IMMUNOSUPPRESSION IN HEAD AND NECK CANCER

The role of p15E-related immunosuppressive factors

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IMMUNOSUPPRESSION IN HEAD AND NECK CANCER

The role of p15E-related immunosuppressive factors

Immuunsuppressie in hoofd hals kanker
De rol van p15E-verwante immuunsuppressieve faktoren

PROEFSCHRIFT

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Contents

Abbreviations

ADCC	antibody dependent cellular cytotoxicity
AIDS	aquired immunodeficiency syndrome
ASV	avian sarcoma virus
BSA	bovine serum albumin
CK	cytokeratin
ConA	concavalin A
CSF	colony stimulating factor
CTL	cytotoxic T-lymphocyte
DMEM	Dulbecco's modified Eagle's medium
DNA	desoxyribonucleic acid
DNCB	dinitro-chloro-benzene
DTH	delayed type hypersensitivity
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ELISA	enzyme linked immunosorbent assay
EBV	Epstein-Barr virus
ERV	endogenous retrovirus
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FeLV	feline leukemia virus
FLV	Friend leukemia virus
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FSV	feline sarcoma virus
HERV	human endogenous retrovirus
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HPV	human papilloma virus
HRP	horse reddish peroxidase
HTLV-I	human T-lymphotropic virus
IFN	interferon
IL	interleukin
KLH	keyhole limpet hemocyanin
LAK-cell	lymphokine activated killer cell
LMWF	low molecular weight factor
LPS	lipopolysaccharide
LTR	long terminal repeat
min	minutes
MAb	monoclonal antibody
MHC	major histocompatibility complex
MST	median survival time
MuLV	murine leukemia virus

NFDM	non-fatty dry milk
NK-cell	natural killer cell
O/N	overnight
PAb	polyclonal antibody
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
PGE	prostaglandin E_2
PPD	purified protein derivative
RLV	Rauscher leukemia virus
RNA	ribonucleic acid
RT	reverse transcriptase
SDS	sodium dodecyl sulfate (in SDS gel electrophoresis)
SCC-HN	squamous cell carcinoma of the head and neck
SCIF	squamous carcinoma inhibitory factor
SER	suppressive E-rosette receptor
SIC	serum immune complex
SU	surface
TAA	tumor associated antigen
TGF	transforming growth factor
TIL	tumor infiltrating lymphocyte
TM	transmembrane
TNF	tumor necrosis factor
TNM	in TNM classification: tumor, nodes, metastases
TSA	tumor specific antigen

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General introduction

General introduction

Cancer is the second cause of death for both younger people and older people in the Western world. Although about 50% of patients with diagnosed cancer can be cured with the conventional modalities of cancer treatment, the other half will die of the disease. The chances of survival from cancer strongly correlate with the type of cancer and with the stage of the disease at the time of diagnosis. After a malignant tumor has been diagnosed, the therapy of choice will also depend upon the type of tumor and the tumor stage.
Surgery is the most widely used approach in cancer therapy. Surgical excision is both quick and effective and it accounts for the largest number of cures. Unfortunately, this form of treatment has several drawbacks. Removal of the tumor mass visible to the surgeon does not guarantee elimination of all tumor cells. In order to remove all tumor tissue, the surgeon may be forced to cut out healthy tissue and, in doing so, may severely damage the patient's functioning or appearance.
Surgery can be combined with radiation therapy to eliminate residual tumor cells or microscopic invasions of malignant cells in surrounding tissues. Radiation therapy can be preferable to surgery in some instances. Because healthy tissues usually recover from radiation exposure more readily than cancerous cells, radiation therapy can preserve the anatomical structures surrounding the tumor. Still, it should be noted that radiation therapy will cause problems, most of which will disappear when therapy cycles are completed. Radiation therapy is especially important for tumors not accessible for surgery (e.g. some brain tumors) or for treating cancers of the larynx (voice box). In this way cancer of the larynx can be cured without impairing the patient's ability to speak.
Chemotherapy is a systemic therapy, required when the tumor cells have already spread throughout the body, or when no solid tumor is present, as is the case in leukemia. A drawback of chemotherapy are the side effects, preliminary caused by the fact that normal dividing cells are also killed by the chemotherapeutic agent.

Such "classical" therapeutic regimens are constantly subject to changes, based on the results of clinical trials. In these trials, new protocols combining surgery, radiation therapy and chemotherapy are developed in order to improve therapeutic outcome (1).
Completely new anti-cancer therapies come into view, as research has lead to a better understanding of the molecular basis and the biological consequences of cancer. Over the past years our knowledge of the transformation of normal cells into tumor cells and the interactions between tumor cells and cells of the immune system has substantially increased. New insights will undoubtfully lead to the development of new tools for early diagnosis and new successfull therapies.

Scope of the thesis

Head and neck cancer is well known to have a negative influence on the immune system of the patient. Over the past decades, the immunological state of patients with head and neck cancer has been described (reviewed by Wustrow and Mahnke in 1996 (2)). In general, it appears that the immune system in these patients is suppressed. However, our knowledge of the exact mechanism of this immunosuppression is still limited. The knowledge of the molecules involved in immunosuppression will help us to understand the pathophysiology of head and neck cancer, to develop new diagnostic tools and probably even to find new therapeutic strategies for the treatment of head and neck cancer.

