies (table 2). Binding of antibodies was detected using the immuno-alkaline phosphatase (AP) anti-alkaline phosphatase (APAAP) method (DAKO, Glostrup, Denmark). Naphtol-AS-MX-phosphate (0.30 $mg \cdot mL^{-1}$; Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) and new fuchsine (160 $mg \cdot mL^{-1}$ in 2 M HCl; Chroma-Gesellschaft, Köngen, Germany) were used as substrate. The specificity of the antibodies was checked using a protein concentration-matched nonrelevant monoclonal antibody and PBS. Double staining of Foxp3 (rat immunoglobulin (Ig)G2a) and CD3 or CD25 (both mouse IgG1) was performed using AP-conjugated goat anti-rat (Sigma-Aldrich Chemie B.V.) and rat APAAP (DAKO) followed by horseradish peroxidase conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL, USA). Naphtol-AS-MX-phosphate and 1 mM Fast Blue substrate were used as substrate for AP and NovaRed was used as substrate for horseradish peroxidise, according to the manufacturer's instructions (Vector, Burlingame, CA, USA). Alexa Fluor 647 labelled anti-human CD4 and fluorescein isothiocyanate conjugated CD25 (both BD Biosciences, San Jose, CA, USA) were used at a dilution of 1:100 and 1:20, respectively. Signals were captured on a Zeiss confocal laser-scanning microscope (LSM510NLO; Carl Zeiss B.V., Sliedrecht, the Netherlands).

Tumour growth of murine mesothelioma after in vivo *depletion of CD4+CD25+ T-cells in BALB/c mice*

Female 6–10-week-old BALB/c (H-2d) mice (Harlan, Zeist, the Netherlands) were housed under pathogen-free conditions at the animal care facility of Erasmus MC (Rotterdam, the Netherlands). Experiments were approved by the local ethical committee for animal welfare (Erasmus University Committee of Animal Experts, Rotterdam, the Netherlands) and complied with the Guidelines for the Welfare of Animals in Experimental Neoplasia by the United Kingdom Coordinating Committee on Cancer Research. The AB1 cell line, a mouse mesothelioma cell line, was kindly provided by B.W.S. Robinson (School of Medicine and Pharmacology, University of Western Australia, Sir Charles Gairdner Hospital, Perth, Australia). For *in vivo* depletion of CD4+CD25+ T-cells, 0.5 mL

TABLE 1 The 80 cytokines probed for on the RayBio human cytokine array membranes

Cytokine	Abbreviation	Systemic name
Angiogenin	Ang	
B-lymphocyte chemoattractant	BLC	CXCL13
Brain-derived neurotrophic factor	BDNF	
Chemokine-β-8-1	Ck-β-8-1	CCL23
Eotaxin, eotaxin-2 and -3		CCL11, CCL24, CCL26
Epidermal growth factor	EGF	
Epithelial neutrophil-activating protein-78	ENA-78	CXCL5
Fibroblast growth factor-4 to -9	FGF-4 to -9	
Fms-like tyrosine kinase-3 ligand	Flt-3 ligand	
Fractalkine	FKN	CX3CL1
Glial-derived neurotrophic factor	GDNF	
Granulocyte chemotactic protein-2	GCP-2	CXCL6
Granulocyte colony-stimulating factor	GCSF	
Granulocyte-macrophage colony-stimulating factor	GM-CSF	
Growth-related oncogene and growth-related oncogene-α	GRO and GRO-α	CXCL1
Haematopoietic growth factors, hepatocyte growth factor	Both HGF	
I-309		CCL1
Insulin-like growth factor-1	IGF-1	
Insulin-like growth factor binding protein-1 to -4	IGFBP-1 to -4	
Interferon-γ	IFN-γ	
Interferon-γ-inducible protein-10	IP-10	CXCL10
Interleukin-1α, interleukin-1β, interleukin-2 to -16	IL-1α, IL-1β, IL-2 to -16	
Leptin		
Leukaemia inhibitory factor	LIF	
Lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed on T-lymphocytes	LIGHT	
Macrophage colony-stimulating factor	MCSF	
Macrophage-derived chemokine	MDC	CCL22
Macrophage inflammatory protein-1β, -1δ, -3α	MIP-1β, -1δ, -3α	CCL4, CCL15, CCL-20
Mesoderm-inducing factor	MIF	
Monocyte chemoattractant protein-1 to -4	MCP-1 to -4	CCL2, CCL8, CCL7, CCL13
Monokine induced by IFN-γ	MIG	CXCL9
Neurotrophin-3 and -4	NT-3 and -4	
Neutrophil-activating peptide-2	NAP-2	CXCL7
Oncostatin M	OSM	
Osteoprotegerin	OPG	
Placenta growth factor	PlGF	
Platelet-derived growth factor-BB	PDGF-BB	
Pulmonary and activation-regulated chemokine	PARC	CCL18
Regulated on activation, normal T-cell expressed and secreted	RANTES	CCL5
Stem cell factor	SCF	
Stromal cell-derived factor-1	SDF-1	CXCL12
Thrombopoietin	TPO	
Thymus and activation-regulated chemokine	TARC	CCL17
Tissue inhibitor of metalloproteinase-1 and -2	TIMP-1 and -2	
Transforming growth factor-β1 to -β3	TGF-β1 to β3	
Tumour necrosis factor-α and –β	TNF-α and -β	
Vascular endothelial growth factor	VEGF	

of anti-CD25 antibody (PC61) ascites fluid (kindly provided by G. Oldenhove, Université Libre de Bruxelles, Brussels, Belgium) was given intraperitoneally 25 days before tumour cell inoculation to each mouse (n=12). The ascitis fluid was purified by affinity chromatography on protein A-Sepharose 4B as described by LOWENTHAL *et al*. [6]. As a control the authors used 0.5 mL of PBS (n=12). On day 0, mice were subjected to a lethal dose of 0.5×10^6 AB1 tumour cells. The occurrence of tumour growth, body weight, physical well-being and survival were measured for 2 months, as described previously [2].

TABLE 2 Source and specificity of antibodies used for the immunohistochemical staining of mesothelioma sections

Antibody	Present on	Source
BDCA2	Plasmacytoid DCs	Miltenyi Biotech
BMK-13	Resting and activated eosinophils	Monosan/Sanbio
CD1a	DC	DAKO
CD3	pan-T lymphocytes	DAKO
CD4	T-helper/inducer lymphocytes, monocytes	DAKO
CD8	T-suppressor/cytotoxic lymphocytes	DAKO
CD11c	Monocytes, granulocytes, NK cells, macrophages, DC	BD Biosciences
CD14	Monocytes (macrophages/granulocytes)	DAKO
CD15	Neutrophils (monocytes)	DAKO
CD16	NK cells, neutrophils and basophils	DAKO
CD24	B-cells, neutrophils, DCs	DAKO
CD25	Activated T-cells and, at a lower density, activated B-cells	DAKO
CD31	Blood vessels and microvessels	DAKO
CD68	Macrophages (antigen-presenting cells)	DAKO
CD209	Monocyte-derived DCs (DC-specific ICAM-3-grabbing nonintegrin)	R&D Systems
Chymase	Mast cells	Chemicon
Foxp3	Regulatory T-cells	eBioscience
HBME1	Mesothelial cells	DAKO
HLA DR DQ DP	MHC class II-expressing cells	DAKO
RCK108	Normal and malignant epithelial cells	DAKO
5B5	Fibroblasts	DAKO

DC: dendritic cell; NK: natural killer; ICAM: intercellular adhesion molecule; MHC: major histocompatibility complex. The location details of each manufacturer are as follows. Miltenyi Biotech: Bergisch Gladback, Germany; Monosan/Sanbio: Uden, the Netherlands; DAKO: Glostrup, Denmark; BD Biosciences: San Jose, CA, USA; R&D Systems: Minneapolis, MN, USA; Chemicon: Temecula, CA, USA; eBioscience: San Diego, CA, USA.

Statistical analysis

Data are expressed as mean±SD. Comparisons between groups were made using the Mann–Whitney U-test for independent samples. A two-tailed p-value <0.05 was considered significant. Data presented as a percentage of tumour-free animals were analysed with Kaplan–Meier survival curves, using the log-rank test to determine statistical significance.

RESULTS

Expression profile of pleural effusions and cell line supernatants on cytokine and chemokine array

An antibody-based cytokine array system (RayBiotech, Inc.) was used to determine cytokine expression profiles in pleural effusion (n=6) and in the supernatant of mesothelioma cell lines derived from the same patients (n=4). No cytokine expression was found in the negative (medium) control. Cytokine expression levels in pleural effusions and corresponding supernatant of mesothelioma cell lines are summarised in table 3. Forty-nine cytokines were undetected in both effusion and supernatant of cultured mesothelioma cell lines. Hepatocyte growth factor (HGF), macrophage inflammatory protein (MIP)-1δ, MIP-3α, neutrophil-activating peptide (NAP)-2, and pulmonary and activation-regulated chemokine (PARC) were exclusively present in pleural effusions and were not detected in mesothelioma cell supernatant (fig. 1). Therefore, it is possible that these cytokines are produced and secreted in pleural effusions by stromal cells and/or inflammatory cells. Some effusions also expressed cytokine levels of eotaxin-2, interleukin (IL)-12, leptin, mesoderm-inducing factor (MIF) and oncostatin M. In contrast, supernatants of mesothelioma cell lines contain high levels of growth-related oncogene (GRO) and RANTES (regulated on activation, normal T-cell expressed and secreted). These proteins seem to be mainly secreted by tumour cells and the amount present in effusion may thus be correlated with the amount of tumour cells.

Cellular recruitment in human mesothelioma tumours

The local release of cytokines and chemotactic factors by tumour cells and surrounding stroma suggests an accumulation of leukocytic infiltrate in the vicinity of the tumour by recruitment from circulating blood cells. Immunohistochemical techniques were therefore performed to determine the inflammatory component in solid tumour tissue. Mesothelioma tumour tissue can be regarded as complex tissues, composed of localised HBME-1 positive tumour fields, separated by stroma and many CD31+ blood vessels (fig. 2). Although some patient-to-patient heterogeneity was noted, leukocyte infiltration was always detected. Macrophages (CD68) and natural killer (NK) cells (CD16) constitute the major part of the inflammatory cell infiltration. Interactions between cancer cells and host immune T-cells (pan-T (CD3), T-helper/inducer (CD4), and T-suppressor/cytotoxic lymphocytes (CD8)) were visualised inside, at the rim and in the stroma of mesothelioma specimens. However, DCs (using antibodies for Langerin, fascin, S100, CD1a, BDCA2 and CD209), eosinophils (BMK-13), mast cells (anti-chymase), B-cells (CD24), and neutrophils (CD15) were rarely detected (fig. 3).

TABLE 3 Cytokines investigated in pleural effusions and corresponding mesothelioma cell lines

Detected[#]	Mean PF±SD:mean SN±SD
PF ↑ ↑	
HGF	1.50±1.50:0
MIP-1δ	1.67±0.52:0*
MIP-3α	0.83±1.33:0
NAP-2	2.33±0.52:0*
PARC	3.50±0.55:0*
PF ↑	
Ang	3.17±0.41:2.00±0*
Eotaxin-2	0.33±0.84:0
IGFBP-1	1.50±1.05:0.50±0.57
IGFBP-2	1.83±0.98:1.25±1.5
IL-12	0.17±0.41:0
IP-10	1.00±0.89:0.75±1.50
Leptin	0.50±0.84:0
MIF	0.67±1.04:0.25±0.5
OSM	0.17±0.41:0
PIGF	0.83±0.75:0.50±1.00
PF ↔ SN	
Osteoprotegrin	0.83±0.75:0.75±0.50
VEGF	0.67±0.82:0.75±0.96
MCP-1	2.33±0.51:2.50±1.73
TGF-β2	0.17±0.41:0.25±0.50
ENA-78	0.50±1.22:0.50±1.00
TIMP-1	2.67±0.51:2.50±1.29
IL-6	4.50±0.84:3.50±2.38
MIP-1β	1.00±0.63:0.75±1.50
SN ↑	
BDNF	0:0.25±0.50
IGFBP-3	0.33±0.51:0.75±0.50
IGFBP-4	0.83±0.75:1.25±0.96
TIMP-2	2.50±0.55:3.25±0.50
IL-8	3.66±1.51:4.25±0.50
SN ↑ ↑	
GRO	1.50±0.84:2.75±0.5*
RANTES	0.17±0.41:1.25±0.96

For cytokine abbreviations see table 1. PF: pleural fluid; SN: cell line supernatant. ↑ ↑ : strong increase; ↑ : moderate increase; ↔: equally expressed in PF and SN. [#]: cytokines that were not detected were BLC, Ck-β-8-1, EGF, eotaxin, eotaxin-3, FGF-4 to -9 Flt-3 ligand, fractalkine, GCP-2, GCSF, GDNF, GM-CSF, GRO-α, I-309, IFN-γ, IGF-1, IL-1α, -1β and -2 to -16, LIF, LIGHT, MCP-2 to -4, MCSF, MDC, MIG, NT-3 and -4, PDGF-B, SCF, SDF-1, TARC, TGF-β1 and -β3, TNF-α and -β, and TPO. *: $p<0.05$.

Treg can be discriminated based on the expression of CD4 and CD25, combined with the transcription factor Foxp3. Mesothelioma tissue sections were analysed by fluorescence microscopy for the phenotypic evidence of CD4+CD25+ double positive cells. T-cells were particularly detected at the rim of tumour areas CD4+CD25+ (fig. 4g and h). The transcription factor Foxp3, identified by SAKAGUCHI *et al.* [7] as a hallmark of naturally arising CD4+CD25+ Treg cells, confirmed the presence of these cells in the vicinity of the tumour (fig. 4a). Double stainings showed that the expression of Foxp3 strongly correlated with CD3 (fig. 4b) and CD25 expression (fig. 4c and d).

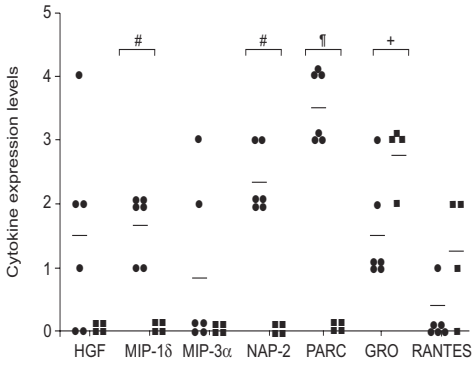

FIGURE 1. Expression levels of cytokines present in effusion (●) and in the supernatant of mesothelioma cell lines (■) assayed by human cytokine antibody arrays. The relative expression levels were determined by density scoring as described in the Methods section. Horizontal bars represent mean values. HGF: hepatocyte growth factor; MIP: macrophage inflammatory protein; NAP: neutrophil-activating peptide; PARC: pulmonary and activation-regulated chemokine; GRO: growth-related oncogene; RANTES: regulated on activation, normal T-cell expressed and secreted. [#]: $p=0.006$; [¶]: $p=0.007$; [+]: $p=0.040$.

Effects of in vivo *CD4+CD25+ T-cell depletion on tumour growth*

The mesothelioma mouse model described in a previous study [2] was used to examine the impact of CD4+CD25+ T-cell depletion on tumour progression. Preliminary studies indicated that in BALB/c mice, CD25 expression was restricted to CD4+ T-cells and represented a small fraction of total cells in the blood, lymph nodes and spleen (2–10%). Kinetic studies have shown that injection of the depleting antibody led to the selective loss of CD4+CD25+ T-cells for ⩾30 days and that replenishment of the population was observed 50 days after treatment [8]. The present authors first investigated the effect of *in vivo* administration of anti-CD25 ascites fluid on the CD25+ population in blood, as revealed by flow cytometry. After a single *i.p.* injection of 0.5 mL PC61 ascites fluid, this population of cells decreased in blood from 3.5 to 1% after 25 days (fig. 5).

In this protocol, BALB/c mice were injected *i.p.* with PBS or PC61 ascites fluid 25 days before tumour cell inoculation. On day 0, all mice were injected *i.p.* with a lethal dose of 0.5×10^6 AB1 tumour cells. First signs of terminal illness (typically formation of ascites, ruffled hair or marked loss of condition) appeared after 6 days in both groups (fig. 6). Mice were subjected to extensive autopsy that always showed solid tumour formation within the peritoneal cavity, accompanied in a few cases by thick, yellow-stained ascites. The nature of the solid tumours varied from numerous small nodules spreading throughout the mesentery and peritoneal lining to a single large mass. Within 40 days, all mice from the PBS group showed evidence of ill health or overt tumour growth. The administration of anti-CD25 antibodies prolonged the median survival from 19 to 33 days. Strikingly, five of the 12 mice (41%) treated with depleting antibody PC61 remained tumour-free for 2 months. Mice were then sacrificed and

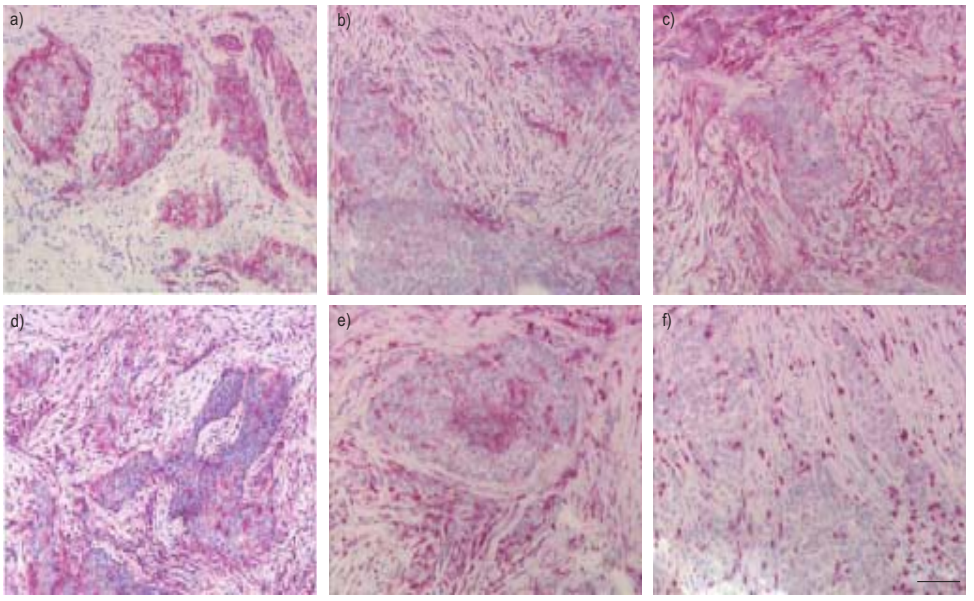

FIGURE 2. Tumour cells were localised in tumour fields as visualised by HBME-1 (a) and cytokeratin staining (RCK108; data not shown). Blood vessels (CD31; b) were present mainly at the periphery of the tumour and are considered a key step in tumour growth. Macrophages (CD68; c), natural killer cells (CD16; d), and T-lymphocytes, both T-helper/inducer (CD4; e) and T-suppressor/cytotoxic (CD8; f) cells, constitute the major part of the inflammatory cell infiltration. Scale bar=100 μm.

checked for tumour growth. No tissue abnormalities or formation of tumours could be detected.

DISCUSSION

Cancer, and in particular mesothelioma, is a chronic disease. When mesothelioma becomes clinically visible 20–40 yrs after asbestos exposure, tumour cells and their products have already been interacting with and affecting host cells for a considerable time to ensure the survival of the tumour. The paths that mesothelial cells take on their way to becoming malignant is unknown and probably highly variable depending on several host factors, including environmental factors, polymorphisms and mutations in susceptibility genes, age and immunity. Tumour development also depends on factors in the micro-environment. Interactions between malignant cells, stromal cells, extracellular-matrix components, various inflammatory cells, and a range of soluble mediators contribute to tumour development and progression. Mesothelioma tumour of patients with advanced-stage disease is composed of more than just cancer cells; it consists of an intricate network of cell types, including endothelial cells that comprise blood vessels and stromal cells. Many immunological cell types surround and penetrate the cancer cell areas, yet tumours escape immune destruction. There is accumulating evidence that cancer cells can even recruit and subvert normal cell types to serve as active collaborators in their neoplastic programme [9]. Understanding these multiple factors that come into play at the tumour micro-environment level may help to better understand and design immunotherapy protocols. The present authors therefore took an unbiased look at which inflammatory cell types were present inside mesothelioma, and which cytokines and chemokines were produced by mesothelioma cell lines and were present in corresponding pleural fluid. One remarkable observation of the present authors' immunohistological studies was that mesothelioma is heavily infiltrated with many immune effector cells. Macrophages, NK cells, and

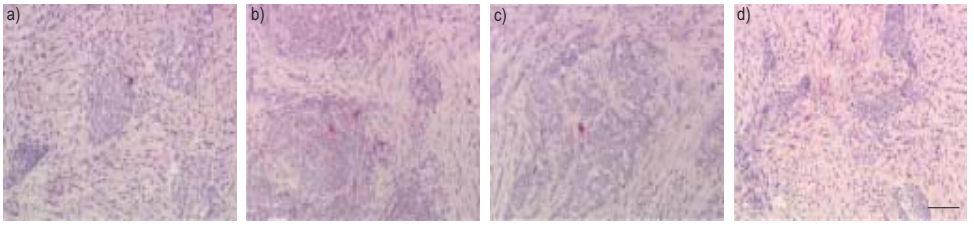

FIGURE 3. Dendritic cells (langerin (a), CD1a (b)), eosinophils (BMK-13; c) and B-cells (CD24; d) were rarely detected. Cells were counter-stained with haematoxylin. Scale bar=100 μm.

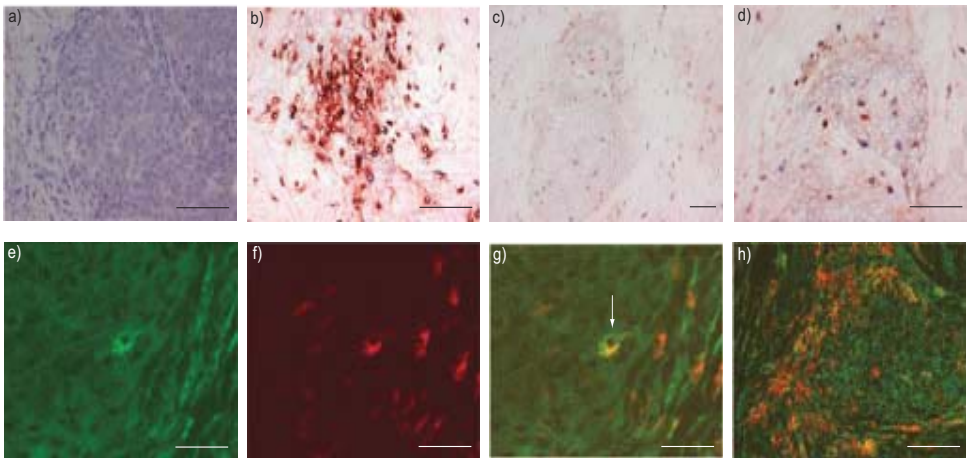

FIGURE 4. a) The transcription factor Foxp3 as a hallmark of naturally arising CD4+CD25+ T-regulatory cells was expressed in the vicinity of the tumour (red staining). Cells were counter-stained with haematoxylin. Expression of Foxp3 (blue staining) strongly correlates with b) CD3 (red staining) or c) and d) CD25 expression (red staining). There was no counter-staining in these cells. Fluorescent microscopy on mesothelioma tissue sections showing e) fluorescein isothiocyanate-labelled CD25+ cells, f) Alexa Fluor 647-labelled anti-human CD4+ cells and g) CD4+CD25+ double positive cells (indicated by arrow). g and h) show the appearance of CD4+CD25+ double positive cells at different magnifications. Scale bars=100 μm (a–d, h) and 25 μm (e–g).

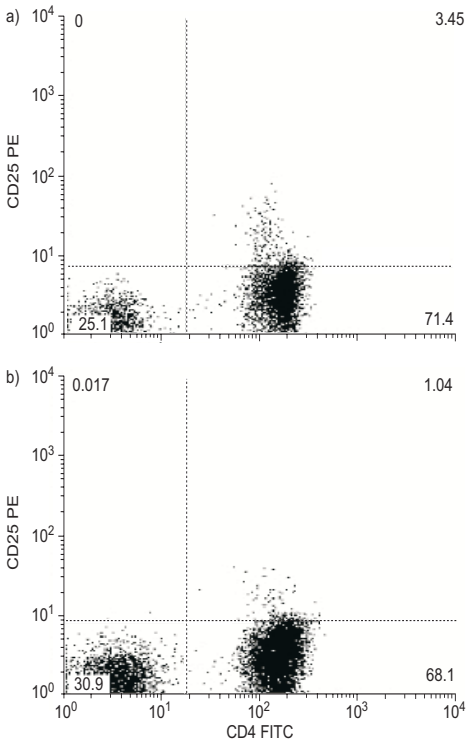

FIGURE 5. Flow cytometry analysis of blood from BALB/c mice that were a) untreated or b) treated with 0.5 mL PC61 ascites fluid on day 25 after antibody administration. PE: phycoerythrin; FITC: fluorescein isothiocyanate.

T-lymphocytes, both T-helper/inducer (CD4) and T-suppressor/cytotoxic (CD8) cells, constituted the major part of the inflammatory cells. Not surprisingly, the present protein array demonstrated the production of many chemokines in both MM cell line supernatant and pleural fluid (interferon-inducible protein-10, MIF, monocyte chemoattractant protein-1, epithelial neutrophil-activating protein-78, MIP-1β, IL-8, GRO, RANTES) or exclusively in pleural fluid (MIP-1δ, MIP-3α, NAP-2, and PARC) with the potential to attract these cell types.

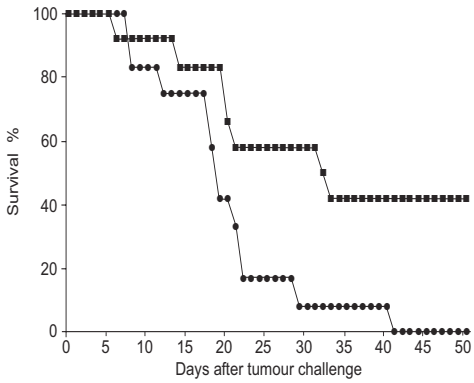

FIGURE 6. Kaplan–Meier survival plot showing the effect of depleting CD4+CD25+ T-cells by the administration of anti-CD25 antibody (PC61) in the development of malignant mesothelioma in a mouse model. Twenty-five days before tumour cell inoculation, mice were injected intraperitoneally with PBS (●, n=12) or depleting antibody PC61 (■, n=12). On day 0, mice were subjected to a lethal dose of 0.5×10^6 AB1 tumour cells. Mice were scored when profoundly ill to UK coordinating Committee on Cancer Research regulations and by the Code of Practice of the Dutch Veterinarian Inspection. $p<0.028$ determined by log-rank test.

The role of macrophages, T-cells and other immune cells in mesothelioma biopsies is still unknown. The influx, probably caused by changes in the microenvironment of the tumour, can favour inflammation, angiogenesis and tumour growth, thus leading to tumour progression. It can also negatively impact on tumour growth; for example, the presence of macrophages in tumour islets of nonsmall cell lung cancer (NSCLC) and the presence of infiltrating effector memory T-cells in colorectal cancer is associated with an increased survival [10, 11]. Understanding the outcome of the anti-tumoural immune response, leading to tumour progression or regression, and the role of individual cell types on outcome is of major importance for the design of future clinical trials.

Eosinophils, mast cells, B-cells and neutrophils were rarely detected. Mast cell infiltration and their density in tumour islets have been described as predictors of survival in NSCLC [10]. The difference in mast cell density in NSCLC and mesothelioma may be caused by discrepancies in the type, stage and size of the tumours and the methods used to assess these cells. WELSH *et al.* [10] used anti-tryptase to detect all mast cells, whereas the present authors stained for the chymase+ mast cell. This discrepancy between tryptase and chymase has also been illustrated in malignant breast tumours [12]. Clinical follow-up data comparing chymase+ and tryptase+ in human mesothelioma biopsies in combination with other immune cell markers may reveal more information regarding their prognostic significance.

As with several other cancer types, many immune effector cells were seen within the tumour whilst tumours were still not rejected. One of the aims of the current investigation was to determine the presence of factors within both the mesothelioma and surrounding stromal cells that could suppress the immune response to the tumour. In addition to factors involved in angiogenesis (angiogenin, vascular endothelial growth factor (VEGF), GRO) and leukocyte attraction (chemokines), several factors were discovered that might suppress the anti-tumoural immune response, either by suppressing the antigen-presenting capacity of DCs or the effector arm of the immune system. One of the factors found in pleural fluids of MM patients was HGF, also known as scatter factor. HGF is a multifunctional factor involved both in development and tissue repair, as well as pathological processes such as cancer and metastasis [13]. It was previously shown that HGF has a predominant role in mesothelioma cell invasion, simultaneously stimulating adhesion, motility, invasion and regulation of matrix metalloproteinase and tissue inhibitor of metalloproteinase levels [14]. Strikingly, it was shown that HGF has the potential to induce GRO and VEGF (also found in the present analysis) in a number of tumour models, including NSCLC [15]. HGF might be a critical mediator of immune suppression in MM as it was recently shown that HGF suppresses the maturation status and antigen-presenting capacity of lung DCs [16]. Similarly, the present authors discovered that VEGF was secreted by MM cell lines and was present in pleural effusions. VEGF's main role is inducing angiogenesis to the benefit of the tumour, but it has long been known to critically suppresses the function of DCs in inducing an anti-tumoural response by keeping these cells in an immature state or inhibiting their differentiation from monocytes [17].

Another striking observation of the present study was that CD1a+ DCs could not be found inside the MM biopsies taken. Additional DC markers (langerin, fascin, S100, BDCA2, and CD209) were applied because of the debate on the use of CD1a [18, 19], and confirmed this rare detection of DC in mesothelioma biopsies. This is in contrast to many other tumours, such as breast cancer or NSCLC, in which CD1a+ DCs are found within tumour lesions [20]. One possible explanation for the lack of DCs in MM would be the presence of high levels of IL-6 produced by the MM cells, as shown in the present study. IL-6 has also been shown by others to be universally expressed in MM cell lines [21]. IL-6 suppresses the development of DCs from CD34+ progenitors and from monocytes *in vitro* [22]. Studies in multiple myeloma patients have demonstrated that high-level IL-6 is responsible for a lack of circulating DCs in these patients [23]. In mice and humans, IL-6 also keeps DCs in a persistently immature state and promotes the differentiation of macrophages from monocytes [22]. The absence of DCs inside tumours has been shown to affect tumour progression in a number of cancers, including lung cancer [24]. DCs inside tumours might be crucial for activating effector CD4 and CD8 cells to exert their effector function locally inside the tumour [25]. The absence of DCs might partially explain why tumours are not killed despite the presence of effector cells inside the tumour. Consequently, increasing the levels of DC differentiation and growth factor granulocyte macrophage colony-stimulating factor have been used in a number of cancers as a strategy to enhance the anti-tumoural response [26].

Another explanation for the presence of immune effector cells inside tumours despite a lack of anti-tumoural response, is the presence of naturally occurring Treg cells that suppress the anti-tumoural T-cells. In a mouse model of spontaneous tumour development it was shown that this is one of the predominant ways in which tumours evade immune recognition [4]. Naturally occurring Treg cells play an important role in maintaining immunological balance by suppressing a wide variety of immune responses to self-antigens, infectious agents and tumours [27]. This subset of CD4+ T-cells express high levels of CD25 (IL-2 receptor α chain), are naturally anergic and require stimulation through the T-cell receptor for induction of their cell-mediated suppressive function. The forkhead transcription factor Foxp3 is particularly important in the development of these cells [28]. An increased frequency of Treg cells has been observed in peripheral blood and tissues of patients with cancer [29]. In ovarian carcinoma, high numbers of infiltrating CD4+CD25+Foxp3+ Treg cells were associated with worse prognosis, and these cells directly suppressed infiltrating CD4 and CD8 cell function [30]. Similarly, in the present study the authors have demonstrated that human mesothelioma biopsies harbour significant numbers of CD4+CD25+ T-cells, and that these CD25+ cells also express the Treg transcription factor Foxp3. Others studies have also described an increase in CD4+CD25+ cells in MM pleural effusion [31].

To address the function of these Treg cells, the present authors turned to the murine transplantable mesothelioma AB1 mouse model [2]. In this model, tumour growth was significantly reduced and survival increased when Treg cells were depleted using a CD25-depleting antibody prior to tumour

implantation. The median survival was prolonged from 19 to 33 days. It was noted that five of the 12 treated mice (41%) remained tumour-free for 2 months. This is reminiscent of other experimental tumour models in which Treg depletion using the same antibodies led to increased tumour rejection [32, 33]. One way in which Treg cells mediate their suppressive function is through signalling of the transforming growth factor (TGF)-β receptor of CD8 T-cells [34]. In the present study, TGF-β2 could be detected in the pleural fluid and MM supernatant, but it is not currently known whether TGF-β is produced by CD4+CD25+ Treg cells. Another suppressive cytokine produced by Treg cells is IL-10. Although the present authors were unable to detect IL-10 in the protein array, a weak staining for IL-10 on immunohistology was observed (data not shown). As well as Treg cells and immunosuppressive cytokines in the tumour micro-environment, there are other possible reasons why the immune response against mesothelioma is generally so ineffective. Low or absent production of specific antigens, crypticity of epitopes or downregulation of MHC expression keeps the immune system ignorant of the tumour. Presentation of antigen without adequate co-stimulation may induce clonal anergy or cause effector cells to develop a non-destructive response or one which leads to cell death. Future experiments in mice will explore the functional significance of these cytokines in Treg-mediated immunosuppression. Currently it can only be speculated why high levels of Treg cells with suppressive function are found inside mesothelioma. The present authors have previously described high levels of heat shock protein-70 in MM-derived tumour fractions [5]. It has shown that self-heat-shock protein-derived peptides have the potential to expand Foxp3+CD4+CD25+ Treg cells [35]. Moreover, high levels of cyclooxygenase 2 and prostaglandin-E_2 have been found in mesothelioma, and are correlated with worse prognosis [36]. Tumour-derived prostaglandin-E_2 specifically induces the Foxp3 gene expression and Treg cell function in human CD4+ CD25- T-cells [37].

One way in which the present data might be employed is in the design of better immunotherapeutic trials for mesothelioma. The present authors have recently shown that immunotherapy using tumour-pulsed dendritic cells is effective in preventing the outgrowth of murine mesothelioma, but that success is limited in mice with larger tumour burden [2]. Inhibiting the immunosuppressive milieu of the tumour by tumour debulking or by blocking vascular endothelial growth factor, interleukin-6 or hepatocyte growth factor activation and/or signalling might be used as an adjunct to dendritic cell immunotherapy. Even more challengingly, the inhibition of T-regulatory function by low-dose cyclophosphamide, specific Toll-like receptor 8 agonists, COX2 inhibition or immunotoxins directed at CD25 might be used in combination with dendritic cell immunotherapy to increase the success rate of mesothelioma tumour eradication. Clearly, pre-clinical studies in mouse malignant mesothelioma models will have to demonstrate the success of such a combined approach before a clinical trial is designed.

ACKNOWLEDGEMENTS

The authors would like to thank G. Oldenhove (Université Libre de Bruxelles, Brussels, Belgium) for providing PC61.

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Chapter 10

Immunotherapy of murine malignant mesothelioma using tumor lysate-pulsed dendritic cells

10

American Journal of Respiratory and Critical Care Medicine 2005; 171:1168-1177

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Immunotherapy of Murine Malignant Mesothelioma Using Tumor Lysate–pulsed Dendritic Cells

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Rationale: **Exploiting the immunostimulatory capacities of dendritic cells holds great promise for cancer immunotherapy. Currently, dendritic cell–based immunotherapy is evaluated clinically in a number of malignancies, including melanoma and urogenital and lung cancer, showing variable but promising results.** ***Objective:*** **To evaluate if pulsed dendritic cells induce protective immunity against malignant mesothelioma in a mouse model.** ***Methods:*** **Malignant mesothelioma was induced in mice by intraperitoneal injection of the AB1 mesothelioma cell line, leading to death within 28 days. For immunotherapy, dendritic cells were pulsed overnight either with AB1 tumor cell line lysate, AB1-derived exosomes, or *ex vivo* AB1 tumor lysate, and injected either before (Days −14 and −7) at the day of (Day 0) or after (Days +1 and +8) tumor implantation.** ***Main Results:*** **Mice receiving tumor lysate–pulsed dendritic cells before tumor implantation demonstrated protective antitumor immunity with prolonged survival (> 3 months) and even resisted secondary tumor challenge. Tumor protection was associated with strong tumor-specific cytotoxic T-lymphocyte responses. Adoptive transfer of splenocytes or purified $CD8^+$ T lymphocytes transferred tumor protection to unimmunized mice *in vivo*. When given after tumor implantation in a therapeutic setting, pulsed dendritic cells prevented mesothelioma outgrowth. With higher tumor load and delayed administration after tumor implantation, dendritic cells were no longer effective.** ***Conclusions:*** **We demonstrate in this murine model that immunotherapy using pulsed dendritic cells may emerge as a powerful tool to control mesothelioma outgrowth. In the future, immunotherapy using dendritic cells could be used as adjuvant to control local recurrence after multimodality treatment for malignant mesothelioma.**

Keywords: cancer; DC-based vaccines; dendritic cells; immunotherapy; mesothelioma

Malignant mesothelioma (MM) arises primarily from the surface serosal cells of the pleural, peritoneal, and pericardial cavities and is a highly aggressive neoplasm. MM of the pleura is most often seen in patients with a history of occupational asbestos exposure. Although the worldwide usage of asbestos has been reduced considerably, incidence and mortality related to MM continue to rise, because of the long latency period of 20 to 40 years between exposure and first symptoms (1, 2). With median survival durations of 10 to 17 months from onset of symptoms, the prognosis is poor (3, 4). To date, there is no standard curative therapy for MM. Surgical approaches such as pleurectomy and extrapleural pneumonectomy alone result in high local recurrence rates and questionable survival benefit. Additional treatments (chemotherapy, radiotherapy, gene therapy, photodynamic therapy, multimodality approaches) result in only limited improvements in response and survival (3, 5–9).

The possibility to harness the potency and specificity of the immune system underlies the growing interest in cancer immunotherapy. One such approach uses dendritic cells (DCs) to present tumor-associated antigens (TAA) and thereby generate tumor-specific immunity (10–12). DCs are extremely potent antigen-presenting cells specialized for inducing activation and proliferation of $CD8^+$ cytotoxic T lymphocytes (CTL) and helper $CD4^+$ lymphocytes (13). This unique property has prompted their recent application as therapeutic cancer vaccines. In the design and conduct of DC-based immunotherapy trials, several important considerations influence induction of a successful protective response (14). First is the source of tumor antigen that can be loaded onto DC. In case of unknown tumor antigens, as for MM, the source of antigen is, by necessity, a tumor cell lysate, apoptotic tumor cells, whole tumor–derived RNA, or tumor-derived exosomes (15). Second is the way in which DCs are activated, because immature DCs can tolerize the antitumoral response (16). Other important variables are dose, frequency, timing, and route of administration (17–22). Taking into account these variables, most studies have shown that injection of mature tumor antigen-pulsed autologous DCs into tumor-bearing hosts induces protective and therapeutic antitumor immunity in experimental animals and for some malignancies in patients (22, 23).

These promising results using DC-based immunotherapy have prompted us to test the hypothesis that autologous DC–presenting tumor antigen might also induce a protective immune response in MM. A mouse model for MM allowed us to prove this hypothesis and to study the impact of antigen source, DC maturation status, and timing of administration on outcome. Our results suggest that DC-based immunotherapy might be effective against this aggressive cancer in which TAA remain undefined. This study should pave the way for a clinical feasibility trial using autologous DCs as a therapeutic adjuvant in the treatment of patients with MM.