The main goals of the experimental work described in this thesis are:

1. To characterize the immunosuppressive factors associated with head and neck cancer, produce monoclonal antibodies against these factors and to investigate the relationship of these factors with retroviral immunosuppressive proteins.
2. To study the distribution of such factors in several tumor types and in normal tissues.
3. To develop a diagnostic test for the detection of these factors in patient sera.
4. To study the therapeutic applicability of monoclonal antibodies against these factors in an animal model.

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Cancer and the immune system

Cancer and the immune system

Malignant transformation of cells

Cancer is a disease, caused by malignant transformation and uncontrolled growth of normal cells. Both induction of proliferation and inhibition of proliferation are normal processes in the physiology of the cell. These processes are tightly regulated by the cellular genes. Cancer arises when genes involved in controlling cell proliferation are damaged. When a single cell accumulates a number of mutations (usually over many years), this cell may escape from the growth regulatory mechanisms and transform into a malignant cell.
Two important types of genes control cell proliferation: (a) oncogenes and (b) tumor suppressor genes. Cellular oncogenes encode growth factors, growth factor receptors and nuclear proteins, each playing a central role in the control of normal cellular proliferation. Tumor suppressor genes encode proteins which prevent uncontrolled cell growth (reviewed by Weinberg in 1996 (3)).

Malignant transformation of cells may be caused by viral infection (e.g. papilloma virus, playing a role in the development of cervical cancer and probably also in head and neck cancer), by chemicals (e.g. asbestos, causing mesothelioma), or by radiation (e.g. ultraviolet rays, causing skin cancer). Several genetic changes have to take place before a cell escapes the normal control mechanisms. One or more of these genetic changes may result in alterations of the cellular characteristics. Tumor cells are often different in shape ("transformation"), which enables recognition of these cells by the pathologist.
Also, tumor cells may produce proteins, which were not produced before the malignant transformation (reviewed by van den Eynde in 1995 (4)). When new antigens (proteins recognized by the immune system) arise in the malignant transformation process, these are called "tumor specific antigens" (TSA). TSA may arise *e.g.* after viral transformation of epithelial cells, as in Human Papilloma Virus (HPV) induced cervix carcinoma. This infection results in the expression of new HPV antigens at the cell surface (5). Also, tumors from non-viral origin, like melanomas, express new, specific, antigens at their cell surface (6). For example, the MAGE-1 gene is aberrantly expressed in melanoma, resulting in the expression of the new MZ2-E antigen (7), which can be recognized by cytotoxic T-cells (8). Mutations in tumor suppressor genes (p53), causing immortalization of the mutated cell, or mutations in oncogenes (*Ras*), causing uncontrolled cell growth, may also result in the generation of tumor specific antigens, which can be recognized by the immune system (9).

Immunological reactivity against malignant cells

One would expect that the immune system is able to recognize the tumor by its tumor specific antigens, elicit an immune response and finally, eliminate the malignant cells. This *immune surveillance* hypothesis, postulated by Burnett and Thomas, already in 1970, however, only applies to the situation where tumors express antigens which are not expressed on other cells. Unfortunately, most antigens expressed by tumor cells are "tumor associated antigens" (TAA), which are also expressed on normal cells. As a consequence, the immune system will be tolerant to such antigens.

Apart from initial recognition, the immune system should also be able to elicit a specific immune response to the malignant cells. The immune system has several cell types at its disposal for the recognition and destruction of tumor cells (Table 1). Some of the cells of the immune system are able to kill tumor cells even without specific recognition (*e.g.* activated macrophages or natural killer (NK) cells). T-cells are able to recognize tumor specific antigens at the surface of the tumor cell, presented in the context of the human leucocyte antigens*. After recognition, cytotoxic T-cells kill tumor cells by cytolysis. In contrast, helper T-cells, after recognition of the tumor cell, produce lymphokines**, molecules which activate other immune cells like macrophages (10) and NK-cells.

Activated macrophages kill tumor cells through cytolysis after cell-cell contact (11,12). Activated macrophages can react indiscriminantly against "immunogenic", "weakly immunogenic" and "non-immunogenic" tumors (13).

NK-cells are able to kill tumor cells through antibody dependent cell mediated cytotoxicity (ADCC) and such cells can be stimulated to become "lymphokine activated killer" cells (LAK-cells). LAK-cells and TIL-cells ("tumor infiltrating lymphocytes") are able to kill tumor cells whether or not such cells express tumor specific antigens (14). Mechanisms involved in tumor cell killing are illustrated in Fig. 1.