METHODS

See the online supplement for more details regarding laboratory animals and cell lines, chromium-release assay, IFN-γ enzyme-linked immunospot (ELISPOT), and the adoptive transfer of splenocytes and $CD8^+$ cytotoxic T cells.

Animals and Cell Lines

Animal experiments were approved by the local Ethical Committee for Animal Welfare and complied with the guidelines for the U.K. Coordinating Committee on Cancer Research (UKCCCR) (24), and by the Code of Practice of the Dutch Veterinarian Inspection. The AB1 cell line, a mouse mesothelioma cell line, was kindly provided by Professor Bruce W. S. Robinson of the Queen Elizabeth II Medical Centre, Nedlands, Australia.

(*Received in original form January 13, 2005; accepted in final form February 28, 2005*)

Supported by the Stichting Asbestkanker Rotterdam and the Mesothelioma Applied Research Foundation. B.N.L. is supported by a VIDI grant from the Dutch Organization for Scientific Research (NWO).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 171. pp 1168–1177, 2005
Originally Published in Press as DOI: 10.1164/rccm.200501-057OC on March 11, 2005
Internet address: www.atsjournals.org

Source of Tumor Antigen Derived from AB1 Tumor

A detailed description of the preparation of tumor antigens appears in the online supplement. The cell suspension of AB1 cells was disrupted by four cycles of freeze/thawing followed by sonication. For preparation of tumor cell lysate from established tumors *ex vivo*, tumors from eight mice with tumor growth were mechanically dispersed followed by freeze/thawing and sonication as previously described. AB1-derived exosomes were isolated as described earlier for human MM cell lines (25).

Culture Conditions of Bone Marrow–derived DC Subtypes Used for Vaccination

A description of the preparation of DC subtypes is detailed in the online supplement.

DC subtype I. These DCs were generated with only minor adaptations from a previously described protocol by Inaba and coworkers (26), and modified by De Veerman and coworkers (27). In short, the precursor DC population was obtained by flushing femurs and tibias of naive mice, depleted of red blood cells and purified using microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (26). The resultant population was cultured for 8 days in DC culture medium (*see* online supplement) and 10 ng/ml recombinant Flt3-L (kindly provided by C. Maliszewski, Amgen, Seattle, WA) (28).

DC subtype II. These were essentially identical to DC subtype I but no Flt3-L was added to the DC culture medium mix.

DC subtype III. These DCs were generated with only minor adaptations from a previously described protocol by Lutz and colleagues (28).

Tumor Antigen Loading and Induction of Maturation

After 8 days of culture in the previously described conditions, tumor cell lysate was added to the DC cultures, to the equivalent of three AB1 cells per DC. In most experiments, after 8 hours, 2 μg/ml CpG motifs (immunostimulatory sequences-oligodeoxynucleotides, a gift from Prof. E. Raz, University of California, San Diego, CA) were added in some of the cultures to allow complete maturation while incubated overnight. The quality of the DC preparation was determined by cell counting, morphologic analysis, and cell surface marker expression by flow cytometry, as previously described (29–32). Briefly, cells were stained with a monoclonal antibody mix containing major histocompatibility complex (MHC) II–fluorescein isothiocyanate, CD11c-allophycocyanin, and phycoerythrin-conjugated maturation markers CD80, CD86, and CD40. Dead cells were excluded by propidium iodide staining.

Experimental Protocols to Demonstrate Induction of Antitumoral Immunity

The next day, DCs for vaccinations were harvested by gentle pipetting and purified by Lympholyte-Mammal (Cedarlane, Hornby, ON, Canada) density gradient centrifugation, washed three times in phosphate-buffered saline (PBS), and resuspended at a concentration of 1×10^6 viable cells in 500 μl PBS. DCs were delivered into the peritoneal cavity of BALB/c mice; control mice received identical numbers of unpulsed DCs (PBS-DC) or PBS alone. The vaccinations were performed according to the following protocols (*see also* Table 1).

Protocol 1: treatment with tumor lysate–pulsed DCs before tumor implantation. On Day −14, 18 mice were vaccinated with 10^6 DC subtype I pulsed with AB1 tumor cell line lysate in 500 μl PBS, of which 12 mice received CpG motif–matured DCs. A corresponding group of mice was vaccinated with 10^6 unpulsed DC subtype I (n = 12) or PBS alone (n = 12). The vaccination procedure was repeated 1 week later (Day −7). On Day 0, the mice were inoculated into the peritoneum with 0.5×10^6 AB1 cells in 500 μl PBS. Mice were examined daily for evidence of ill health or overt tumor growth. Mice were killed if profoundly ill, according to UKCCCR regulations, and were scored as a death in survival analysis. All mice were underwent extensive autopsy. In mice that survived for a prolonged period after active DC immunotherapy, a second tumor challenge with 0.5×10^6 AB1 cells was given intraperitoneally after 3 months.

Protocol 2: treatment with tumor lysate–pulsed DCs at the day of tumor implantation. Two groups of 12 mice were injected intraperitoneally with 500 μl of a mixture of 10^6 CpG-maturated DC subtype I and 0.5×10^6 AB1 cells in PBS or AB1 cells alone, and the occurrence of tumor growth, body weight, physical well-being, and survival were followed.

Protocol 3: treatment with tumor lysate–pulsed DCs after tumor implantation and effect of DC subtypes on outcome. At 1 and 8 days after injection with 0.5×10^6 AB1 cells, two groups of 12 mice were injected with PBS, or CpG-matured DC subtype I, and the occurrence of tumor growth, body weight, physical well-being, and survival were measured for the next 3 months. To test whether the conditions of DC culture influenced the success of immunotherapy, two groups of six mice received CpG-matured DC subtype II and subtype III after tumor implantation.

Protocol 4: effect of high tumor load on success of treatment with tumor lysate–pulsed DCs after tumor implantation. On Days 1, 3, and 5 after intraperitoneal injection with 1×10^6 AB1 cells, mice (n = 4) were injected intraperitoneally with 1×10^6 CpG-maturated DC subtype III and the occurrence of tumor growth, body weight, physical well-being, and survival were measured.

Protocol 5: source of antigen used to pulse DCs. On Days 1 and 8 after intraperitoneal injection with 0.5×10^6 AB1 cells, mice (n = 6) were treated with 1×10^6 CpG-maturated DC subtype III loaded with AB1 tumor cell line lysate, *ex vivo* AB1 tumor lysate, or AB1-derived exosomes. The occurrence of tumor growth, body weight, physical well-being, and survival were measured for a month.

Statistical Analysis

Data presented as percentage of tumor-free animals were analyzed with Kaplan-Meier survival curves, using the log-rank test to determine statistical significance. Statistical analysis was performed using SPSS (SPSS, Inc., Chicago, IL).

RESULTS

Phenotype of Bone Marrow–derived DCs and Effects of Antigen Pulsing

As previously shown (26, 27, 33), culture of lineage-negative bone marrow cells in granulocyte-macrophage colony–stimulating factor (GM-CSF) and Flt3-L leads to DC differentiation, as shown by the almost universal expression of CD11c and MHC class II. To examine the phenotype of DCs after exposure to AB1 tumor cell line lysate or *ex vivo* tumor lysate, we performed flow cytometry using DC maturation markers CD40, CD80, and CD86. Moreover, the effect of adding the innate immune-activating unmethylated CpG motifs on DC maturation was investigated. As shown in Figure 1, the overnight addition of AB1 tumor cell lysate to bone marrow–derived DC subtype I at Day 9 of culture induced the upregulation of CD40 and CD80 (and also CD86, data not shown), compared with unpulsed DCs, in accordance with the induction of maturation. The addition of CpG motifs during this overnight period leads to an even further mature phenotype of DCs, expressing high levels of CD40, CD80, CD86, and MHC II. This effect of CpG and tumor cell line lysate or *ex vivo* tumor lysate occurred irrespective of whether DCs were grown in the absence of Flt3-L (DC subtype II) or were generated from total bone marrow cells in the presence of GM-CSF (DC subtype III). DC subtype I, whether exposed or not to CpG motifs to induce further maturation, were used for experiments exploring the potential of DC immunotherapy.

Immunization with Tumor Lysate–pulsed DCs before Tumor Implantation Prevents Mesothelioma Outgrowth

At Days 14 and 7 before tumor cell injection, naive mice were injected intraperitoneally with PBS, 1×10^6 untreated DC subtype I, or DC subtype I treated with AB1 tumor cell line lysate with or without CpG motifs (Figure 2). On Day 0, all mice were injected intraperitoneally with a lethal dose of 0.5×10^6 AB1 tumor cells. The first signs of terminal illness (typically formation of ascites, ruffled hair, or marked loss of condition) appeared after 10 days in the PBS-treated group receiving tumor challenge. Within 30 days, all mice from this group showed evidence of ill health or overt tumor growth. Mice were subjected to extensive autopsy, which always showed solid tumor formation within the

TABLE 1. SCHEMATIC REPRESENTATION OF THE FIVE VACCINATION PROTOCOLS TO DEMONSTRATE INDUCTION OF ANTITUMORAL IMMUNITY

Injection with DC Subtype* (*no. experiments* × *no. mice/experiment*)	AB1 Injected at Day 0/ Time Schedule of DC Injection
Protocol 1	
PBS (2 × 6)	Days −14 and −7
Unpulsed DCs (I) (2 × 6)	
AB1 tumor cell line lysate–pulsed DCs (I) (1 × 6)	
AB1 tumor cell line lysate–pulsed DCs + CpG (I) (2 × 6)	
Protocol 2	
PBS (2 × 6)	Day 0
AB1 tumor cell line lysate–pulsed DCs + CpG (I) (2 × 6)	
Protocol 3	
PBS (2 × 6)	Days +1 and +8
AB1 tumor cell line lysate–pulsed DCs + CpG (I) (2 × 6)	
AB1 tumor cell line lysate–pulsed DCs + CpG (II) (1 × 6)	
AB1 tumor cell line lysate–pulsed DCs + CpG (III) (1 × 6)	
Protocol 4	
PBS (1 × 4)	Day +1
AB1 tumor cell line lysate–pulsed DCs + CpG (III) (1 × 4)	Day +1
AB1 tumor cell line lysate–pulsed DCs + CpG (III) (1 × 4)	Day +3
AB1 tumor cell line lysate–pulsed DCs + CpG (III) (1 × 4)	Day +5
Protocol 5	
PBS (1 × 6)	Days +1 and +8
AB1 tumor cell line lysate–pulsed DCs + CpG (III) (1 × 6)	
AB1 *ex vivo* tumor lysate–pulsed DCs + CpG (III) (1 × 6)	
AB1-derived exosome–pulsed DCs + CpG (III) (1 × 6)	

Definition of abbreviations: DC = dendritic cell; PBS = phosphate-buffered saline.
* DC subtype (*see* Culture Conditions of Bone Marrow-derived DC Subtypes Used for Vaccination).

peritoneal cavity, which was accompanied in a few cases by thick, yellow-stained ascites. The nature of the solid tumors varied from numerous small nodules spreading throughout the mesentery and peritoneal lining to a single, large mass. Strikingly, mice immunized with AB1 tumor cell line lysate–pulsed DCs showed prolonged survival, and all mice remained tumor free for more than 3 months. The protective effect occurred both in the group receiving CpG-matured antigen-pulsed DCs and in the group receiving less mature, unmanipulated antigen-pulsed DCs. In a separate group of DC-protected mice, four mice were killed at 2 months and checked for tumor growth. No tissue abnormalities or formation of tumors could be detected. Enhanced survival in 5 of 12 mice (42%) was also seen in mice injected with unpulsed DCs, suggesting that these unpulsed DCs could induce tumor protection in a nonspecific manner.

To check if DC immunization induced longlasting protective immunity, some protected mice receiving DC immunotherapy and tumor challenge were injected for a second time 3 months after the first tumor challenge with a repeated injection of 0.5×10^6 AB1 tumor cells. In these protected mice, a second tumor challenge did not lead to MM outgrowth, and mice again survived for an extended time of at least 3 months (survival curve not shown).

Immunization with Tumor Lysate–pulsed DCs at the Day of Tumor Implantation Promotes Mesothelioma Outgrowth

Poor prognosis with accelerated death occurred when tumor lysate–pulsed CpG-matured DC subtype I was administered simultaneously with AB1 tumor cells through a single intraperitoneal injection. In animals treated in this way, median survival was 13 days, whereas in mice receiving only tumor challenge, median survival was 20 days (Figure 3).

Immunization with Tumor Lysate–pulsed DCs after Tumor Implantation Prevents Mesothelioma Outgrowth

We next examined if DC immunotherapy given after tumor challenge would inhibit MM outgrowth. When tumor lysate–pulsed CpG-matured DC subtype I was injected 1 and 8 days after a lethal dose of AB1 cells, survival was much improved. In DC-treated animals, median survival was more than 2 months (66% of animals were still alive at 2 months) compared with only 23 days in mice receiving only AB1 cells (Figure 4). We also tested whether different culture conditions used to generate DCs would lead to better success of immunotherapy and protect more mice from death. Treatment with tumor lysate–pulsed CpG-matured DC subtypes II and III enabled the complete control of MM outgrowth, with all mice surviving beyond 2 months.

We then examined the effect of prior tumor load on the success of DC immunotherapy. Because the previously described experiment showed that DC subtype III was the most effective to control MM outgrowth after tumor transplantation, we performed all succeeding experiments with this DC subtype. To allow faster tumor growth, we injected 1×10^6 instead of 0.5×10^6 AB1 tumor cells intraperitoneally, leading to death within 12 days (median survival, 10 days). Immunotherapy of MM had a better outcome when DCs were injected early in tumor development, indicating that tumor load plays an important role in survival (Figure 5). When DCs were injected 1 day after tumor cell injection, median survival was prolonged to 23 days. When DC immunotherapy was initiated 3 days after tumor implantation, median survival was 21 days, whereas when DC immunotherapy was delayed until 5 days after tumor implantation, median survival was only 13 days. In these experiments, where high tumor load was associated with delayed treatment with only one DC injection, death occurred in all animals by 35 days, irrespective of treatment.

Source of Antigen Used to Pulse DCs

Very few TAA have been described in MM, and therefore a source for exogenous tumor peptides is unavailable (34). Therefore, we tested the efficacy of DC immunotherapy using DCs loaded with different "crude" sources of tumor antigens, which include AB1 cell lysate, tumor tissue lysate isolated *ex vivo*, and AB1-derived exosomes. As seen in Figure 6, DC subtype III

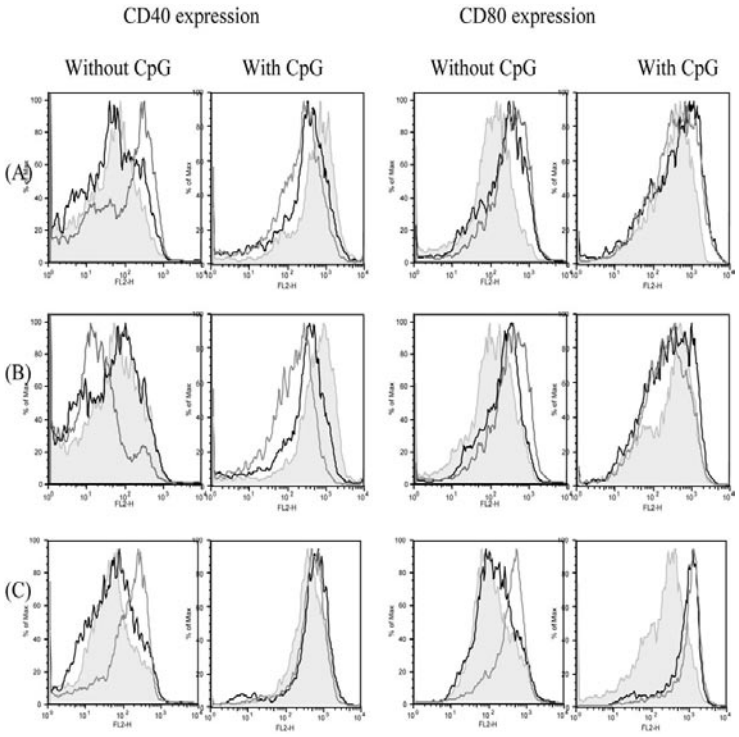

Figure 1. As assessed by flow cytometry, expression of the maturation markers CD40 and CD80 on dendritic cell (DC) subtype I (*A*), DC subtype II (*B*), and DC subtype III (*C*) unpulsed (*gray-filled*), pulsed with AB1 tumor cell line lysate (*gray line*), or pulsed with lysate extracted from established tumors *ex vivo* (*black line*). Expression of these maturation markers was also measured after the addition of CpG motifs.

pulsed with these different sources of antigen was also effective in prolonging survival when given 1 and 8 days after tumor implantation. In this experiment, AB1 cell line lysate–pulsed DCs induced the best overall survival, followed by *ex vivo* tumor cell lysate and AB1-derived exosome–pulsed DCs.

Successful Tumor Lysate–pulsed DC Immunotherapy Is Associated with Cytotoxic T-Cell Induction

Splenocytes obtained from protected animals that had resisted a tumor challenge and from naive mice were used in a ^{51}Cr-release assay and IFN-γ ELISPOT assay (Figure 7). AB1-specific CTL responses were measured in the ^{51}Cr-release assay (Figure 7A). Corrected percentage lysis of AB1 cells by splenocytes taken from protected mice was significantly elevated compared with naive mice (mean, 24% vs. < 1%; $p < 0.05$). The production of IFN-γ by splenocytes after DC immunotherapy and tumor protection was measured using ELISPOT. The number of IFN-γ–producing spleen (and lymph node) cells was markedly increased in protected mice, up to 20 times higher compared with naive mice (Figure 7B). The addition of tumor cells to the ELISPOT assay did not lead to a further enhancement of number of IFN-γ spots, illustrating that IFN-γ release was spontaneous after DC immunotherapy. Thus, both ^{51}Cr-release and IFN-γ ELISPOT supported an overall increase of CTL activity in DC-protected mice.

Transfer of Splenocytes or CD8$^+$ T Cells from DC Immunotherapy–protected Mice Transfers Tumor Protection

To prove that CD8$^+$ CTL induced by DCs were mediating protection from tumor outgrowth, we evaluated the efficacy of adoptive transfer of splenocytes and CD8$^+$ T cells in the prevention and treatment of MM (Figure 8). Intravenously injected splenocytes (10×10^6 cells) were given 7 days before and 2 days after a lethal dose of AB1 cells (Figure 8A). Splenocytes from protected mice dramatically increased the survival of mice compared with splenocytes from naive mice when injected 7 days before AB1 injection. Treatment with splenocytes 2 days after AB1 injection did not increase survival. Intravenously injected CD8$^+$ T cells increased survival when given 7 days before or 2 days after AB1 injection (Figure 8B).

DISCUSSION

There is no widely accepted curative approach for MM and treatment is usually complicated by a high local recurrence rate, despite aggressive surgery and novel attempts to improve local control (35). Multimodality approaches, including extrapleural pneumonectomy followed by chemoradiotherapy, have been of some benefit in prolonging survival of highly selected subgroups of patients, at the expense of considerable toxicity, but they have had a relatively small impact on the majority of the patients diagnosed (8). Therefore, new therapeutic strategies are urgently needed. Alternative therapies based on the intrapleural injection of adjuvants (e.g., bacille Calmette-Guérin) or photodynamic therapy using photosensitizers remain unsatisfactory because they have shown little potential for improving local control or overall survival, and often are quite toxic. Immunotherapy approaches, such as systemic, intrapleural, or intralesional administrations of interferons (IFN-α, IFN-β, IFN-γ) or interleukins (IL-2, GM-CSF, IL-12), are in an experimental stage for patients

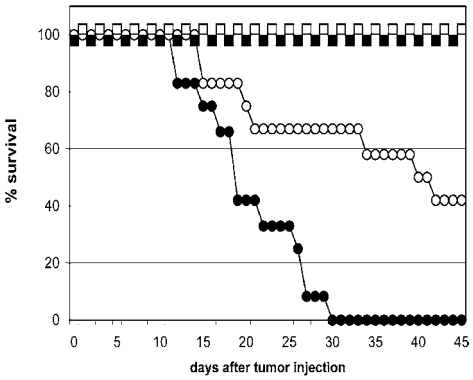

Figure 2. Effect of DC-based immunotherapy before tumor implantation (Protocol 1, *see* Table 1). Kaplan-Meier survival plot shows the effect of tumor lysate–pulsed DCs pretreatment in the prevention of MM outgrowth. On Days –14 and –7, mice were injected intraperitoneally with phosphate-buffered saline (PBS; *closed circles*, n = 12), unpulsed DCs (*open circles*, n = 12), or DCs pulsed with tumor lysate with or without CpG motifs (*open squares*, n = 12, and *closed squares*, n = 6, respectively). On Day 0, mice were subjected to a lethal dose of 0.5×10^6 AB1 tumor cells. Mice were scored when profoundly ill to U.K. Coordinating Committee on Cancer Research regulations and by the Code of Practice of the Dutch Veterinarian Inspection. There were no further changes in survival for up to 3 months in the treated mice. Tumor lysate–pulsed DCs (± CpG motifs), unpulsed DCs median survival of 39 days versus PBS median survival of 18 days; $p < 0.00001$ and $p < 0.0024$ by log-rank test.

with mesothelioma (36–41). Systemic administration of cytokines has resulted in considerable toxicity in both human and murine models and intrapleural delivery produces a localized immune reaction with tumor regression in only a minority of patients (39, 42, 43). The strategy of using gene therapy to directly introduce various cytokine genes into cells has been performed (40, 44, 45). It provides an increased local concentration of cytokines while minimizing the systemic toxicities, and significant tumor reductions in animals were demonstrated. However,

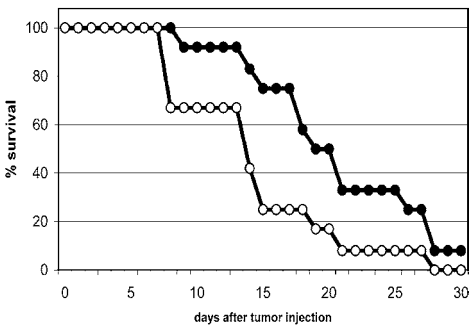

Figure 3. Simultaneous injection of tumor cells alone (*closed circles*, n = 12) or in combination with DC subtype I (*open circles*, n = 12; Protocol 2, *see* Table 1). $p < 0.0385$ by log-rank test.

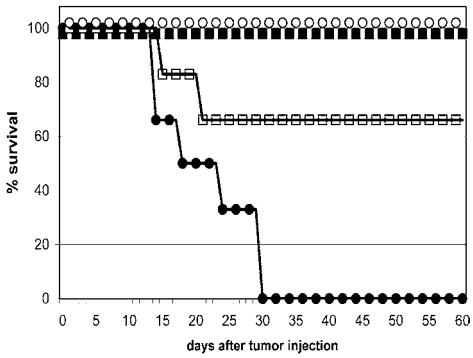

Figure 4. Kaplan-Meier survival plot shows the effect of tumor lysate–pulsed DCs in the treatment of malignant mesothelioma (MM; Protocol 3, *see* Table 1). At 1 and 8 days after injection of AB1 tumor cells, mice were injected intraperitoneally with PBS (*closed circles*, n = 6), DC subtype III (*open circles*, n = 6), or DC subtypes I and II cultured in the presence or absence of Flt3-L (*open squares*, n = 6, and *closed squares*, n = 6, respectively).Tumor lysate–pulsed DC subtype I and III, DC subtype II versus PBS DC; $p < 0.007$ and $p < 0.0439$ by log-rank test.

this research has not gone beyond the laboratory. Other studies are at the early stages of preliminary clinical trials and are not standard mesothelioma treatment. The major drawback of these strategies is that they are passive forms of immunotherapy, which will probably yield only temporary benefit, in contrast to strategies aimed at inducing an active immune response to cancer cells, such as vaccination strategies using stimulated DCs. Therefore, in this article, we have explored a new way of controlling the outgrowth of MM, which is to use the natural adjuvant capacities of DCs, the most powerful antigen-presenting cells of the immune system (13). DC-based immunotherapy is emerging as a non-toxic, efficient, and broadly useful immunotherapy strategy for the treatment of patients with cancer (46). DCs are instrumental in inducing activation and proliferation of $CD8^+$ CTL, some-

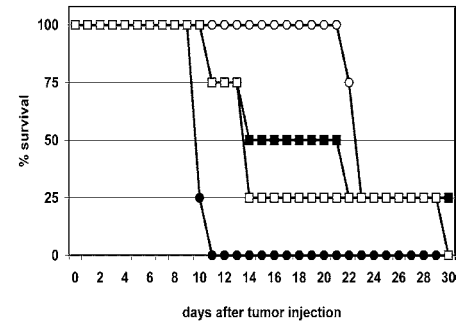

Figure 5. Kaplan-Meier survival plot shows the effect of tumor lysate–pulsed DCs in the treatment of MM (Protocol 4, *see* Table 1). On consecutive days after injection of 1×10^6 AB1 tumor cells, mice were injected intraperitoneally with PBS (*closed circles*, n = 4), DC subtype I on Day 1 (*open circles*, n = 4, $p < 0.0058$), Day 3 (*closed squares*, n = 4, $p < 0.0171$), or Day 5 (*open squares*, n = 4, $p < 0.0171$). p values defined by comparison with the untreated group.

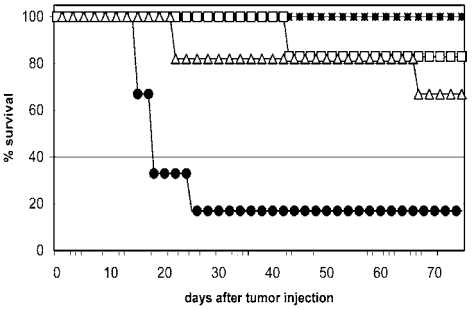

Figure 6. Kaplan-Meier survival plot showing the effect of injection on Days 1 and 8 after injection of a lethal dose of AB1 cells with DCs pulsed with the following: PBS (*closed circles*, n = 6), AB1 cell lysate (*closed squares*, n = 6), tumor tissue lysate (*open squares*, n = 6), and AB1-derived exosomes (*open triangles*, n = 6; Protocol 5, *see* Table 1). AB1 cell lysate–pulsed DCs, tumor tissue lysate–pulsed DCs, and AB1 exosome–pulsed DCs versus PBS-pulsed DCs; $p < 0.0043$, $p < 0.0101$, and $p < 0.0355$, respectively.

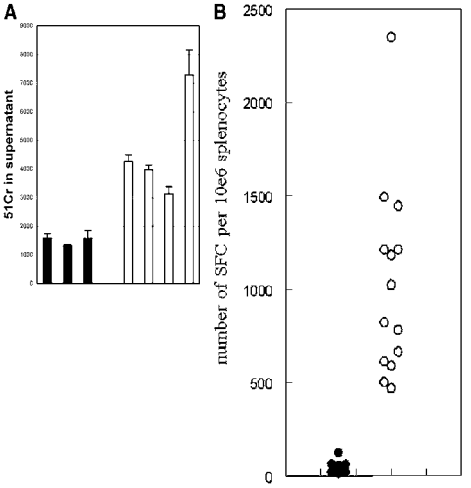

Figure 7. (*A*) The amount of ^{51}Cr released was determined from lysed AB1 target cells by splenocytes from naive mice (n = 3, *black bars*) and 2 months after tumor injection of DC-treated mice (n = 4, *white bars*). Percentage of lysis was calculated using the formula: corrected % lysis = 100 × (experimental release − spontaneous release [target cells incubated in medium alone])/(maximum release–spontaneous release). For naive mice, the corrected % lysis was 1, 0, and 1%, and for DC-treated mice, 21, 19, 13, and 44%, respectively. (*B*) The mean number of IFN-γ–producing spot-forming cells (SFC) in ELISPOT wells was enumerated with an automated spot-counting system and mean number of SFC per 10^6 splenocytes is shown (naive mice: *black dots*, n = 9; DC-treated mice: *white dots*, n = 14).

times even bypassing the need for CD4 help. In patients with cancer and in tumor-bearing mice, DC function is suppressed through release of tumor-derived soluble factors that inhibit the differentiation, maturation, and therefore immunostimulatory function of DCs, leading to a defective induction of CTL responses (47–53). A major advantage of DC-based immunotherapy is that DCs can be generated in large amounts *in vitro*, in the absence of this suppressing environment, and subsequently injected in a mature state to induce CTL responses.

It is now well established that tumor cells contain many antigens that can be recognized by the host immune system. DC immunotherapy was shown to be efficient as a cancer vaccine for tumors when pulsed with known TAA, such as the melanoma antigen–derived family of tumor antigens. In the case of tumors without known TAA, DCs have been pulsed with necrotic or apoptotic tumor material or with tumor-derived RNA, also leading to efficient immunity. For MM, few TAA are known like SV-40 large T antigen, a family of testis-associated antigens, Wilms' tumor-1 protein, mesothelin, calretinin, telomerase, survivin, and topoisomerase II, but these antigens are not expressed on the membranes of all tumors and are therefore not suitable as an antigen source for DC pulsing. None of these TAA have been evaluated as a source of peptides to pulse DCs or in a cancer vaccine trial (34, 54). Vaccinating against a single or a few TAA is limited by peptide restriction to a given HLA type and the induction of CTL without Th1 response. Also, tumor lysate–priming strategies are advantageous in providing the full antigenic repertoire of the tumor and, particularly, unique tumor antigens, which will theoretically decrease the ability of tumors to evade the immune response by downregulation of a single antigen. Ebstein and coworkers (34) have recently shown that human DCs pulsed with dead MM cells were able to induce a cytotoxic T-cell response *in vitro* directed against the tumor, particularly when DCs were loaded with apoptotic tumor material, illustrating that MM cells contain unknown TAA that can lead to an antitumoral immune response. Although this strategy was shown to be efficacious *in vitro*, it has not been shown that tumor antigen–pulsed DCs would have an antitumoral effect against MM *in vivo*.

Reliable animal models can provide useful preclinical information about DC immunotherapy and are critical for evaluating and defining tumor immunology paradigms because they provide an *in vivo* milieu that cannot be reproduced *in vitro* (55). The injection of crocidolite asbestos–induced AB1 mesothelioma cells into the peritoneal cavity of syngeneic mice provides a valid experimental model for human MM (56–60). This murine model demonstrates all the histologic and pathologic features of the human disease and, like most solid tumors, is only weakly immunogenic, without any known TAA. We used this MM model and demonstrated that DCs pulsed with lysed murine MM cells induced protective immunity to MM challenge *in vivo*. The first signs of terminal illness after intraperitoneal injection of 0.5×10^6 AB1 cells occurred between 2 to 4 weeks, but mice receiving tumor lysate–pulsed DCs were protected for months and even resisted a secondary challenge with tumor, illustrating the induction of long-lived immunity. In support of the induction of a systemic antitumoral immune response, we examined the lymph nodes and spleen to see if CTL activity was induced by DC immunotherapy in protected mice. The ^{51}Cr-release and IFN-γ ELISPOT assays are widely used for measuring antigen-specific CTL cytotoxicity and for immunologic monitoring of cancer vaccine trials (61, 62). Splenocytes obtained from treated animals lysed target AB1 tumor cells *in vitro* with enhanced efficacy, compared with naive animals, confirming the presence of enhanced numbers of CTL and/or natural killer (NK) cells. Moreover, after DC immunotherapy, there was a strong increase in the number of IFN-γ–producing cells in the spleen of protected mice, most likely activated CD4 and CD8 cells.

Strikingly, mice treated twice with unpulsed DCs before a lethal dose of tumor cells also showed prolonged survival in

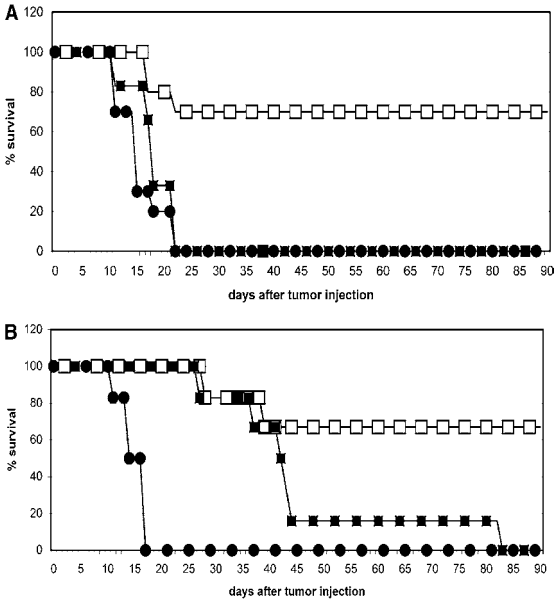

Figure 8. Adoptive transfer of splenocytes (*A*) and $CD8^+$ cells (*B*) isolated from the spleen of naive (*circles*, n = 10) or protected mice (*squares*). (*A*) Seven days before AB1 injection (*open squares*, n = 10) and 2 days after AB1 injection (*closed squares*, n = 6), mice were injected intraveneously with 10×10^6 splenocytes. (*B*) Mice were treated 7 days before AB1 injection with 1.5×10^6 $CD8^+$ cytotoxic T cells (*open squares*, n = 6) or 2 days after AB1 injection with 3.5×10^6 $CD8^+$ cytotoxic T cells (*closed squares*, n = 6).

42% of the mice, although no source of tumor antigen was provided to these cells. This phenomenon was previously described in 1985 by Knight and coworkers (63). Several explanations are possible. First, it was recently shown that DCs not exposed to tumor antigen had the capacity to strongly enhance antitumoral NK cell activity through direct activation of NK cell cytotoxicity, thus activating the innate immune response to various tumors (64). This response occurs even in the complete absence of CD8 cells and requires $CD4^+$ T cells and DC–NK cell contact (65). DC-activated NK cells could therefore kill MM cells directly, in the absence of tumor antigen. Activated NK cells are known to kill the AB1 murine MM cells and human MM cells (66, 67). Second, because both DCs and AB1 cells were grown in fetal bovine serum–containing medium, we suspect that serum proteins might have provoked this response. Some of these proteins are antigenic and thus could induce a strong antitumoral immune response (68). To avoid this problem, we attempted to grow AB1 cells and DCs in serum-free conditions, which turned out to be troublesome. Finally, injected DCs could survive for 1 week after injection and could potentially take up tumor cell fragments, thus leading to tumor antigen presentation and to a tumor-specific immune response.

The induction of proper DC maturation is an important factor in the design of DC immunotherapy trials, because antigen presentation by immature DCs might tolerize for TAA and potentially enhance tumor growth (16). Therefore, clinical trials evaluating the potential of DC immunotherapy have included protocols to induce DC maturation. Most commonly, DCs are exposed to a cocktail of maturation cytokines or are exposed to monocyte-conditioned medium. In our system, the pulsing of DCs with lysed tumor cells already led to DC maturation, in the absence of any additional cytokines. It has been shown that necrotic tumor cells can enhance DC maturation and immunogenicity by providing so-called danger signals to DCs, one important factor being uric acid (69). To further enhance maturation of DCs, we exposed DCs to innate immune system–activating signals during the period of antigen pulsing. Synthetic, unmethylated CpG oligodeoxynucleotides (CpG) are considered potent activators of DC function and maturation *in vitro* and *in vivo*, by acting on the innate Toll-like recepter 9, expressed by myeloid DCs and plasmacytoid DCs in mice and plasmacytoid DCs in humans (70–72). Unmethylated CpG motifs have been evaluated as immunotherapeutic adjuvants in a number of preclinical cancer models, where they have led to enhanced induction of CTL and NK cell responses (73). The immunostimulatory effect of CpG motifs on DCs leads to upregulation of surface costimulatory molecules and increased cytokine production, thus further enhancing the ability of DCs to stimulate T-cell responses (74). CpG motifs are preferred in clinical trials because other potent stimulators of DC maturation, like LPS and tumor necrosis factor α, have toxicity concerns or are expensive to produce, respectively (74). In bone marrow–derived DCs, CpG motifs further enhanced DC maturation, but because of the highly efficacious induction of DC maturation induced by tumor cell lysate *per se*, and the consequent 100% protection of all mice from tumor challenge, we could detect no additional survival benefit by using CpG-matured DCs for immunotherapy. Because the maturational stage of the DCs ultimately may have significant effects in generating antitumoral T-cell and NK cell responses, CpG motifs were used as stimulators of DC maturation in subsequent protocols.

Having established that DC immunotherapy had the potential to induce a protective tumor-specific immune response, we next investigated whether DCs given after tumor challenge had the capacity to eliminate or slow down tumor growth. When DC immunotherapy was given 1 and 8 days after tumor implantation, protection from tumor growth was dependent on the way in which DCs were generated. The DC subtype I (grown from lineage-negative precursors in GM-CSF and Flt3-L) was effective in 66% of mice, whereas DCs grown in GM-CSF and generated from lineage-negative cells (DC subtype II) or from whole bone marrow cells (DC subtype III) protected all mice from tumor

growth. It therefore seems that addition of Flt3-L reduced the efficacy of DCs to reduce established tumor growth. Other studies demonstrated that cytokines GM-CSF and Flt3-L, and a combination of these, influence the heterogeneity of DCs generated from bone marrow cells (33, 74–76). The *in vitro* administration of the cytokine Flt3-L to bone marrow progenitor cultures generates immature plasmacytoid-like DCs, which could induce tolerogenic effects on T cells (77, 78). Although our phenotypic analysis of cultured DCs did not show dramatic differences in the level of expression of the costimulatory molecules CD40, CD80, or CD86, it is possible that addition of Flt3-L still induced subtle differences in the immunostimulatory potential of DCs used for immunotherapy. In particular, one possibility that we have not explored is whether different DC subsets might directly influence tumor growth by producing cytokines or chemokines with direct antitumoral activity (e.g., IL-12, interferon-inducible protein–(IP)-10, monoxine induced by γ interferon).

In contrast to the curative effect when tumor lysate–pulsed DCs were given 1 and 8 days after tumor challenge, a poor prognosis occurred when tumor cells and DCs were injected simultaneously via the peritoneal route. The observation of a paradoxic tumor-enhancing effect of simultaneous administration of DCs and tumor cells is not without precedent and may be caused by several factors. First, high levels of cytokines or soluble mediators produced by MM cells could downregulate cellular immune responses induced by DCs. Next, tumor cells might cluster with DCs, which, through their highly motile nature, might lead to more widespread dissemination and attachment of cancer cells to the mesothelial surface. Finally, and most interesting, it was recently shown in experiments where DCs were mixed with tumor cells *in vivo* that DCs can transform into endothelial cells, thus enhancing tumor vasculogenesis and tumor growth (79).

To make DC immunotherapy clinically applicable, an easily accessible source of tumor antigen is an absolute requisite. Therefore, we analyzed whether extracts made from *in vivo* established tumors would be as efficacious as the primary AB1 cell line lysate to load DCs. Furthermore, growth of the AB1 tumor *in vivo* under the selective pressure of the immune system might have selected loss-of-antigen variants during the immunoediting phase of tumor growth (80). However, DCs loaded with *ex vivo*–isolated tumor lysate were as efficacious as AB1 cell lysate–loaded DCs in mediating protection from tumor outgrowth. In a clinical setting, tumor cells for preparing a lysate might be obtained from surgical resection specimens, from thoracoscopic biopsy material, and from cancer cells isolated from pleural effusions. Another source of tumor antigen that has received great attention lately is exosomes. Exosomes are endosomal-derived vesicles that are secreted by almost all nucleated cell types, including tumor cells, B cells, and DCs. Seminal articles by Wolfers, Chaput, and Andre and colleagues have demonstrated that tumor-derived exosomes are a rich source of shared tumor antigens that can be used for loading onto clinical grade DCs (81–83). Several early-phase vaccine trials involving exosomes as a source of tumor antigen are currently underway. We have recently shown that MM cell lines secrete exosomes, rich in heat shock proteins 70 and 90, possibly explaining why exosomes are a source of tumor-derived antigens (25). Moreover, the pleural fluid of patients with MM contains large amounts of exosomes, which could potentially serve as a source of tumor antigen in a clinical trial, similarly to what was described for ascites fluid in ovarium carcinoma (84). When DCs were loaded with AB1 cell line–derived exosomes and injected after tumor implantation, they were able to reduce tumor outgrowth, illustrating the feasibility of such an approach.