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*Human Leucocyte Antigens (HLA) are encoded by the genes of the major histocompatibility complex (MHC). HLA class I antigens are expressed on all nucleated cells and on thrombocytes. HLA class II antigens are expressed on antigen presenting cells (*e.g.* B-cells, macrophages and dendritic cells) and on activated T-cells. Antigens presented at the cell surface are recognized in the context of HLA molecules. Thus, peptides derived from intracellular antigens (oncogene products, viral antigens) associate with HLA class I antigens and are recognized by cytotoxic CD8+ T-cells. Antigens which have been processed by antigen presenting cells associate as peptides with HLA class II antigens and are recognized by helper CD4+ T-cells.

** Lymphokines are biological or pharmacological active proteins produced by lymphocytes. As not only lymphocytes produce such biological active proteins, the term lymphokine was changed into cytokine. Examples of cytokines are: interferon (IFN), interleukins (IL) and tumor necrosis factor (TNF)

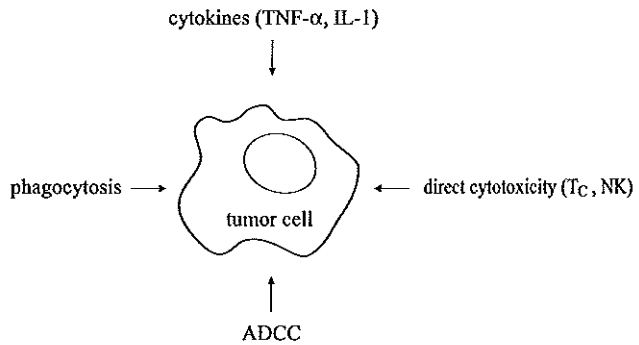

Fig. 1 Mechanisms involved in tumor cell killing: direct or antibody-dependent cellular toxicity, cytokine mediated cytolysis or phagocytosis. Taken from "Medische Immunologie" by Benner *et al.* (15).

Tumor cell escape from immunological defence mechanisms

The fact that transformed cells may continue their process of uncontrolled cell growth, thereby giving rise to malignant tumors, indicates that either the recognition and/or destruction mechanisms in the immune system are dysregulated.
Tumors escape elimination by host defences through a variety of means, including host alterations in antigen expression, decreased expression of human leucocyte antigens and secretion of immunosuppressive factors.
Tumor cells prevent recognition of antigens at the cell surface by cells of the immune system at least in three different ways:

1. HLA class I downregulation is a frequently observed event. Between 40 and 90% of tumors derived form HLA+ epithelia are HLA deficient (17). Cytotoxic T-lymphocytes recognize antigens only in the context of HLA class I antigens, implicating that HLA class I negative tumor cells escape T-cell cytotoxicity.
2. Reducing the expression of tumor specific antigens by modulation of the antigens from the cellular membrane will prevent their immune recognition.
3. Blocking antibodies, which bind tumor specific antigens, may prevent the recognition of these antigens by circulating T-cells, thereby preventing an immune response.

After specific recognition of a malignant tumor cell, an immune response can only be elicited when the immune system is properly activated. Suppression of immune activation will also prevent the destruction of tumor cells. Patients with advanced cancer are frequently found to exhibit impaired immune responses. Both lymphocyte, as well as macrophage and NK-cell functions are suppressed in these patients. Major factors involved in suppression of immune reactivity against tumor cells are soluble products, produced by the tumor cells (18). An important notion is that immunosuppression particularly occurs in patients with head and neck cancer (2,19-25).

Table 1. Cells involved in the immune response against tumor cells (15,16).

Cell type		Actions
lymphocytes	T-cells	*T-helper cells (CD4+) recognize antigen on antigen presenting cells (B-cells, macrophages and dendritic cells) in the context of MHC II. *Produce cytokines to stimulate all other immune cells (*e.g.* IL-2, IL-4, IL-5, IL-6, IL-9) *T-cytotoxic (CD8+) cells recognize antigen on tumor cells in the context of MHC I and subsequently kill these cells trough cytolysis *T-cytotoxic cells kan also kill through ADCC (see NK-cells) *T-suppressor cells produce cytokines which are inhibitory for other immune cells (*e.g.* IL-10, IL-13)
	B-cells	*Present antigen to T-cells in the context of MHC II *Produce antigen specific antibodies
	natural killer cells (NK-cells)	*Bind antibody coated tumor cells through Fc receptor - IgG binding and kill through antibody dependent cellular toxicity (ADCC) *Produce interferon (IFN-γ which acts synergisticly with TNF-α)
	lymphokine activated killer cells (LAK-cells)	*Lymphokine (IL-2) activated lymphocytes bind to tumor cells and kill through non-specific cytolysis (LAK-cells) *Lymphokine (IL-2) activated tumor infiltrating lymphocytes (TIL) are present in the tumor and kill tumor cells
	large granular lymphocytes (LGL)	*Kill through antibody dependent cellular cytotoxicity (ADCC)
phagocytes	Macrophages	*Present antigen to T-cells in the context of MHC II *Lymphokine activated macrophages kill tumor cells through release of toxic cytokines (*e.g.* tumor necrosis factor, TNFα and IL-1) *Produce immunoregulatory cytokines (stimulatory and inhibitory *e.g.* PGE2)

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Head and neck cancer

Head and neck cancer

In broad terms, head and neck cancer represents malignancies in the region above the clavicles. Most significant are cancers arising from the lining of the mouth (oral cavity), throat (pharynx) and voice box (larynx), collectively referred to as the upper aerodigestive tract (Fig. 1).

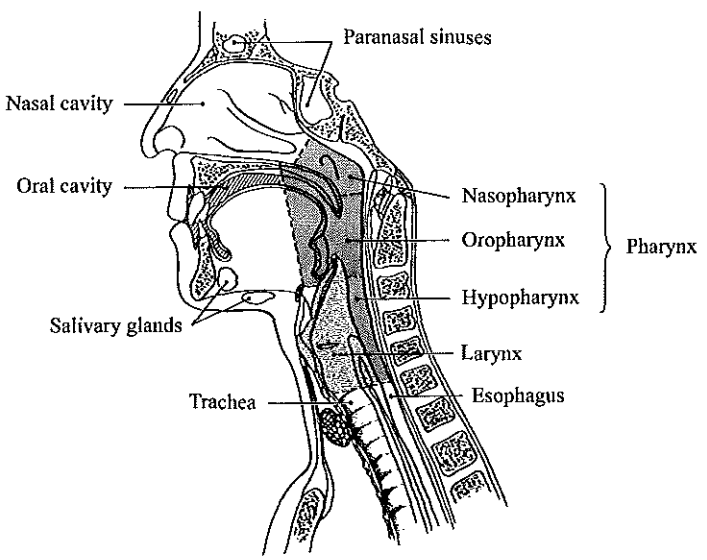

Fig. 1. Sagittal section of the upper aerodigestive tract (26).

Tumors of these sites comprise about 5% of all malignant tumors in men and about 2% in women, resulting in more than 500.000 new cases per year worldwide (26,27). The great majority of tumors of the head and neck originate from the epithelial cells of the mucosal surface lining the upper aerodigestive tract and are called carcinoma. Histologically these tumors are almost always defined as squamous cell carcinoma (squamous cell carcinoma of the head and neck: SCC-HN) (28).

Etiology of head and neck cancer

Significant etiologic factors associated with SCC-HN are heavy smoking and excessive alcohol drinking. More than 90% of the patients have a history of previous tobacco use and alcohol exposure (29). Burning of tar produces a variety of substances which are broken down into carcinogenic compounds that bind to DNA. People who smoke 25 cigarettes per day have over 16 times more chance of developing head and neck cancer as compared to people who do not smoke. Smoking

filtered cigarettes seems to be associated with around half the risk of smoking untipped cigarettes (30). Alcohol potentiates genetic damage induced by carcinogens by inhibiting DNA repair (31), or may act indirectly by promoting hepatocellular dysfunction, resulting in diminished detoxification of oral carcinogens.
Other risk factors for head and neck carcinoma include vitamin A deficiency, poor oral hygiene (32) and viral infections (herpes simplex virus type I, human papilloma virus or Epstein Barr virus) (33-38).
Human papilloma viruses form a large family of DNA viruses. These viruses are trophic to squamous epithelium for their replication. Several cases of head and neck carcinomas are clearly HPV related, for example in patients with long-standing recurrent respiratory papillomatosis in which one of the papillomas has undergone malignant conversion. The combination of a long-term infection with HPV and a second carcinogenic factor (*e.g.* smoking or radiation) increases the probability of malignant conversion of the pre-existing benign lesion (39). Another mechanism of HPV induced carcinogenesis could be the inactivation of p53 or retinoblastoma tumor suppressor gene products by HPV proteins. HPV oncoprotein E6 binds and degrades p53 (40) and E7 antigens bind the retinoblastoma gene product (41). Although the exact role of HPV in head and neck cancer has not been elucidated, 23% of head and neck cancers, analyzed in several studies, were positive for HPV (39).

Head and neck cancer therapy

Two modalities of therapy, surgery and radiation therapy, have well-established roles in the treatment of carcinoma of the head and neck. Choice of therapy depends on many factors such as the site and extent of the primary lesion, the likelihood of complete surgical resection, the presence of lymph node metastases, invasion of adjacent soft tissue structures and bone, impairment of function, etc. In general, lower stage tumors (stage I or II, without metastases, see Table 1), are effectively treated by either surgical excision or irradiation, whereas more advanced lesions (stage III or IV, see Table 1) are best treated with a combination of surgery and radiation therapy. In patients with small primary lesions (T1, T2) surgery and radiation therapy results in a 5 year survival of 70-90%. However, these patients have a substantial risk of developing a second primary malignancy in the upper aerodigestive tract of 4-7% per year (43).
About 60% of primary head and neck cancers are already locally advanced (T3, T4) with cure rates of 15 - 40%. In these patients, local recurrence, the development of regional metastases to the cervical lymph nodes (30 - 80%) and distant metastasis (10-30%) occur in high frequency. Radiation therapy is used to reduce the risk of loco-regional recurrence after resection of the primary tumor.