In our studies evaluating the therapeutic efficacy of tumor lysate–loaded DCs given after a larger tumor challenge, MM had a better outcome when DCs were injected early in tumor development, indicating that tumor load played an important role in survival. Although the potency of immunotherapy treatment decreased when DCs were injected later, mice still showed an improved prognosis compared with no treatment, but eventually tumors escaped immune surveillance and all mice died. It is now well established that larger tumor mass is associated with an immunosuppressive milieu that has the capacity to suppress the effector arm of the antitumoral immune response (CTL response inside the tumor) and the inductive arm of the immune response (i.e., the potential of antigen-presenting DCs to induce CTL responses). To prove that CTL induced by DCs were mediating antitumor responses, the efficacy of adoptive transfer of splenocytes and CD8 cells was evaluated in the prevention and treatment of MM (Figure 8). Intravenously injected, purified $CD8^+$ T cells from protected mice dramatically increased survival compared with $CD8^+$ T cells from naive mice when given 7 days before and, to a lesser extent, 2 days after AB1 injection. The suppressive tumor microenvironment might explain the decreased efficacy when CD8 cells are given after tumor administration. Splenocytes from protected mice increased the survival of mice when injected 7 days before AB1 injection but not 2 days after tumor injection. In both cases, 10×10^6 splenocytes were injected. The percentage of $CD8^+$ T cells in this splenocyte preparation was between 2 and 7%, mounting to 2×10^5 to 7×10^5 CD8 cells. Approximately seven times more purified $CD8^+$ T cells were used in the purified CD8 fraction experiment. The lower amount of effector $CD8^+$ T cells in the splenocyte preparation in combination with the immunosuppressive effect exerted by the tumor can explain the difference in efficacy. Another explanation why splenocytes are less effective would be the presence of T cells with regulatory capacity (regulatory T cells) within the splenocyte inoculum. We have preliminary evidence that a population of $CD4^+CD25^+$ regulatory T cells enhances mesothelioma outgrowth by suppressing the antitumoral immune response (J.P.J.J.H., unpublished observations). In our most recent studies on human MM, we have found that tumors are infiltrated with large numbers of CD4 and CD8 T cells, yet tumors escape immune destruction (unpublished observations). Preliminary data on microarray RNA expression profiles and proteomic expression suggest that high levels of several immunomodulatory substances and chemokines, which may interfere with the maturation and/or function of DCs, are also present in the supernatant of MM cell lines and the corresponding patient's pleural fluid (manuscript in preparation). As shown for other tumors, mesothelial tumors produce a number of regulatory factors (e.g., vascular endothelial growth factor, IL-6, IL-10, macrophage-colony stimulating factor, prostaglandin E_2, and transforming growth factor β) that effectively suppress the function of DCs (49–51, 85). Understanding the multiple factors that come into play at different time points of the treatment process may help to better understand and design immunotherapy protocols.

In conclusion, we demonstrate in this article the potency of DC immunotherapy in the control of MM outgrowth. Our study should pave the way to a clinical trial addressing the safety and feasibility of using tumor lysate–pulsed or exosome-pulsed DCs to induce tumor-specific CTL responses in patients with MM. Although such a trial will be initiated first in patients with end-stage disease after chemotherapy, we hypothesize that DC-based immunotherapy would have its greatest effect when given at times when tumor load is minimal—for example, after extrapleural pneumonectomy. Finding the optimal conditions to deliver clinical DC immunotherapy to patients with MM will be our challenge for the future.

Conflict of Interest Statement: J.P.J.J.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.G.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.C.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; B.N.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Monique Willart and Thomas Soullié for their technical assistance during our mouse experiments, and Tanja Nikolic for her assistance in the statistical analysis. Joke Zuijderwijk is thanked for advice and technical assistance in ELISPOT studies and Kris Thielemans for providing GM-CSF.

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Chapter 11

Chemotherapy followed by dendritic cell-based immunotherapy in a patient with mesothelioma

Preliminary case report

Joost P.J.J. Hegmans, I.Jolanda M. de Vries, Henk C. Hoogsteden, Carl G. Figdor, Bart N. Lambrecht, and Joachim G.J.V. Aerts

Case report
Chemotherapy followed by dendritic cell-based immunotherapy in a patient with mesothelioma

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ABSTRACT

Malignant pleural mesothelioma (MM) is a highly devastating tumor in clinical practice with a median survival from the first signs of illness of 9 months (1). As of this time, there are no curative options. Treatment for MM is consequently considered purely palliative and has the goals to improve quality of life and to slow down disease progression. Currently the combination of pemetrexed and cisplatin is considered standard of care for patients with MM, as an increase in median survival of 3 months was noticed (2-6). Because of this limited success, novel therapeutic regimens are urgently needed. Specific immunological treatment of mice did prolong the survival in a murine model for MM. Therefore, we are performing a phase I clinical study which evaluates the safety and efficacy of dendritic cell-based immunotherapy in patients with MM. In this preliminary report, results are shown of the first MM patient ever treated with autologous tumor lysate-loaded, monocyte-derived dendritic cells (DCs). DCs are considered as the most potent antigen presenting cells and can activate the patient's immune system to induce tumor regression (7, 8). We have combined chemotherapeutic agents and DC-based immunotherapy in an adult male patient who gave consent to such treatment. After ALIMTA/cisplatin chemotherapy, he received three vaccinations of autologous DCs that were simultaneously pulsed with autologous tumor lysate and the immunogenic protein keyhole limpet hemocyanin (KLH). The patient received 50x10^6 mature DCs per injection, up to three injections with an interval of two weeks. The first results demonstrate that these vaccinations were well tolerated and induced strong anti-KLH immunological responses and that the whole treatment showed successful results both clinically and radiologically. We feel that the treatment can be given safely to more patients and hope to finalize the phase I study in 10 patients by the end of 2007.

INTRODUCTION

A 68-year-old man was admitted to our department with complaints of weight loss of 5 kg and right-sided back pain at the thoracolumbar junction for 3 months. Dyspnea prevented him from performing regular exercise. A physical examination was performed at initial presentation. Breath sounds were decreased on the whole right lung. Chest X-ray and computed tomography (CT) scans showed a significant collection of pleural fluid on the right side. After drainage of the pleural fluid, pleural thickening and an absence of any lesions in the contralateral pleural space was detected, suggestive of pleural or pulmonary malignancy and highly suspicious for malignant pleural mesothelioma (MM) (Figure 1). The patient mentioned a history of asbestos exposure during his occupation as a fitter. As the first pleural effusion did not decipher a diagnosis, a thoracoscopy was performed. A cytological examination of the obtained biopsies revealed the findings typical of epithelial MM. In addition, immunohistochemical studies were performed using antibodies

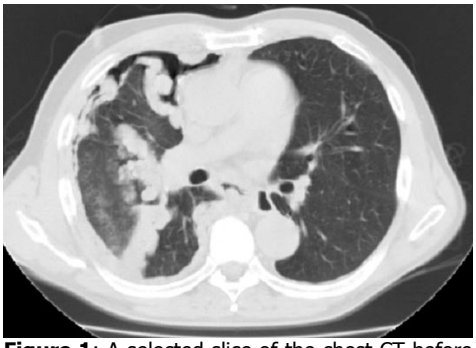

Figure 1: A selected slice of the chest CT before treatment showing a right-sided pleural thickening and calcification.

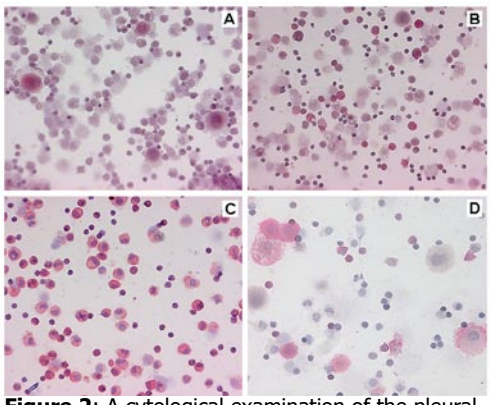

Figure 2: A cytological examination of the pleural effusion indicating the typical findings of MM. Immunohistochemistry using antibodies for cytokeratin 5/6 (A), thrombomodulin (B), vimentin (C), and calretinin (D) at 200x magnification.

to carcinoembronic antigen (CEA), Ber-EP4, cytokeratin 5/6, thrombomodulin, N-cadherin, vimentin, HBME-1, calretinin, and Wilms' tumor 1 (WT-1) protein (all DAKO, Glostrup, Denmark). Malignant cells demonstrated positivity for cytokeratin 5/6, thrombomodulin, vimentin, and calretinin (Figure 2). The adenocarcinomatous markers (CEA and Ber-EP4) and other markers were negative.

PREPARATION OF CELL LYSATES FROM PLEURAL EFFUSION CELLS

The treatment was approved by the Medical Ethics Review Committee (METC) of the Erasmus MC, Rotterdam, The Netherlands (Figure 3). Part of the study was performed at the Department of Tumor Immunology of the NCMLS, Nijmegen (Figure 4). During thoracentesis, 2 L of dark-yellow, highly viscous pleural fluid was gently aspirated and collected in sterile flasks without anticoagulant or other additives. Effusion was transported immediately to the cleanroom facility and centrifugated at 400xg for 15 min at room temperature (RT) and the resulting supernatant was discarded. The red blood cells in the cell pellet were removed by hypotonic lysis using sterile water. Cells were counted ($250x10^6$ cells) and resuspended at a concentration of $50x10^6$ cells per ml physiological saline. Cells were lysed by repeated freezing in liquid nitrogen and thawing at RT (6 cycles) followed by 100 Gy of irradiation. The resulting tumor extract was stored in aliquots at −80°C. Serum and an aliquot of the tumor extract was negatively tested for infectious agents (HIV, HTLV I&II, HBV, HCV) and bacterial contamination.

CHEMOTHERAPY

The patient received four cycles of chemotherapy consisting of 1000 mg (500 mg/m^2) premetrexed (ALIMTA, Eli Lilly and Company, Fegersheim, France) and 150 mg (75 mg/m^2) cisplatin every 4 weeks according to the treatment schedule described (2-6). Dietary supplementation with low-dose folic acid and vitamin B12 prior to and during the treatment was given to limit toxicity (10). During the four cycles of chemotherapy, the patient did not experience any serious adverse events, neither non-hematological nor hematological toxicities were encountered. The patient had a partial response after his last chemotherapy and has since returned to his usual activities.

DENDRITIC CELL CULTURE

Six weeks after the last administration of the chemotherapy, a delayed type hypersensitivity (DTH) skin-test was performed to investigate if the drugs still exerted their influence on the patients' immune system by using 7.5 Lf of purified tetanus toxoid (NVI, Bilthoven, The

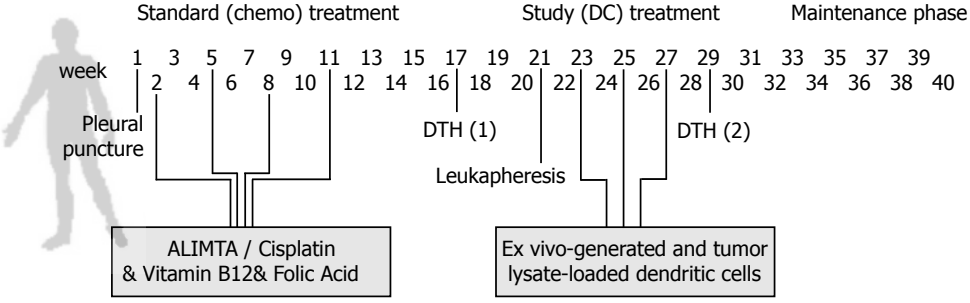

Figure 3: Synopsis of the clinical protocol consisting of a combined treatment with chemotherapy followed by active immunotherapy using autologous tumor lysate-loaded dendritic cells.

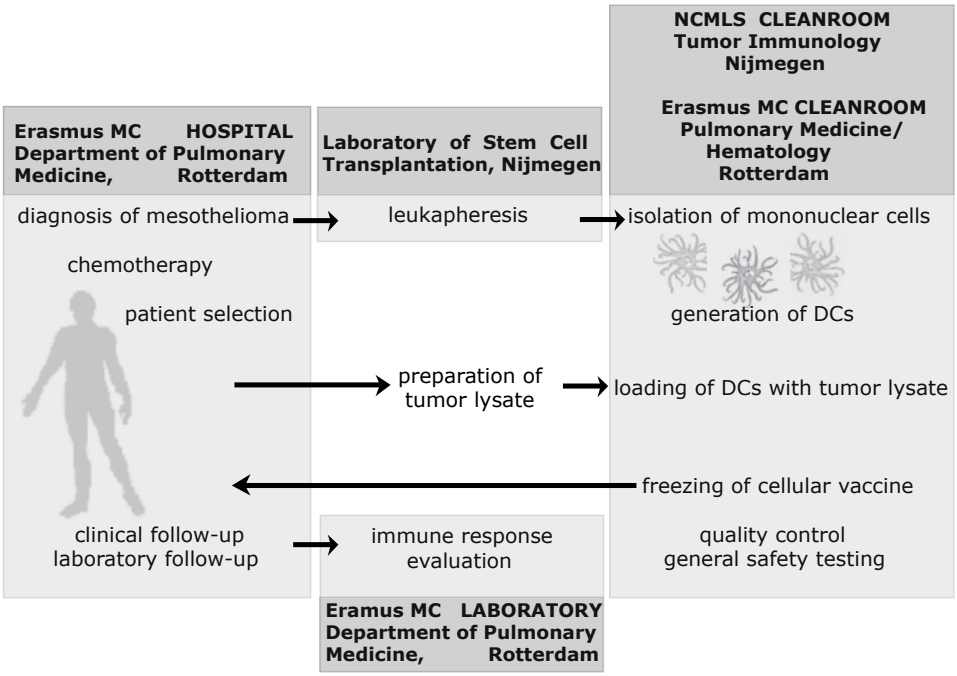

Figure 4: The treatment of the patient was organized in close collaboration with the Department of Tumor Immunology of the NCMLS (Nijmegen), where part of the study was performed.

Netherlands) as positive control and 50 µl of saline as negative control. A positive skin test reaction on tetanus toxoid was measured whereby the erythematous induration exceeded 18 mm in diameter 48 hours after subcutaneous injection. Five week later, we used a previously described method to generate clinical grade mature dendritic cells in conformity with GMP (Good Manufacturing Practice) guidelines (8, 11). In brief, a concentrated 150 ml leukocyte fraction was generated through a 4-h restricted peripheral blood leukapheresis, processing 9 L of blood (COBE Spectra Apheresis System, Gambro BCT, Zaventem, Belgium). Peripheral blood mononuclear cells were then enriched using counter-flow centrifugal elutriation (Elutra, Gambro BCT, Zaventem, Belgium) as described by Berger et al. (12). Monocytes were further purified by PureCell (Medicult, Denmark) density gradient centrifugation and resuspended at a concentration of $5x10^6$ cells/ml in XVIVO-15 (Cambrex Bio Science, Verviers, Belgium) supplemented with 2% pooled human AB serum (DC-culture medium). The next day, half of the medium was removed and replaced by the same volume of DC-culture medium supplemented with 1000 IU/ml interleukin (IL)-4 (CellGenix, Freiburg, Germany) and 1600 IU/ml granulocyte macrophage-colony stimulating factor (GM-CSF; CellGenix). After 6 days of culture, semi-adherent and non-adherent cells were harvested by pipetting. Cells ($1x10^6$) were seeded per well of a 6-well plate in fresh DC-culture medium supplemented with 500 IU/ml IL-4, 800 IU/ml GM-CSF, 10 µg/ml KLH (Calbiochem, La Jolla, CA, USA), and tumor cell lysate (1 tumor cell equivalent to 3 DCs). The next day the maturation cocktail was added (prostaglandin E2 [PGE2; 10 µg/ml Pharmacia&Upjohn, Puurs, Belgium], tumor necrosis factor-alpha [TNF-α, 20 ng/ml), IL-1β [5 ng/ml], and IL-6 [15 ng/ml; all CellGenix]). Cells were harvested at day 9 and $50x10^6$ cells used for immediate vaccination; the remaining cells were frozen in DMSO for the second and third vaccination and for the DTH skin test. Release criteria for each batch of DCs were previously described (8).

DC VACCINATION

Three vaccinations were administered with a 2-week interval (Figure 1). Each vaccination, consisting of $50x10^6$ mature KLH and tumor lysate-loaded DCs, and was intradermally (i.d.) and intravenously (i.v.) administrated. Dosage was divided 1/3 i.d. in the forearm and 2/3 though i.v. route by mixing the components in 100 ml of normal saline drip. Constant monitoring was done till 4-h after the administration of vaccine

therapy. No immediate adverse effects were observed. The patient developed self-limited fever a few hours after the vaccinations (with the exception of the first vaccination). Local skin reactions were seen at the site of the intradermal injection one week after the first vaccination. Subsequent vaccinations gave a quicker (2-3 days) and increased induration and erythema. This might suggest that in this patient some form of immunity was induced. Three millimeter skin punch biopsies were taken from the i.d. vaccination site at day 2 and day 14 and at a control site and showed an thickening of the epidermis and infiltrates predominantly consisting of HLA-DR,-DQ,-DP+ cells, macrophages (CD68), and T lymphocytes (CD3, CD4, and CD8) using immunohistochemical techniques (Figure 5).

MONITORING

Vaccination with DCs gave no serious adverse reactions and no clinical or laboratory evidence of any autoimmune reaction (antinuclear antibody [ANA], entractable nuclear antigen [ENA], rheumatoid factor [RF] IgM and anti-cyclic citrullinated peptide [anti-CCP]). There were no substantial changes in the results of routine blood tests (data not shown). Minor changes were detected in MESOMARK expression during the DC treatment. His weight remained constant during the whole treatment. Two weeks after the third DC vaccination a DTH test was performed. Different amounts of tumor lysate, KLH alone, DCs loaded with tumor lysate and KLH, DCs loaded with tumor lysate, and an appropriate positive control (tetanus toxoid) and negative control (saline) were injected intradermally and read 48 hours later. Tumor lysate alone did not induce a skin reaction probably caused by insufficient material. The tumor lysate and KLH-loaded dendritic cells and the helper antigen KLH alone gave erythema and an induration of 22 mm and 15 mm, respectively. Tumor lysate-loaded DC (without KLH) induced erythema but no induration.

The clinical response data were evaluated by computed tomography (CT) scans (Figure 6) and chest X-ray (Figure 7). The patient showed a partial response after chemotherapy that further improved after the DC vaccinations.