Chemotherapy is currently considered a standard therapy for treatment of patients with recurrent cancer or distant metastases (44,45). Chemotherapy has been applied

Table 1. TNM classification of malignant tumors of the head and neck (42)

T - Primary Tumor	TX = Primary tumor cannot be assesed T0 = No evidence of primary tumor Tis = Carcinoma *in situ*
Lip and oral cavity	*buccal mucosa, upper and lower alveolus and gingiva, hard palate, tongue and floor of the mouth*
Tis	Carcinoma in situ
T1	Tumor <= 2 cm in greatest dimension
T2	Tumor > 2 cm, but <= 4 cm
T3	Tumor > 4 cm
T4	Tumor invades adjacent structures (*e.g.* cortical bone, deep muscle of tongue, maxillary sinus, skin)
Pharynx	*oropharynx, nasopharynx and hypopharynx*
Oropharynx nasopharynx	T classification as for the lip and oral cavity
T1	Tumor limited to one subsite of nasopharynx
T2	Tumor invades more than one subsite of nasopharynx
T3	Tumor invades nasal cavity and/or oropharynx
T4	Tumor invades skull and/or cranial nerves
hypopharynx	
T1	Tumor limited to one subsite of hypopharynx
T2	Tumor invades more than one subsite of hypopharynx or an adjacent site, *without* fixation of hemilarynx
T3	Tumor invades more than one subsite of hypopharynx or an adjacent site, *with* fixation of hemilarynx
T4	Tumor invades adjacent structures, *e.g.* cartilage or soft tissues of neck
Larynx	*supraglottis, glottis and subglottis*
supraglottis	
T1	Tumor limited to one subsite of supraglottis, with normal vocal cord mobility
T2	Tumor invades more than one subsite of supraglottis or glottis, with normal vocal cord mobility
T3	Tumor limited to larynx with vocal cord fixation and/or invades postcricoid area, medial wall of piriform sinus or pre-epiglottic tissues
T4	Tumor invades through thyroid cartilage and/or extends to other tissues beyond the larynx, *e.g.* to oropharynx, soft tissues of neck

Glottis

T1	Tumor limited to vical cord(s) with normal mobility
T2	Tumor extends to supraglottis and/or subglottis, and/or with impaired vocal cord mobility
T3	Tumor limited to the larynx with vocal cord fixation
T4	Tumor invades through thyroid cartilage and/or extends to other tissues beyond the larynx, *e.g.* to oropharynx, soft tissues of the neck

subglottis

T1	Tumor limited to the subglottis
T2	Tumor extends to vocal cord(s) with normal or impaired mobility
T3	Tumor limited to the larynx with vocal cord fixation
T4	Tumor invades through cricoid or thyroid cartilage and/or extends to other tissues beyond the larynx, *e.g.* to oropharynx, soft tissues of the neck

N - Regional Lymph Nodes	The regional lymph nodes are the cervical nodes
N0	No regional lymph node metastases
N1	Metastases in a single ipsilateral lymph node (<= 3 cm)
N2	Metastases in a single ipsilateral lymph node (> 3 cm, but <= 6 cm), in multiple ipsilateral lymph nodes (none > 6 cm), or in bilateral or contralateral lymph nodes (none > 6 cm)
N3	Metastases in a lymph node > 6 cm

M - Distant Metastases

M0	No evidence of distant metastasis
M1	Distant metastases

Stage Grouping

0-II	Primary tumor (Tis, T1 or T2), without lymph node or distant metastases
III	Primary tumor (T1 or T2) with N1 metastases; or T3
IV	Primary tumor T4 with N1 metastases; any primary tumor stage with N2 or N3 metastases, or with distant metastases

in many ways, *e.g.* as induction therapy before other forms of treatment, synchronously with radiotherapy, as maintenance therapy after radiotherapy and/or surgery and by intra-arterial infusion. The purpose of chemotherapy is to palliate symptoms and prolong survival. Although chemotherapy is able to induce a significant tumor reduction, no detectable survival benefit for head and neck carcinoma has been demonstrated (46). Active single agents in chemotherapy of head and neck cancer include methotrexate, cisplatin, carboplatin, 5-fluorouracil, hydroxyurea, doxorubicin and bleomycin.
In recent years, the combined use of chemotherapy and radiotherapy has been studied in patients with advanced head and neck cancer. By not using surgery, the damage to vital organs can be reduced. This concept of "*organ preservation*", has matured as a rational option to massive surgery and thus, patients may be spared the morbidity of laryngectomy, glossectomy or resection of the pharynx. Patient survival, using this non-surgical therapy, is comparable to survival after conventional surgery and radiotherapy (28,47).

Most failures of treatment (recurrent tumors) in head and neck cancer patients occur in the primary region, neck nodes or in both. Recurrences are more common in patients with stage III and stage IV disease than in patients with smaller tumors without lymph node metastases. The majority of patients with primary stage III and stage IV disease have been treated with a combination of surgery and radiotherapy. After recurrence, few of these patients can be salvaged with secondary treatment, therefore, most patients die of uncontrolled tumor growth, which is a significant clinical problem.
Because the entire epithelium of the head and neck area is exposed to the carcinogenic effects of tobacco and alcohol, new malignant transformations of epithelial cells may occur in other parts of the upper aerodigestive tract. This observation was in 1953 termed "*field cancerization*" (48).
If patients survive long enough from their local/regional disease, without local recurrence, there is a considerable risk to develop distant metastases (10-40%) (49).