DISCUSSION

In an effort to improve the outcome for patients with MM, we have earlier investigated tumor lysate-pulsed DCs to boost the immune system to specifically eradicate malignant cells in a murine model (13). We demonstrated that mice receiving DC-based treatment prior to tumor implantation developed a protective anti-tumor immunity with prolonged survival and mice even resisted a secondary tumor challenge. This tumor protection was associated with strong tumor-specific cytotoxic T lymphocyte responses. When given after tumor implantation in a therapeutic setting, pulsed DCs prevented MM outgrowth. However, with a higher tumor load or a delayed administration after tumor implantation, DC-based treatment was no

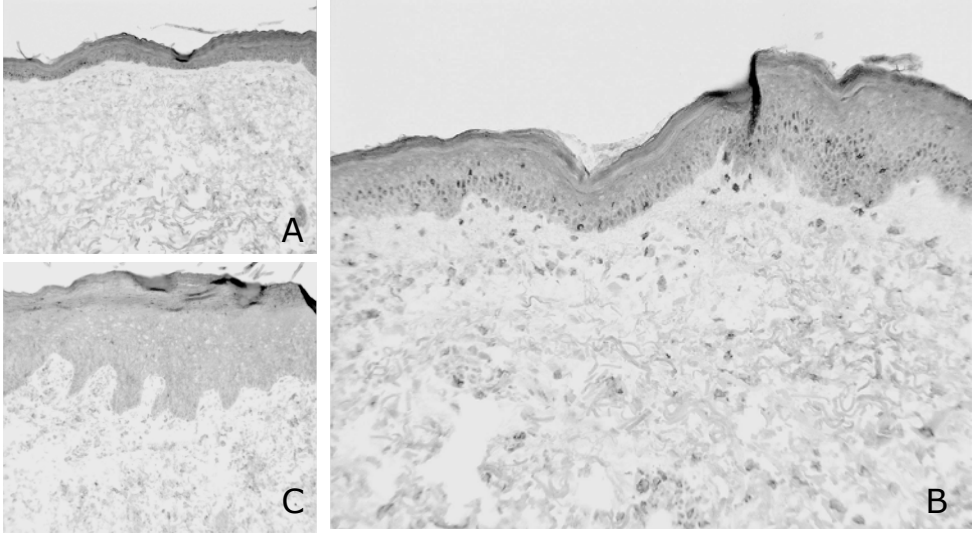

Figure 5: Immunohistochemical staining for CD3 on skin biopsy slides taken from a non-affected skin (A), 2 days (B) and 14 days (C) after DC vaccination from the injection site.

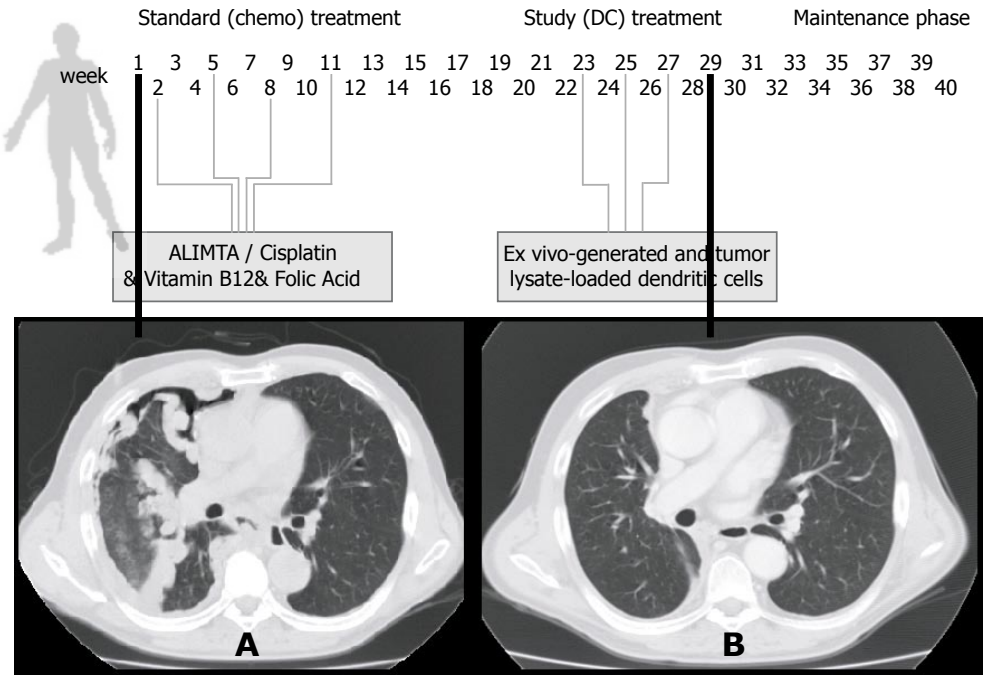

Figure 6: CT scans before (A) and after (B) the whole treatment, comparable in position, revealed a substantial regression of the tumor.

longer effective. This inability was probably caused by the tumor micro-environment consisting of many immunosuppressive cytokines and regulatory T cells (14). We hypothesize that DC-based immunotherapy would have its greatest effect when given at times when tumor load is minimal, for example after extrapleural pneumonectomy or chemotherapy (13). Others have suggested a therapeutic synergy between immunotherapy and the tumor cells that have been killed by chemotherapy (15). Therefore, we have combined chemotherapeutic agents and DC-based immunotherapy to an adult male patient after approval by the local ethical committee.

Nowadays a combination of pemetrexed and cisplatin is given because it is considered standard of care for patients with inoperable MM (2-6). Cisplatin inhibits DNA synthesis and pemetrexed is a multitargeted antifolate that inhibits several enzymes involved in purine and pyrimidine synthesis (16,17). This treatment is the only therapy for mesothelioma that is approved by the US Food and Drug Administration (FDA). The downside of these drugs are the systemic side effects, but dietary supplementation with folic acid and vitamin B12 limits these toxicities (10). Another concern is the toxicity to dividing cells in the bone marrow and peripheral lymphoid tissues. It is known that pemetrexed can cause neutropenia (11% to 58%), leukopenia (13% to 55%), anemia (33%), or thrombocytopenia (9% to 27%). To prevent immunosuppression by these agents through affecting the developing antitumor T cell response, an 12 weeks interval was chosen between chemotherapy and the first DC vaccination. As no specific tumor antigens have been identified in MM so far, DCs were pulsed with autologous tumor lysate prepared by freeze-thawing and irradiation of pleural cells from the thoracentisis at the time of diagnosis. Being self in nature, this tumor material is usually a relatively weak antigen and may benefit from a strong immune response against a foreign antigen as Coley found already in the 18th century. He observed that the injection of bacterial toxins (from bacillus Calnette-Guérin [BCG]) close to the tumor caused strong inflammation associated with tumor regression. Therefore, based on the same principle the immunogenic protein keyhole limpet hemocyanin (KLH) was used in this study as a helper antigen and as a tracer molecule, which allows *in vivo* and *in vitro* monitoring of immunologic responses induced by the vaccinations. KLH and tumor lysate was added at day 6 to the culture, which may lead to the simultaneous

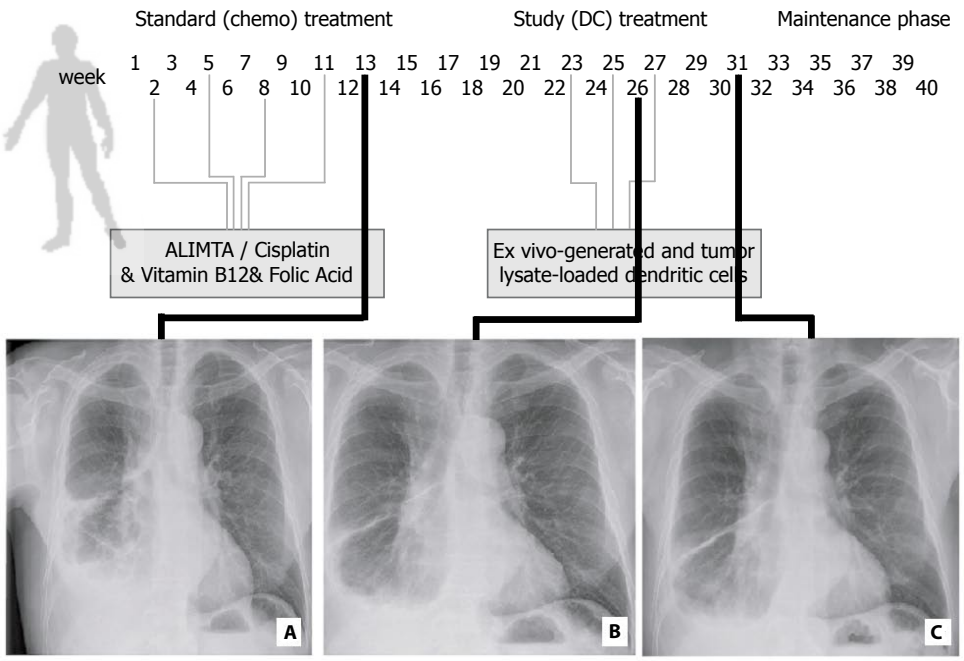

Figure 7: Chest X-rays at different time points during the treatment. Two weeks after the last administration of chemotherapy (A), one week after the second DC-vaccination (B), and four weeks after DC-based immunotherapy (C).

uptake of both antigens by immature DCs. The concomitant presentation leads to the side-by-side activation of T cells specific for the KLH and for the tumor antigen. In this situation, the T cell specific for KLH provide the important signals and will trigger DCs. This activation leads to an upregulation of costimulatory molecules and cytokine secretion. The relatively weak tumor-specific, CD8+ cytotoxic T cell benefits from this stimulatory environment (the helper antigen concept).

In our present report we established the safety and potential efficacy of dendritic cell therapy for the first time in a patient suffering from MM. The results show that injection of DCs was well tolerated without systemic toxicity, with the exception of a low-grade fever and a temporarily local skin reaction after DC injection. The participant experienced no rash or lymphadenopathy or developed any clinical evidence of autoimmunity or rheumatoid disease. CT scan and X-ray revealed substantial regression of the tumor

At this juncture, however, it is difficult to predict the sustained response as well as the decrease in tumor volume in this patient but it shows a new dimension in MM treatment. It is important to emphasize that the patient described in this here is part of a larger study and therefore it is too early to allow firm conclusions to be made. Currently we are investigating the immune status of the serum and blood samples taken during the whole procudure using ELISA and *in vitro* assays of lymphocyte function (proliferation and cytokine responses to KLH and tumor lysate).

ACKNOWLEDGEMENTS

The authors thank Anita Schelen, Daniëlle Hijdra, Mandy van de Rakt, Nicole Meeusen-Scharenborg, Annemiek de Boer, Joost Lesterhuis, and Daniel Benitez-Ribas for advice and technical assistance. This study was funded by the Stichting Asbestkanker Rotterdam and the Mesothelioma Applied Research Foundation (MARF).

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Chapter 12

Summarizing discussion

Chapter 12 Summarizing discussion

The work described in this thesis combines the application of several novel techniques with new immunological insights to improve the diagnosis and treatment of malignant mesothelioma. In this final chapter, the most important findings and conclusions of the previous chapters will be discussed in a broader sense and in the light of recent developments in research on mesothelioma. Concluding remarks are made about proteomic techniques to enhance diagnostic testing for mesothelioma, the influence of the tumor microenvironment, and the manipulation of the immune system to improve the outcome of mesothelioma. The current therapeutic application of dendritic cell-based immunotherapy is described and suggestions for further prospects are given.

12.1 Overview of the experimental work that was performed

12.1.1 *Phage antibody display technology*

Special emphasis was put on the development of phage antibody display technology to discover human antibodies with new specificities directed against antigens in membranes of living cells. The advantages of human phage antibodies compared to conventional (animal) monoclonal or polyclonal antibodies include speed of isolation, no animals are needed, and the fact that antibody specificities can be found with a vast number of antigens, even with non-immunogenic, highly conserved or extremely toxic antigens. Because the immunoglobulin V genes are cloned, subsequent manipulation is possible to change the fine specificity and affinity of these antibodies. Phage antibodies are now tested for their applicability in immunotherapy because they are less likely to provoke a human immune response, are not readily degraded by the liver, and penetrate into tissues much more readily than conventional monoclonal antibodies do. Successful isolation of phage antibodies has been reported mostly using purified antigens but has proven to be much more complicated with complex mixtures of antigens, such as intact cells. A given cell type contains thousands of different epitopes, each capable in theory of binding phage antibodies. To overcome problems involving phage antibody selections on intact cells, we have developed an experimental model system that allowed for optimization and comparison of various selection strategies. The model system described in ***chapter 4*** comprises labeling of intact cells with the fluorescently labeled lipoprotein fluorescein-DHPE. Fluorescein-DHPE can be incorporated into the membrane of living cells, extending the fluorescein head group out of the membrane. Although results are subjected to experimental conditions, the fluorescein-DHPE model system fits the requirements for an appropriate model system for selections on living cells. It is easy to perform (labelling takes 20 min), and requires no complex (molecular) techniques. It is widely applicable for any given cell type and the growth characteristics and protein expression of cells remains unchanged. The lipoprotein fluorescein-DHPE is continuously present and is equally distributed on all cells, while labelling intensity can be regulated by varying the fluorescein-DHPE concentration. Because undesired cells and target cells are closely related, differing by only a single antigen, highest stringency conditions can be tested. A requisite was that the phage antibody specific for the antigen (fluorescein-DHPE) was present in the library. After optimization of key steps in the selection

procedure, we were able to isolate a fluorescein-DHPE specific phage from a synthetic library using intact mesothelioma cells. Unfortunately, due to the lack of financial support, the Phage Display Unit, a core facility operating from the Department of Immunology, was abolished at May 1, 2002. Responsible researchers and technology (patent rights for phage library) were moved to a company (Crucell, Leiden, The Netherlands). Therefore we had to stop our research in finding phage antibodies that could discriminate between normal cells and mesothelioma cells. There are close to 700 antibodies currently tested in clinical and preclinical trials (URL: www.clinicaltrials.gov; [keyword: antibody]). The first antibodies derived from phage display technology have been approved for treatment of rheumatoid arthritis in the US by the Food and Drug Administration (Humira™, antibody specific for human tumor necrosis factor). Monoclonal antibodies (MORAb-009) directed against mesothelin, a cell-surface glycoprotein overexpressed in MM, are currently studied as candidates for targeted therapy. With time, these recombinant antibody technologies will provide a solid basis for the discovery of antibody-based biopharmaceuticals, diagnostics and research reagents for decades to come. However, thorough investigation of the safety issues is necessary to ensure that antibodies have no adverse properties before they are used in man for the treatment of diseases, as the following will highlight. In March 2006, volunteers taking part in a clinical trial involving TeGenero's drug TGN1412 (a humanised agonistic anti-CD28 monoclonal antibody) resulted in hospitalization of six volunteers, at least four of whom suffered from major organ failure (1,2).

12.1.2 *Proteomics (2D DIGE / SELDI-TOF mass spectrometry)*

Diagnosis of MM is limited because of the difficulty of distinguishing mesothelioma cells from (reactive) mesothelial cells or other metastatic malignant cells that have spread from another organ to the pleural space. Proteomics is the systematic analysis of the protein expression of healthy and diseased tissues and leads to understanding of how the cell actually works and how disease processes operate. The proteome, contrary to the genome, is not a static parameter: it reflects not only the presence of active or inactive (mutated) genes, but also their extent of expression at a specific time point. In addition, the proteome reflects all proteins and peptides that may rise from only one gene, i.e. different cleavage products and proteins with different post-translational modifications. In this thesis, malignant pleural mesothelioma samples were analyzed and compared with other pulmonary diseases presenting with pleural effusion to find protein markers that are potentially informative on the biological behavior of the tumor. These proteins are then called biomarkers and are differentially present in a sample taken from a subject of one phenotypic status (e.g., having mesothelioma) as compared with another phenotypic status (e.g., not having mesothelioma). They can be found (either newly formed, or at increased or decreased amounts) in blood, other body fluids, or in (tumor-) tissues, and are indicative of the presence of a disease. They are produced and sometimes secreted by tumor cells or they can be the result of the body's response to the development of the cancer. Mesothelioma cells express and probably release proteins into the pleural effusion that may have diagnostic value as biomarkers on their own and may provide further insights into the pathological process. In chapter 5 and 6, we utilized proteomic techniques to analyze pleural effusions

in order to discover changes in expression of pleural proteins that either cause, or result from, malignant mesothelioma. ***Chapter 5*** describes the utilization of two-dimensional (2D) fluorescence difference gel electrophoresis (DIGE) to characterize proteins that are differently expressed in pleural effusion and serum of the same mesothelioma patient. This novel technology allows the detection and quantitation of differences in protein abundances between samples using cyanine dye labeling with spectrally resolvable CyDye DIGE Fluor minimal dyes. These dyes are used to fluorescently label up different protein samples prior to mixing them together and running them simultaneously on the same 2D gel. DIGE was combined with mass spectrometry to identify the proteins that had undergone relevant changes. A protein with a molecular mass of 28 kDa and pI of 5.3, identified by MALDI-TOF as apolipoprotein A1 (Apo A1) was detected in serum of patients. This protein migrated differently from the major Apo A1 and represents only a small fraction of the total serum Apo A1 but this isoform was absent in pleural effusions. The number of patients was too small to permit conclusions for the unique mode of biosynthesis and processing of this Apo A1 isoform. Nonetheless, the results are provocative and further work into the function and expression of this protein and other differentially expressed proteins (either up- and down-regulated) will be interesting.

In ***chapter 6***, we studied changes in expression of proteins in pleural effusions to discover biomarkers which might improve the diagnosis of MM. Therefore, protein profiles of complex pleural effusions were determined using surface enhanced laser desorption/ionization (SELDI) time-of-flight (TOF) technology (Ciphergen Biosystems, Fremont, USA) (3,4). Pleural effusion samples were collected from patients with confirmed MM (n=54) and from patients with effusions due to other causes (cardio pulmonary diseases, symptomatic inflammatory diseases and other cancers with pleural involvement (carcinoma, sarcoma, lymphoma, and melanoma)). The five peaks with the best discriminating power identified were at 6614 Da, 6626 Da, 6656 Da, 6821 Da, and 8799 Da. Each of these five peaks was down regulated in the MM group. The first four peaks were characterized as apolipoprotein (Apo) CI or adducts of Apo CI, and the 8799 Da peak as Apo AII. Patients with diminished levels of Apo CI and/or Apo AII were exceedingly likely to have MM versus other conditions.

The identified isoform of Apo AI (2D DIGE) and Apo CI (using SELDI-TOF) are common serum and pleural fluid proteins and changes in their concentrations most likely reflect epiphenomena rather than tumor originating (e.g. altered enzymatic activity). Apo AI is synthesized both in the liver and small intestine and is a major component of high density lipoprotein (HDL) particles. This protein regulates HDL particle size distribution and promotes cellular cholesterol efflux (5). It is a known negative acute phase reactant, of which increased or decreased expression has been described in several cancers (6-12). An isoform of apo AI was also detected by 2DE in serum obtained from individuals with high risk for the development or who were diagnosed with hepatocellular carcinoma (13). Apo C1 is primarily synthesized in the liver and has inhibitory or stimulatory effects on a variety of receptors and enzymes involved in lipoprotein metabolism (5). To our knowledge, there are two reports on apolipoprotein C1 down-regulation in cancer, both SELDI-TOF MS studies in serum of colorectal cancer (6,14). In our setting, the sensitivity and specificity of Apo CI was 76 % and 69 %, respectively. The area under the receiver operating characteristic curve (AUC) for Apo CI (6626 Da) was 0.755. The same samples were also analysed for osteopontin (OPN)(15), human epididymes protein 4 (HE4)(16,17), cytokeratin

19 fragment (CYFRA 21-1)(18,19), and the soluble mesothelin-related protein (SMRP)(20,21). Besides Apo CI (AUC 0.755), CYFRA 21-1 (AUC 0.736) and SMRP (AUC 0.860) were informative in delineating mesothelioma from non-MM pleural effusions. We show that proteomic profiling of pleural effusions from MM patients and non-MM individuals can diagnose mesothelioma with a AUC of 0.755 in our cohort of patients, outperforming OPN, HE4, and CYFRA 21-1. Studies are underway to further investigate the possible role and function of the decrease of these apolipoproteins in the oncogenesis of MM. As a future application we suggest the potential of Apo CI as a candidate biomarker for inclusion as one of the variables in a decision tree for MM diagnosis in pleural effusion fluid cases, next to SMRP for this pathology.