Apart from the prognosis for survival, the devastating effects of the disease and treatment on important bodily functions and the physical implications, are of paramount concern for patients with head and neck cancer. These effects may comprise severe impairment of the individual's ability to chew, swallow, speak, breathe, hear, see, taste and smell. Furthermore, the disease and the result of treatment is often visible and offensive to others, resulting in psychosocial problems and depression (50,51).

Clearly, there is still a constant need to adapt therapeutic strategies for patients with head and neck cancer and to employ more effective, nonsurgical (nonmutilating) methods. With the aim of reducing the number of recurrent and second primary

tumors, the use of retinoids (vitamin A analogs) has been tested in chemoprevention trials. Retinoids have been shown to slow down the development of epithelial cancers, as a result of suppression of the expression of genes associated with squamous differentiation. Preclinical *in vitro* studies and preliminary results of clinical trials have shown a positive effect of retinoids particularly in the prevention of recurrent head and neck cancer (52-55).

The major focus of head and neck oncologists over the past years has been to find new ways to integrate chemotherapeutic agents into the multimodality therapy of patients with advanced lesions (56). Although this has lead to new therapeutic strategies, the results have not had a demonstrable impact upon cancer mortality (57,58). Two-thirds of patients with head and neck cancer have advanced disease at the time of diagnosis, with an expected survival of less than 30%. As the expected survival for patients with early stage disease is over 70%, early, accurate diagnosis is the best way to improve survival rates. Of course, prevention of head and neck cancer by cessation of smoking and less alcohol abuse would have the greatest impact on (the incidence of) head and neck cancer (59,60)!

As new therapeutic strategies using surgery, radiotherapy and chemotherapy will unlikely influence cure rates further, immunotherapy may well become an adjuvant to standard treatment (58). Systemic and locoregional immunotherapy with IL-2 in SCC-HN has been studied in preclinical and clinical research (61,62). Although a host immune response towards the tumor can be elicited, the serious toxicity of large systemic doses of IL-2 was a major problem in these studies. A better knowledge of the exact causes of immunosuppression in head and neck cancer will help in the development of the most effective immunotherapeutic strategies.

Head and neck cancer and the immune system

The remarkable ability of head and neck cancer to cause extensive local tissue damage and regional nodal involvement in the absence of distant metastic spread has lead to the belief that there are some distinctive local immunological characteristics. Local immunosupppression could well be a contributing factor to progressive disease and local recurrence (19,24,30,63-68).
Specific cells of the immune system and subsets of cell types have been studied in detail and immune dysfunctions have predominantly been found in cell types associated with the cellular immune response.

T-cells

T-cells are part of the cellular immune sytem and are able to recognize antigens at the cell surface. After recognition, T-cells are able to kill the recognized cells through cytolysis.

Disturbances of cellular immune functions are the most obvious abnormalities in patients with head and neck cancer. Since 1970, many investigators have published that a positive skin test to antigens like DNCB and PPD correlates with a better prognosis (69,70). However, as skin test results are not easy to interpret (71), and since the correlation with prognosis has been questioned (24,72,73), this method is presently not used to predict tumor prognosis.
Total numbers of peripheral T-lymphocytes are reduced in patients with SCC-HN, indicative for the immunosuppressed state. This decrease in T-cell counts correlates with a poor prognosis (70,74). However, statistically significant correlations with prognosis have been inconsistent and tumor stage and localization should certainly be taken into account (65,75-77). In general, the mean helper/cytotoxic-suppressor (T4/T8) ratio is increased with increasing tumor stage and is significantly elevated in patients with SCC-HN as a group, as compared with normal subjects. This elevated level is the consequence of a decrease in CD8+ cells in stage III and IV disease (64,65,67,78).
When T-cell populations were studied in more detail it became clear that peripheral blood T-cell subset counts did not always correlate with immunohistology of tumor infiltrating cells (64,78,79). Most authors report a relative high number of CD4+ and CD8+ cells around tumor margins of stage I and stage II tumors, indicative for al role of *immune surveillance* in these tumors. T-lymphocyte infiltrates seem to be favorable for patients' prognosis. More evidence for a role of the cellular immune response in early stage carcinomas came from the immunohistological study of mononuclear cell infiltrates around laryngeal and broncheal squamous cell carcinoma. These types of cancer have a comparable etiology and pathogenesis (both tumors are associated with smoking). However, as more than 60% of patients with early stage laryngeal carcinoma survive for 5 years, only 5% for patients with broncheal carcinoma survive. Immune infiltrate is both common and abundant in laryngeal carcinoma, but not in broncheal carcinoma. The cellular infiltrate is likely to give rise to an immune response, causing the difference in prognosis (77).