12.1.3 *Exosomes*

Exosomes are small natural membrane vesicles released by a wide variety of cell types into the extracellular compartment by exocytosis (22). The biological functions of exosomes are only slowly unveiled, but it is clear that they serve to remove unnecessary cellular proteins (e.g. during reticulocyte maturation (23)) and act as intercellular messengers because they fuse easily with the membranes of neighbouring cells, delivering membrane and cytoplasmic proteins from one cell to another. DC-derived exosomes (dexosomes) can transfer antigen-loaded MHC class I and II molecules, and other associated molecules, to naïve DCs and T-cells, potentially leading to the amplification of both adaptive and innate cellular immune responses (24). Because of this, exosomes have been suggested as novel source of cell-free therapeutic cancer vaccines (24-26). The first two phase I trials evaluated in the clinic consisted of autologous dexosomes (patient-specific exosomes released by dendritic cells and loaded with tumor antigen-derived peptides) as immunotherapeutic regimens for melanoma and non small-cell lung cancer (27,28). These studies revealed that dexosome immunotherapy was well tolerated and led to the induction of immune responses, and disease stabilization for several patients. Andre *et al* have shown that tumor-derived exosomes loaded on DCs trigger T-cell-mediated anti-tumor immune responses leading to a strong inter-tumor cross-protection suggesting that these exosomes contain shared tumor-rejection antigens (29). We analysed the production of exosomes *in vivo* in pleural fluid of cancer patients, and from MM cell lines *in vitro* to gain information on their potential biological function(s) and to detect whole native tumor antigens. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was used to characterize the protein composition of the exosomes. In ***chapter 7***, exosomes were isolated from pleural effusions of patients suffering from different cancer types involving the pleura. We encountered difficulties during the purification process due to the high content of complement factors and immunoglobulins in exosome fractions. Another problem was that tumor cells are not the only source of exosomes in pleural effusions, as extracellular shedding is commonly encountered in other cell types, among them mesothelial cells and immune cells. This would argue for the existence of a variety of exosomes from different cellular origins in pleural fluid. Therefore, exosomes secreted by well-characterized human MM cell lines were purified and characterized for protein content (***chapter 8***). MHC class I was found to be present together with high amounts of heat shock protein (HSP) 90 and heat shock cognate protein (HSC) 70. Heat shock proteins act as chaperones, providing a fingerprint of the MM cell's content, and hence these

exosomes may be good candidates for a cancer immune therapy (30-32). HSP in exosomes can be taken-up by dendritic cells and macrophages (perhaps by CD91 receptor-mediated endocytosis (33-35)), and processed for presentation to the immune system in the lymph nodes. Most interestingly was the detection of developmental endothelial locus-1 (DEL-1) in MM-derived exosomes, which can act as a strong angiogenic factor (36,37) and may increase the vascular development in the neighborhood of the tumor. Therefore, MM-derived exosomes may either be involved in the sampling of antigens to antigen presenting cells or in transferring various proteins, such as angiogenic factors, to favour the tumor growth. In ***chapter 10***, mouse MM-derived exosomes were used as source of tumor antigens for dendritic cells, which then mediated $CD8^+$ T-cell-dependent anti-tumor effects but were less capable of inducing an anti-tumor response then tumor lysate was. Our current knowledge of exosomes is still in its infancy but the biologic function is expected to vary by their protein composition and thus the origin of the producing cells. Recent findings suggests that cell-derived vesicles (exosomes are also named membraneous vesicles or microvesicles) could also induce immune tolerance (38,39), suppression of NK function (40), T cell apoptosis (41,42), or metastasis (43,44). By secreting exosomes, tumors are able to accomplish the loss of those antigens that may be immunogenic and capable of signaling to immune cells as well as induce dysfunction or death of immune effector cells (45). A full understanding of the biological significance of tumor-derived exosomes requires additional study but these membrane vesicles could become a new important component in orchestrating the tumors' escape from the host immune system. Meanwhile, the *in vivo* use for vaccination of tumor exosomes should proceed with caution.

12.1.4 *Tumor micro-environment*

The exact sequence of events that take place between asbestos exposure and the first clinical signs of MM 20 to 40 years later is unknown. The paths that mesothelial cells take on their way to become malignant is probably highly variable and dependent of several host factors, including environmental factors, polymorphisms and mutations in susceptibility genes, age and immunity. When a patient is diagnosed with MM, tumor cells and their products have already been interacting with and affecting host cells for a considerable time to facilitate their growth and progression. At that stage the tumor exists in intimate symbiosis with the rest of the body and consists of an intricate network of tumor cells and stroma, including endothelial cells, fibroblasts, lymphocytes, macrophages, and other cell types. These diverse cellular populations that invade and surround a tumor – the so-called tumor micro-environment – shape its development and progression. There is accumulating evidence that cancer cells subvert normal cell types to serve as active collaborators in their neoplastic program (46,47). The cellular components in tumor stroma contributes to tumor immunopathogenesis of human cancers but the exact roles and mechanisms are not well understood (48).
Understanding the various cell types and the range of soluble mediators present in the MM micro-environment may help to better understand tumor development and progression. Therefore, the expression profiles of 80 different cytokines in supernatant of MM cells *in vitro* and corresponding patient's mesotheliomatous pleural effusion was determined. It showed that MM cells are potent sources of a

number of cytokines as described in ***chapter 9***. We found that MM cells and their surrounding stroma generate an immunosuppressive microenvironment capable in suppressing the effector arm of the anti-tumoral immune response (CTL response inside the tumor) and the inductive arm of the immune response, i.e. the potential of antigen presenting DCs to induce CTL responses. To illustrate this, many immune effector cells are present within the tumor while tumors were still not rejected. Tumor-infiltrating T lymphocytes (TILs) and macrophages represent an abundant population in MM tumor tissue (Figure 1). Studies have revealed that tumor-associated macrophages promote tumor growth and metastasis by directly acting on tumor cells (46, 49-52), endothelial cells (53) and on antigen-specific T-cells (54). Recently it has been demonstrated that ovarian carcinoma ascites fluid, which has a comparable cytokine composition as pleural effusions (high IL-6 and IL-10, low IL-4 and GM-CSF) induce surface B7-H4 expression in macrophages that suppress the tumor-associated antigens (TAA) specific T cell immunity (54). Also other immune cells found in the tumor bed, including NK cells and NKT cells, have been shown to dampen T-cell function (55-57). Strikingly, human MM biopsies are largely devoid of dendritic cells (DCs). This is in contrast to many other tumors such as breast cancer or NSCLC, in which DC are found within tumor lesions that correlates with a better prognosis (58-60). Some studies have tried to increase and activate DCs in human MM by local infusion of recombinant GM-CSF (61,62) or subcutaneous administration of GM-CSF together with autologous tumor lysate (63). The proposed mechanism for GM-CSF efficacy is differentiation and attraction of DCs to boost TAA-specific immunity. Although vaccination induced tumor specific immunity, both cellular and humoral in 32% of patients, no major tumor regressions were observed. Because GM-CSF can also block the IL-10-induced B7-H4 expression in macrophages (54), it will be interesting and worthwhile to reexamine if GM-CSF–mediated reduction in macrophage B7-H4 expression accounts for efficacy. We were the first to demonstrate that human MM tissue contains significant amounts of regulatory T (Treg) cells. This subset of $CD4^+CD25^+$ T-cells express the transcription factor forkhead box P3 (FoxP3), glucocorticoid-induced TNF-receptor-related-protein (GITR), and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and is naturally anergic and requires stimulation through the T-cell receptor for induction of their cell-mediated suppressive function (64). Treg cells are also increased in malignant pleural effusions (65). We showed that depletion of these cells by systemic administration using anti-CD25 mAb led to increased survival in a transplantable mouse model of mesothelioma. Needham et al. showed that depletion of Treg cells inside mouse MM tumors with anti-CD25 mAb injected directly into the tumors cause reduced tumor growth (66). Treg cells and other immunosuppressive cells (for example $B7\text{-}H4^+$ macrophages) might be selectively depleted with directed immunotoxins or suppressed by administering a cytokine such as TNF (67-71). These depletions could be utilized in combination with other immunotherapy approaches as multimodality treatments of residual tumor deposits following chemotherapy or surgical debulking of tumors.

12.1.5 *Dendritic cell-based immunotherapy*

The possibility to harness the potency and specificity of the immune system underlies the growing interest in cancer immunotherapy. One such approach uses the patients' own DCs to present tumor-associated antigens and thereby generate tumor-specific immunity (72,73). DCs are extremely potent

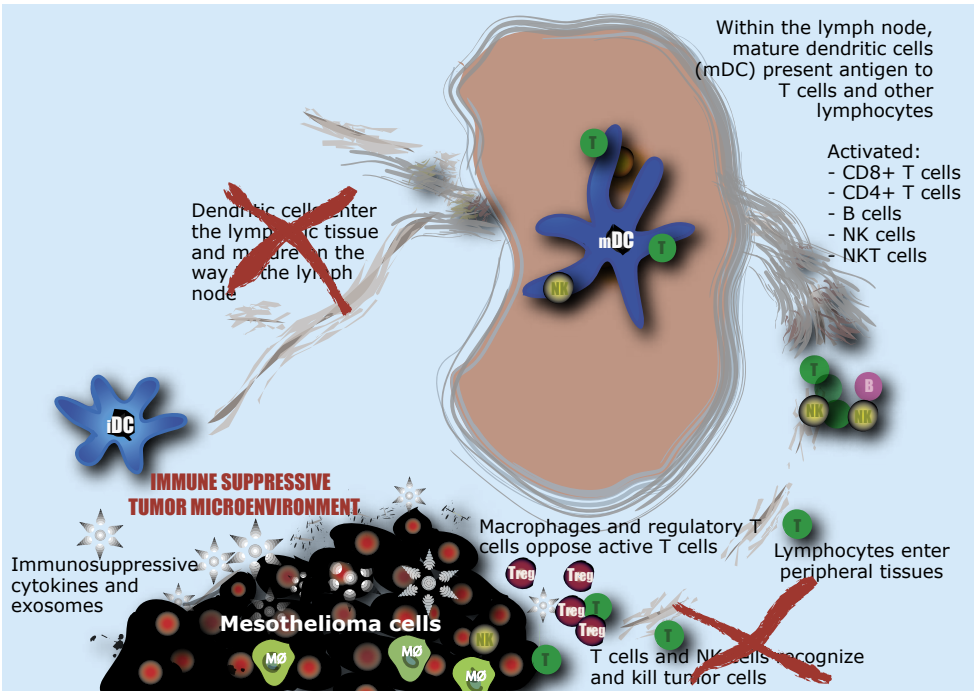

Figure 1: Human mesothelioma cells secrete exosomes and produce an offensive repertoire of cytokines (GRO, RANTES, IL-6, VEGF, etc.) that can suppress the function of dendritic cells and incoming effector cells. Another prominent component of tumors includes stromal cells (fibroblasts and endothelial cells), tumor-associated macrophages, and regulatory T cells which also release a variety of immune modulators (HGF, Angiogenin). All these cells and factors in the tumor micro-environment are contributing to direct the local immune system away from anti-tumor reactions.

antigen presenting cells specialized for inducing activation and proliferation of lymphocytes (74). If the DCs can be made to attack mesothelioma cells, it would mean a non-toxic treatment (unlike chemo- and radiotherapy) with no side-effects, and long-lasting immunological memory (similar to vaccines providing long-term protection against viruses). In ***chapter 10***, we tested the hypothesis that autologous DCs presenting tumor antigens (tumor lysate derived from cell lines, lysate derived from excised tumors, and exosomes) might induce a protective immune response in MM. A mouse model for MM allowed us to prove this hypothesis and to study the impact of antigen source, DC maturation status and timing of administration on outcome. Mice receiving tumor lysate-pulsed DCs prior to tumor implantation demonstrated protective anti-tumor immunity with prolonged survival (> 3 months) and they even resisted a secondary tumor challenge. Tumor protection was associated with strong tumor-specific cytotoxic T lymphocyte responses.

In our studies evaluating the therapeutic efficacy of tumor lysate-loaded DCs given after a larger tumor challenge, MM had a better outcome when DCs were injected early in tumor development indicating that tumor load played an important role in survival. Adoptive transfer of splenocytes or purified $CD8^+$ T lymphocytes transferred tumor protection to unimmunized mice in vivo. In conclusion, the study showed that DC-based immunotherapy in a mouse model for mesothelioma induces tumor-specific cytotoxic T lymphocyte responses leading to an increase in survival.

12.1.6 *Main findings emerging from the experimental work*

Proteomic technologies are useful in the design of more specific tests and to gain further insight into the pathological process of MM (**chapter 5,6,7,8,9**).

2D DIGE revealed that an isoform of Apo AI is differently expressed in serum and pleural fluid from individuals with MM (**chapter 5**).

Apo CI levels are decreased in pleural fluid of MM patients and this protein seems to be a promising marker for diagnosis in addition to SMRP, with superior performance compared to OPN, HE4, and CYFRA 21-1 (**chapter 6**).

MM cells secrete exosomes that contain, among others, MHC class I and heat shock proteins (**chapter 8**).

MM cell-derived exosomes contain developmental endothelial locus-1 (DEL-1), which can act as a strong angiogenic factor (**chapter 8**).

MM cell-derived exosomes can be used as a source of tumor antigens for DCs, which then mediate an anti-tumor response (**chapter10**).

MM cells are potent sources of a number of cytokines (**chapter 9**).

Human MM tissue contains significant amounts of macrophages and $CD4^+CD25^+Foxp3^+$ regulatory T-cells (**chapter 9**).

Depletion of regulatory T-cells led to an increased survival in a mouse model for MM (**chapter 9**).

Human MM biopsies are largely devoid of DCs (**chapter 9**).

An increased survival is observed in mice pretreated with DCs and these mice even resist a secondary challenge with a lethal dose of tumor cells 3 months later (**chapter 10**).

Pulsed DCs given after tumor challenge have the capacity to slow down tumor growth (**chapter 10**).

Pulsed DCs induce tumor-specific cytotoxic T-cell responses in mice (**chapter 10**).

$CD8^+$ T-cells transfer tumor protection to non-immunized mice *in vivo* (**chapter 10**).

Survival of tumor-bearing mice is dependent of the type of DCs and time of vaccination (**chapter 10**).

12.2 Translational research initiated by this work

Based on studies in other types of cancer in humans where beneficial effects were obtained (75), and based on our pre-clinical data in a mouse model for MM (***chapter 10***), we introduced DC-immunotherapy for patients with mesothelioma. After approval by the Institutional Medical Review Board of the Erasmus MC (METC) in December 2005, a phase I clinical trial using DC immunotherapy was started (Table 1). Ten patients are treated with chemotherapy (pemetrexed/ cisplatin) followed by three vaccinations of autologous tumor lysate-loaded monocyte-derived DCs, each dose consisting of $50x10^6$ cells. Chemotherapy is given prior to DC vaccination in order to reduce tumor load, thereby potentially augmenting the efficacy of the vaccination. More information regarding this study can be found on the internet sites http://clinicaltrials.gov, www.trialregister.nl, or www.controlled-trials.com.

Table 1: Synopsis of the phase I clinical study of active immunotherapy using autologous tumor lysate-loaded dendritic cells for the treatment of mesothelioma.

Title	Dendritic cell-based immunotherapy in patients with malignant pleural mesothelioma (MEC-2005-269)
Study phase	Phase I
Objectives	1) To define the safety and toxicity of tumor lysate-pulsed dendritic cells (DCs) in patients with malignant pleural mesothelioma 2) To determine if vaccination with DCs results in a detectable immune response 3) To observe and document anti-cancer activity by clinical evaluation (CT-scan)
Study design	Non-randomized, three-dosing study
Patients	Number: 10 Type of cancer: malignant pleural mesothelioma Stage of disease: end stage, after chemotherapy HLA restriction: none
Investigational agents	Formulation: autologous monocyte-derived DCs pulsed with autologous tumor-lysate Dose: > $50x10^6$ dendritic cells Route of administration: intravenously and intradermally Number of doses: 3 Schedule of doses: every 2 weeks
Special procedures	Cleanroom facility for preparation of DCs and tumor-lysate. Research laboratory to measure the immune response *in vitro*
Study endpoints	Primary: safety and toxicity Secondary: anti-tumor responses *in vitro* and *in vivo* (DTH, laboratory tests, and by clinical response evaluation)

The objectives of this phase I study are to define the safety and toxicity of tumor lysate-pulsed autologous DCs injected in patients with MM after chemotherapy. Secondary end-points include immune responses by skin delayed type hypersensitivity (DTH) reactions on mesothelioma cell lysates and the control antigen KLH (keyhole limpet hemocyanin). Read-out parameters are the side effects, immune responses, anti-tumor responses both *in vivo* and *in vitro* and survival of this treatment.
In chapter 11, the results of the first MM patient ever treated with a combination of chemotherapy and DC-based immunotherapy are described, however, it is too early to allow any conclusions to be made about the effectiveness from this preliminary report. We anticipate to finalize this study in 10 patients by the end of 2007.

12.3 Future prospects

Immunotherapy is undoubtedly a very promising but also very challenging approach in the treatment of cancer. However, the full therapeutic potential has not yet been exploited (76). Variables that may require further optimization and standardization include the most effective source of dendritic cells, the optimal conditioning and maturation stimuli, the optimal route of administration, and the frequency and the optimal dose of DC vaccinations for treatment (76). Pre-clinical studies can further improve the immunological outcome of DC-based immunotherapy and may open up exciting new options for increased efficacy in the clinic. Deciphering the immunosuppressive factors produced by MM cells capable of affecting DCs, effector T lymphocytes, macrophages, NK and NKT cells, may give a better ability to predict tumor behaviour and to refine treatment. In addition, measures that can overcome the suppressive function of T_{reg} cells (77) and tumor-associated macrophages (78) might be used in combination with DC immunotherapy to increase the success rate of MM tumor eradication. Understanding these mechanisms of MM resistance and growth is an important area of research since overcoming the immune suppression has the potential for improved palliation, prolonged survival, and even potential cure for certain mesothelioma patients.

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Nederlandse samenvatting voor leken

Asbest is een verzamelnaam voor een aantal in de natuur voorkomende mineralen, die zijn opgebouwd uit microscopisch kleine naaldachtige vezels. Vanwege de unieke combinatie van een aantal specifieke eigenschappen van deze mineralen – zoals slijtvastheid, chemische bestendigheid, elektrisch isolerend vermogen en onbrandbaarheid – gecombineerd met een lage prijs en goede verwerkbaarheid maakte dat het in de vorige eeuw en met name vanaf de Tweede Wereldoorlog op zeer grote schaal werd toegepast.
Nu roept asbest associaties op met angst, woede, ziekte, pijn en dood omdat vast staat dat omgang met asbest dodelijk kan zijn. Sinds 1993 is er een algeheel asbestverbod van kracht in Nederland, hoewel er nog steeds veel asbesthoudende producten aanwezig zijn in schepen, bouwconstructies, vloer- en dakbedekkingen, isolatie van verwarmingsinstallaties, in oude remvoeringen en koppelingsplaten, enzovoorts. In landen die samen twee derde van de wereldbevolking omvatten gaat het verwerken van asbest nog gewoon door (China, India, Indonesië, Vietnam, Rusland, Canada, Zuid-Afrika, etcetara). De productie bedraagt wereldwijd nog steeds meer dan 2 miljoen ton per jaar. Blootstelling aan asbest kan leiden tot kwaadaardige aandoeningen (mesothelioom en longkanker) en niet-kwaadaardig aandoeningen (asbestose, pleuraplaques, pleuraverbreding en pleurale vochtophoping). Dit proefschrift besteed alleen aandacht aan mesothelioom, ook wel asbestkanker, borst- of longvlieskanker genoemd. Openbaart deze ziekte zich bij een patiënt, dan is sprake van een snel en dodelijk verloop: 80% van de patiënten overlijdt binnen één jaar nadat de diagnose is gesteld en zeer weinig patiënten overleven het tweede jaar na diagnose. In Nederland sterft ieder jaar een toenemend aantal mensen aan mesothelioom tengevolge van blootstelling aan asbest die in een ver verleden (vaak 15 – 40 jaar daarvoor) heeft plaatsgehad. Verwacht wordt dat deze stijgende trend zich nog een groot aantal jaren zal voortzetten en dat uiteindelijk vele duizenden sterfgevallen als gevolg van mesothelioom zullen worden geteld. Patiënten en hun nabestaanden willen vaak erkenning en schadeloosstelling omdat het zeer aannemelijk is dat werkgevers al heel lang op de hoogte waren van de gevaren. Compensatie voor de materiële en immateriële schade van patiënten leidt vaak tot lange juridische procedures. Dit alles maakt dat, ondanks dat het een relatief zeldzame kanker betreft, er veel aandacht voor bestaat in de media. Vrijwel dagelijks berichten media over asbest dat vrijkwam tijdens een brand, of extra kosten voor de sloop van schepen en gebouwen vanwege de aanwezigheid van asbest. De omslag van dit proefschrift geeft slechts een selectie van krantenartikelen weer van het afgelopen jaar met verschillende aspecten van asbest(-kanker).