Although immune surveillance might play a role in early stage disease, immmunosuppression is the predominant phenomenon in advanced cancer. Apart from a reduction in cell numbers, the response of T-cells to mitogens (PHA and Con-A) and antigens is also suppressed (20,21,80,81). T-cell suppression is of clinical relevance because of the interactions of T-cells and T-cell cytokines with other cells of the immune system. The suppressed response of T-cells to antigens and mitogens results in a suppressed production of immunostimulatory cytokines (*e.g.* IL-2) (82). IL-2 is a potent stimulator of T-cell, B-cell, macrophage and natural killer cell proliferation and activation. Immunosuppression resulting in a deminished production of IL-2 will result in suppression of these cell types, all involved in the immune response against tumor cells (see Chapter 2, Table 1).

B-cells

B-cells produce antibodies (immunoglobulins) which recognize foreign antigens. Antibodies are important mediators of the humoral immune system. After antigen binding, antibody dependent cellular cytotoxicity is responsible for the destruction of the antigen-expressing cells. Also, B-cells play a major role in antigen presentation to other immune cells.

No major suppression has been found in the humoral immune system of patients with head and neck cancer. B-cell numbers and B-cell function, as well as immunoglobulin concentrations for IgG, IgM and IgD are equal to those in normal healthy individuals (64,67,78,80). Moreover, antibody responses to tumor antigens have been detected. These responses were merely directed against intracellular antigens, coming into circulation after necrosis of tumor cells. Since these antibodies do not recognize surface membrane antigens they will not play a role in the defence against growing malignant cells (83). However, when antigens and antibodies form complexes in the circulation, these serum immune complexes (SIC) influence cellular cytotoxic reactions. Antibody dependent cell mediated cytotoxicity (ADCC) will be reduced when the effector cells bind immune complexes instead of antigen coated tumor cells (80). Elevated levels of circulating immune complexes are associated with poor prognosis (84).

Elevated IgA levels were also described, either before or after therapy (80). IgA antibodies are able to bind viral antigens, thereby preventing a cellular cytotoxic reaction against tumor cells expressing viral antigens. Levels of specific IgA to viral antigens of EBV in nasopharyngeal cancer correlate with tumor load (21,85). In these tumours, determination of elevated levels of EBV-specific IgA is used for early tumor detection and in monitoring the disease during and after treatment.

Elevated IgE levels are correlated with a good prognosis (21). IgE might mediate immune defence mechanisms as it does in allergy (86). Interestingly, the incidence of malignancies seems to be less frequent in allergic people (87,88).

NK-cells

NK-cells are able to kill tumor cells through antibody dependent cell mediated cytotoxicity (ADCC) and can be stimulated to become LAK-cells (see Chapter 2, Table 1). Patients with squamous cell carcinoma of the head and neck have decreased numbers of NK-cells in peripheral blood. Moreover, these cells show reduced cytotoxic activity (25,76,89). However, no correlations were detected between reduced cell numbers or activity and the tumor stage. Only in advanced tumor stages a correlation between reduced NK-cell numbers and prognosis was found (89).

Interestingly, reduced NK-cell function, as determined in *in vitro* assays, was found to be correlated with a high risk of developing metastases (90). Tumor cells produce NK-cell suppressive factors which inhibit NK-cell cytotoxicity, thereby enabling metastases to develop (66,68,91). On the other hand, squamous cell carcinoma cells are able to induce NK-cell activation, thereby preventing metastatic spread (92). In cases where host defence mechanisms are compromised in other ways, for example

after radiation, the risk of metastases may again increase, providing an explanation for the increased incidence of metastasis observed when surgery is combined with postoperative radiotherapy (24).

Macrophages

Macrophages develop tumor cell cytolytic capacities after activation by T-cell cytokines. They also produce tumor growth inhibitory factors and thus play an important role in the defence against tumor cells and in the prevention of tumor growth. On the other hand, macrophages produce growth factors and promote angiogenesis, thereby promoting tumor growth (93).

Although macrophage infiltration in SCC-HN was reported (94), no correlations with prognosis or metastatic spread were found. This can be explained by the fact that macrophage count per se gives no information about macrophage function. As macrophage function cannot be elucidated using immunohistology, a tumor growth inhibiting or potentiating role cannot be established. Monocyte/macrophage function is dependent on the balance between activating and inhibitory signals (95,96). Patients with head and neck cancer show reduced monocyte and macrophage functions. Macrophages of these patients show decreased MHC-antigen expression, resulting in decreased antigen presentation to T-cells, decreased IL-1 secretion and reduced activation of T-helper cells to generate IL-2 (97). Defective monocyte chemotaxis, depressed monocyte polarization and decreased cytotoxicity towards tumor cells have also been reported (98-100).

Immunotherapeutic experiments show the importance of immunoregulatory factors and of mononuclear cells in surveillance against cancer. In patients with head and neck cancer, an increase in immune cell infiltrate and tumor regression was seen after treatment with a prostaglandin synthesis inhibitor, indomethacin (101-103). Prostaglandins are macrophage or tumor derived factors, which have immunosuppressive properties and play a role in tumor-induced immunosuppression (see Table 2). Other major inhibitors of monocyte and macrophage functions are low molecular weight factors, related to the retroviral immunosuppressive protein p15E, to be discussed in more detail in the next section.