Het maligne (kwaadaardige) mesothelioom is een tumor van de pleura (borstvlies), het peritoneum (buikvlies) of het pericard (hartvlies). De meest voorkomende klachten waarmee de patiënt met een pleura mesothelioom zich presenteert zijn kortademigheid, pijn op de borst, gewichtsverlies en hoestklachten. Op de röntgenfoto is dan vaak pleuravocht te zien al of niet in combinatie met verbreding van de pleura. Om de diagnose maligne mesothelioom te kunnen stellen en uit te sluiten dat het geen uitzaaiing van een andere kankervorm betreft, wordt pleuravocht en/of pleuraweefsel verkregen middels thoracoscopie. Op dit materiaal wordt cytologisch onderzoek en histologische diagnostiek verricht. Dit is erg lastig omdat de incidentie van tumor cellen in pleuravocht

vaak te laag is en omdat mesothelioom cellen weinig specifieke eigenschappen hebben die onderscheid met bijvoorbeeld adenocarcinoomcellen of reactieve mesotheelcellen mogelijk maken. Er worden diverse therapieën toegepast zoals chirurgie, radiotherapie, immunotherapie en chemotherapie maar deze leiden slecht tot een beperkte verbetering van de overleving. Het doel van het in dit proefschrift beschreven onderzoek was dan ook om de diagnostiek en de behandeling van mesothelioom te verbeteren.

Om het onderzoek naar de verbetering van de diagnostiek en de behandeling te kunnen verrichten zijn tientallen pleuravochten en tumorweefsels verwerkt die veroorzaakt werden door mesothelioom of door andere aandoeningen (adenocarcinoom / sarcoom / nier-falen etc.). Tien goed gekarakteriseerde mesothelioom tumor cellijnen zijn hieruit verkregen. Deze zijn in eerste instantie gebruikt om de diagnostiek te verbeteren door specifieke antistoffen te isoleren uit een faag-antilichaam bank. Antistoffen kunnen zeer specifiek eiwitten op bijvoorbeeld het oppervlak van cellen herkennen en zodoende gebruikt worden voor diagnostiek of zelfs therapie door het aankoppelen van toxische stoffen. Een model systeem maakte het mogelijk om de belangrijkste stappen in de selectie procedure op levende cellen te optimaliseren [hoofdstuk 4]. Het opheffen van de ondersteunende eenheid en vraagtekens rond de patent rechten zorgde ervoor dat dit deel van het onderzoek werd gestopt.
Met behulp van verschillende nieuwe eiwittechnieken (2D DIGE [hoofdstuk 5]; SELDI [hoofdstuk 6]) is getracht specifieke eiwitmarkers in het pleuravocht te vinden die karakteristiek zijn voor mesothelioom. Middels 2D DIGE vonden we een iso-vorm van Apolipoproteine AI in het serum, maar niet in het pleuravocht van dezelfde patiënt. Met de SELDI-TOF technologie vonden we dat andere leden van de Apolipoproteinen familie (Apolipoproteine CI) verlaagd zijn in het pleuravocht en dat deze, in combinatie met andere markers, gebruikt kunnen worden om mesothelioom te onderscheiden van andere oorzaken van pleuravocht.
Exosomen zijn kleine membraanblaasjes die selectief door cellen worden uitgescheiden met een mogelijke functie in de communicatie tussen cellen. Het was bijzonder moeilijk om deze membraanblaasjes zuiver uit het pleuravocht te isoleren [hoofdstuk 7], daarom is de secretie van exosomen door mesothelioom cellijnen verder onderzocht [hoofdstuk 8]. MALDI-TOF analyse wees uit dat deze exosomen eiwitten bevatten die belangrijk zijn voor de antigeen presentatie, migratie en adhesie. Ook werd het eiwit "developmental endothelial locus-1" (DEL-1) gevonden dat bloedvaten in de directe omgeving van de tumor (de zogenaamde tumor micro-omgeving) zou kunnen induceren en daarmee de groei van de tumor kan bevorderen. Hoofdstuk 9 behandeld andere eiwitten en cellen in de micro-omgeving van de tumor. Talrijke cytokinen (signaaleiwitten) die belangrijk zijn voor de immunosuppressie (remming van het afweersysteem) en angiogenese (bloedvatvorming), zijn aanwezig in pleuravochten, waarbij een groot aantal ook geproduceerd en gesecreteerd worden door mesothelioom cellen. Ook worden er grote hoeveelheden cytokinen gevonden die leukocyten (afweercellen uit het bloed) kunnen aantrekken en activeren. Naast vele T lymfocyten en macrofagen werden er ook regulatoire T cellen in of in de directe omgeving van een tumor gevonden. In een proefdier model voor mesothelioom neemt de overleving toe als deze regulatoire T cellen gedepleteerd worden doordat ze een efficiënte anti-tumor respons kunnen onderdrukken. In hoofdstuk 10 wordt voor het eerst aangetoond dat dendritische cellen (DC) de groei van mesothelioom kan remmen in een proefdiermodel. Dendritische cellen zijn dan

in staat om een anti-tumor respons te induceren door cytotoxische T lymfocyten te activeren en te laten prolifereren. Muizen zijn beschermd tegen een letale dosis van tumor cellen als ze eerst op de juiste manier gepulste dendritische cellen krijgen toegediend. Worden ze echter toegediend bij een bestaande tumor dan is de werking afhankelijk van de tumor grootte. In december 2005 hebben we toestemming gekregen van de medisch-ethische toetsingscommissie om deze therapie, de zogenaamde DC-immunotherapie, in een fase I studie bij mesothelioom patiënten te onderzoeken. Hoofdstuk 11 behandeld de voorlopige resultaten van de eerste mesothelioom patiënt die ooit behandeld is met een combinatie van chemotherapie en DC-immunotherapie. Hoewel de resultaten veelbelovend zijn, zijn meer patiënten en testen nodig om de efficiëntie van DC-immunotherapie te bepalen.

De rol en de toepasbaarheid van specifieke Apolipoproteinen in de diagnostiek van mesothelioom zal verder onderzocht worden in samenwerking met commerciële partners (Ciphergen Biosystems en Fujirebio Diagnostics). Het onderzoek dat verder in dit proefschrift wordt beschreven heeft de kennis vergroot over de eigenschappen van het maligne mesothelioom en de rol en toepasbaarheid van het immuunsysteem hierin. De huidige fase I klinische studie waarbij autologe (van de patient zelf) dendritische cellen worden geïnjecteerd na chemotherapie zal informatie geven of het afweersysteem zodanig kan worden gestimuleerd dat tumor cellen worden vernietigd. Verder onderzoek naar het beïnvloeden van het immunosuppressieve karakter van mesothelioom (bijvoorbeeld het blokkeren van de werking van interleukine-6, HGF en/of van regulatoire T lymfocyten) zou nieuwe aanbevelingen kunnen geven om immunotherapie bij patiënten met mesothelioom verder te optimaliseren.

List of abbreviations

2-DE	two-dimensional electrophoresis
Amp	ampicillin
AP	alkaline phosphatase
APC(s)	antigen presenting cell(s)
Apo	apolipoprotein
AUC	area under curve
BSA	bovine serum albumin
CD	cluster of differentiation
CHAPS	cholamidopropyldimethyl ammonio propanesulfonate
CTL(s)	cytotoxic T lymphocyte(s)
d	day
DC(s)	dendritic cell(s)
DEL-1	developmental endothelial locus-1
DIGE	differential gel electrophoresis
DTH	delayed-type hypersensitivity
DTT	dithiotreitol
EDTA	ethylene-diamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
EM	electron microscopy
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
Flt3-L	Fms-like tyrosine kinase 3-ligand
g	gravity
GM-CSF	granulocyte-macrophage colony stimulating factor
h	human or hour
HBSS	Hanks' balanced salt solution
HE4	human epididymes protein 4
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HS	human serum
HSC	heat shock cognate protein
HSP	heat shock protein
i.d.	intradermal(ly)
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
i.p.	intraperitoneal(ly)
IPG	immobilized pH gradient
i.v.	intravenous(ly)
Kan	kanamycin
kDa	kilo dalton
KLH	keyhole limpet hemocyanin
m	mouse
mAb	monoclonal antibody
MALDI	matrix-assisted laser desorption ionization
MHC	major histocompatibility complex
MM	malignant (pleural) mesothelioma
MS	mass spectrometry
MW	molecular weight
NK	natural killer
NKT	natural killer T
OPN	osteopontin
PAGE	polyacrylamid gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PF	pleural fluid
PhAb	phage antibody
pI	isoelectric point
PMR	Pulmonary Medicine Rotterdam
ROC	receiving operating characteristics
RPMI	Roswell Park Memorial Institute
RT	room temperature
ScFv	single-chain variable antibody fragment
SD	standard deviation
SDS	sodium dodecyl sulphate
SELDI	surface enhanced laser desorption ionization
SMRP	soluble mesothelin-related proteins
TAA	tumor-associated antigens
Tet	tetracycline
TGF	transforming growth factor
Th	T helper
TIL	tumor-infiltrating lymphocyte(s)
TNF	tumor necrosis factor
TOF	time of flight
Treg	regulatory T
Tris	tris(hydroxymethyl)-amino-methane
U/ml	units per milliliter
VEGF	vascular endothelial growth factor

Dankwoord

Als u op deze pagina bent geraakt door al het voorgaande gelezen te hebben zult u begrijpen dat ik dit alles niet had kunnen volbrengen zonder steun van anderen. Diegenen wil ik nu dan ook van harte bedanken. Allereerst en vooral alle patiënten die toestemming gaven om hun "overtollig" pleuravocht en weefsel te gebruiken voor dit onderzoek. Dit kontakt met patiënten gaf deze vreselijke ziekte een menselijk gezicht waardoor achter ieder stukje weefsel een echt persoon ontstond. Deze bijzondere dimensie aan het laboratorium werk betekende voor mij een extra stimulans. Hierbij wil ik hen, en de betrokken longartsen, chirurgen en verpleegkundigen hartelijk danken.

Voor de financiële bijdrage van dit onderzoek wil ik de Stichting Asbestkanker Rotterdam en de Amerikaanse patiënten organisatie MARF hartelijk danken. De heren van de Stichting Asbestkanker: dhr Van Rijckevorsel, dhr Schippers en dhr Van de Ven voor het opzetten, inzamelen van het geld en het zich belangeloos inzetten voor deze stichting. Zonder dit initiatief zou het onderzoek niet hebben plaats gevonden. Ik hoop dat we nog lang de geldkwesties en de voortgang van de experimenten samen kunnen bespreken onder het genot van een broodje kroket met een glaasje wijn. MARF, and in particular Mrs J. Wayne and Mr. C. Hahn, for granting our research in 2003 and in 2005, and organising impressive meetings between scientists, physicians, and patients.

Een aantal personen wil ik in dit dankwoord graag met nadruk noemen. Allereerst wil ik mijn beide promotoren, prof.dr. Henk Hoogsteden en prof.dr. Bart Lambrecht bedanken. Beste Henk, het is alweer bijna 10 jaar geleden dat ik bij je thuis de overgang van Immunologie naar Longziekten kwam bespreken. Van deze overstap heb ik nooit spijt gehad. Bedankt dat je mij de mogelijkheid hebt gegeven om deze promotie uit te voeren en de vrijheid die je gaf bij de invulling hiervan. Beste Bart, bedankt voor het in mij gestelde vertrouwen. Je moest zelf nog promoveren toen je bij ons in Rotterdam kwam werken, nu ben je al professor, en terecht! Jouw enorme medische ervaring en wetenschappelijke kennis van allerlei longaandoeningen in het algemeen en dendritische cellen in het bijzonder is fenomenaal. Bedankt voor je aanwijzingen en verbeteringen van manuscripten en projecten ondanks je overvolle agenda.

De overige leden van de kleine en grote commissie: prof.dr. Sillevis Smitt, prof.dr. Looijenga, prof.dr. Thielemans, prof.dr. Figdor, dr. Luider, dr. Gregoire en dr. Aerts wil ik bedanken voor het beoordelen van mijn proefschrift en het opponeren.

Beste prof.dr. Figdor, het is voor mij een genoegen om u in mijn commissie te hebben (Spinoza prijs 2006, red.). Ik ben dankbaar dat we de klinische studie in samenwerking met uw laboratorium onder deskundige leiding van Jolanda de Vries en met hulp van het sympatieke cleanroom team: Mandy van de Rakt, Nicole Meeusen-Scharenborg, Annemiek de Boer, Joost Lesterhuis, Inge Boullart en Daniel Benitez-Ribas kunnen uitvoeren. Ik was aangenaam verrast van de vriendelijkheid waarmee ik ontvangen werd en ben onder de indruk van de collegialiteit en goede communicatie binnen de groep. Het is altijd weer een plezier naar Nijmegen te gaan.

Dr. Luider, beste Theo, bedankt voor je hulp met de MALDI-TOF en het zelfstandig mogen gebruiken van de geavanceerde apparatuur om proteomics te kunnen doen.

Dr. Joachim Aerts, bedankt voor je veelvuldige en snelle overleg bij de METC aanvraag en de huidige klinische studie. Het is verbazingwekkend te zien hoe je je privé-leven (met weer een nieuwe spruit), het klinische werk en wetenschappelijk onderzoek weet te combineren. Jij weet snelheid met kwaliteit te combineren.

Ook wil ik nog een aantal mensen bedanken uit mijn Immunologie periode. Errol Prens, Roger Troost en Martijn Schrama (en hun echtgenotes natuurlijk) voor de vriendschap die, na al die jaren, nog onverminderd standhoudt.

Veel mensen heb ik gedurende mijn tijd bij Longziekten zien komen en sommige ook weer zien gaan: Alex, Annabrita, Bianca, Bregje, Brigit, Claudia, Corine, Daniëlle, Elke, Femke, Fransje, Gerard, Gert-Jan, Hamida, Harmjan (oud-kamergenoot, adviseur computeraangelegenheden en winnaar van vele "euro-dubbeltjes" weddenschappen), Hendrik-Jan, Hermelijn, Ivette, Jan-Bas, Jorrit, Kamal, Karolina, Kassiani, Leonie, Lous, Marco, Margaretha, Martin, Menno, Mirjam (mijn kamergenoot, je maakte onze kamer tot een tweede thuis), 'ons' Moniqueske, Nanda, Paco, Peter, Pim, Rahila, Rebecca, Sophia, Taco, Tanja, Thomas, Victor en andere die ik vergeten mocht zijn. Allen bedankt voor de interesse in mijn onderzoek, adviezen maar bovenal de gezellige tijd tijdens de koffie (klokslag 10.10 uur en 15.00 uur), eten in de bedrijfskantine, de weddenschappen (choko toffs - Harmjan, zure matjes - Peter, alcohol - Thomas, spekjes - Danielle, negerzoenen - Menno, en dat er nog vele "helden" mogen volgen), de labuitjes en tijdens de congressen.
Een aantal wil ik nog eens apart noemen. Jan-Bas Prins, ik wil je bedanken dat je me gevraagd hebt om mee naar Longziekten te gaan om een nieuw laboratorium op te zetten. Het was buitengewoon interessant te ervaren wat daarbij komt kijken. Jij, samen met Henk gaven de aanzet tot mesothelioom onderzoek wat ik bij Bart heb kunnen voortzetten, uiteindelijk resulterend in dit proefschrift.
Sjaak Burgers, het was prettig en gezellig om met je van gedachten te wisselen over mesothelioom tijdens de congressen in Brescia, Las Vegas en Chicago.
Martin Bard, mijn Franse collega, voor het samen opzetten van de exosoom isolatie en de MALDI-TOF analyses. Dit heeft geresulteerd in twee manuscripten en diverse hoofdstukken over "proteomics in kanker". Je was een fijne collega en ik ben onder de indruk hoe jij zo snel hebt kunnen promoveren in een vreemd land op een "tweede" onderwerp. Ook de overige mede-auteurs op de artikelen wil ik via deze weg nogmaals bedanken voor hun bijdrage.

En natuurlijk mijn paranimf Annabrita Schoonenberg-Hemmes, zonder jou had dit boekje er heel anders uitgezien. Helaas voor het onderzoek ben je met Richard naar Finland geëmigreerd maar het is goed te zien dat jullie het daar prima naar jullie zin hebben. Milloin odotatta perheenlisäystä (tik-tak)? Je bent een kei en onze gezamenlijke artikelen, die de basis van de huidige klinische studie vormt, is voor een groot gedeelte jouw verdienste. Ook was je de gangmaker van het lab voor het organiseren van uitjes. Ik ben blij dat je even terug bent uit Finland om mij als paranimf terzijde te staan.

Ook mijn andere paranimf, Harald Stamhuis, wil ik bedanken voor zijn steun op deze dag. Als sergeant "verkenner" en sergeant "wasstraat" waren we al op elkaar aangewezen om door onze militaire diensttijd te komen. Bij beide is daarna nog veel gebeurd maar we hebben altijd kontakt gehouden en het is altijd heerlijk om naar jullie in Friesland te gaan (Fryslân boppe) of als jullie naar ons komen. Ik heb diep respect voor jouw (en Attsje's) nuchterheid, hartelijkheid, relativerings- en doorzettingsvermogen.

Pap en mam, hoewel ik al lang ben uitgevlogen is het altijd weer fijn terug in het warme nest te komen.
Lieve Kirsten, je weet de opvoeding van onze kleine meid prima te combineren met je eigen carrière. Bedankt voor je begrip en steun met name tijdens de laatste fase van het onderzoek.
Nu dan nog een speciaal woordje voor mijn lieve dekselse donderstraal: Amber. Op dit moment begrijp je nog niet goed waar papa nou al die tijd mee bezig is. Jij liet me echter inzien om met aandacht te leven en dat de gelukkigste momenten in de pure allerdaagse kleine dingen zitten. Straks gaan we eerst nog even lekker kroelen, keleure, danse of puzzele, noemies eten of een nammetje zoeken – wat jij wil!

Het proefschrift is klaar maar gelukkig gaat het onderzoek gewoon door. Ik kijk uit naar de opzet van een Rotterdamse DC-immunotherapie-unit, de resultaten van de fase I klinische studie en het voorbereiden van een sterk vervolg door middel van preklinische studies (samen met Joachim en met hulp van Daniëlle Hijdra en Margaretha Lambers).
Mijn hoop voor de toekomst is dan ook dat we eens kunnen zeggen dat mesothelioom niet langer meer dodelijk is.

Joost

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Curriculum Vitae

The author of this thesis was born on May 31, 1967 in Heesch. He completed his study in 1990 at the Higher Laboratory School (HLO) in Oss (degree: Bachelor's, specialization: biochemistry – biotechnology). He received his training at the Department of Tumorbiology, Radboud University Nijmegen, where he studied genes encoding ovarian carcinoma-associated antigens. After his military service in 1991, he joined the Department of Immunology in Rotterdam studying immunological aspects of psoriasis, a common inflammatory skin disease characterized by epidermal hyperproliferation. This work was performed under supervision of Prof.dr. E.P. Prens and Prof.dr. R. Benner.
He was involved in the initiation of the research laboratory of the Department of Pulmonary Medicine by Prof.dr. H.C. Hoogsteden in 1997. Research on malignant mesothelioma is performed under supervision of Prof.dr. B.N. Lambrecht and is financially supported by "Stichting Asbestkanker Rotterdam" and the Mesothelioma Applied Research Foundation (MARF). He is currently employed by the same department, responsible for introducing clinical dendritic cell-based immunotherapy in Rotterdam.

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