Tumor-derived immunosuppressive factors

As immune dysfunctions accompanying head and neck cancer have been described in detail, research now is focussing on the identification of factors responsible for this immunosuppression. Identification of immunosuppressive factors may result in new therapeutic strategies to restore immune reactivity. Table 2 presents an overview of immunosuppressive factors associated with (head and neck) cancer, which cause local and regional suppression of the immune system.

Table 2. Immunosuppressive proteins associated with human cancer

Protein	Origin	Immunosuppressive effect	Refs.
p15E-related factors	Macrophages, tumor cells.	Immunosuppressive effects are comparable to those ascribed to retroviral p15E. See chapter 4.	(172-174, 184-187)
IL-10	T-cells, B-cells, macrophages, human carcinoma cell lines.	Downregulation of cell mediated immunity: inhibition of T-helper cell, NK-cell and macrophage cytokine production, inhibition of monocyte/ macrophage class II MHC expression, monocyte dependent T-cell proliferation, inhibition of monocyte killing.	(188-190)
TGF-β	Macrophages, bone, platelets, tumor cells, TIL.	Inhibition of B- and T-cell activation and proliferation, of LAK and CTL generation, of NK cytolytic activity, macrophage activation (respiratory burst) and downregulation of HLA-DR.	(191,192)
IFN-α,-β	Lymphocytes, macrophages.	Suppression of monocyte maturation and macrophage tumoricidal activity, suppression of T-helper cells.	(193-194)
CSF	tumor cells	Induction of macrophages possessing immunosuppresssive activity	(18)
PGE2	Macrophages, lymphoid cells, tumor cells.	Inhibition of B- and T-cell proliferation, of LAK and CTL generation, NK cytolytic activity and T-cell and macrophage effector functions.	(195-197)
Gangliosides	Macrophages, melanoma, liver.	Inhibition of generation of cytotoxic T-cells, of activation and growth of T-helper cells, of NK-cell cytotoxicity, of antibody formation.	(198,199)
SER	Macrophages	Suppression of E-rosette formation (SER), of T-cell proliferation	(200)
Acute phase proteins	Liver, tumor cells	(e.g. haptoglobin, α1-acid glycoprotein, α1-antitrypsin, C-reactive protein). Inhibition of lymphocyte proliferation and activation.	(201)
SCIF	SCC-HN	Squamous cell inhibition (SCIF), inhibition of LAK-activity.	(202)

4

Retroviral p15E and p15E-related immunosuppressive factors

Retroviral p15E and p15E-related immunosuppressive factors

Over the past decade, retroviral p15E-related factors* have shown to be important immunomodulators in head and neck cancer.
P15E-related factors are related to the low molecular weight protein p15E, a molecule present in the envelope of retroviruses. Retroviral p15E is an important factor in retrovirus-related immunosuppression. Retroviruses, retroviral p15E and p15E-related factors associated with human cancer are described in this chapter.

Retroviruses

Retroviruses are small, single strand RNA-containing viruses. They are characterized by their ability to translate RNA into complementary DNA, using the enzyme reverse transcriptase. The virus family includes murine, feline and human retroviruses.
The simple retroviral genome consists of three genes: *gag*, *pol* and *env*. The *gag*-gene encodes the internal structural proteins of the virion, the *pol*-gene encodes the reverse transcriptase and protease enzymes and the *env*-gene encodes the proteins of the retroviral envelope. Retrovirus-encoded proteins are named according to their size, as estimated from their migration in SDS gels; according to modifications, such as phosphorylation or glycosylation; and sometimes according to their gene of origin. For example, the 70-kilodalton *env* encoded glycoprotein is gp70*env* and the 15-kilodalton *env* encoded protein is p15E. Precursor proteins are designated by the prefix "Pr". A simplified nomenclature was introduced by Leis in 1988. Retroviral proteins are now also named according to their location in the virion and their function (104). Retroviruses have a long terminal repeat (LTR) in their genome, which contains domains that play a role in the integration of the viral DNA into host DNA and regulatory domains for DNA transcription.
After viral infection of host cells, the viral RNA genome is reversily transcribed to produce a double-stranded DNA copy which can integrate into the host chromosome to form a provirus. The structure of the provirus is 5'-LTR-*gag*-*pol*-*env*-LTR-3'. The genome of the murine leukemia virus was fully characterized and taken as an example for the description of the retroviral genome (Fig. 1) (105).

The provirus is transcribed to produce genomic RNA which is translated to produce the viral proteins, required for the assembly of new viral particles. Without transcription, the provirus can remain in the host DNA for prolonged periods. If the infected host cell is a germ cell, the viral genetic material will be inherited by all subsequent generations as an endogenous retrovirus. Such endogenous proviruses are widely distributed in nature.

* Also referred to as p15E-related factor, although it is by no means certain that the immunosuppression caused by this factor can be ascribed to one protein.

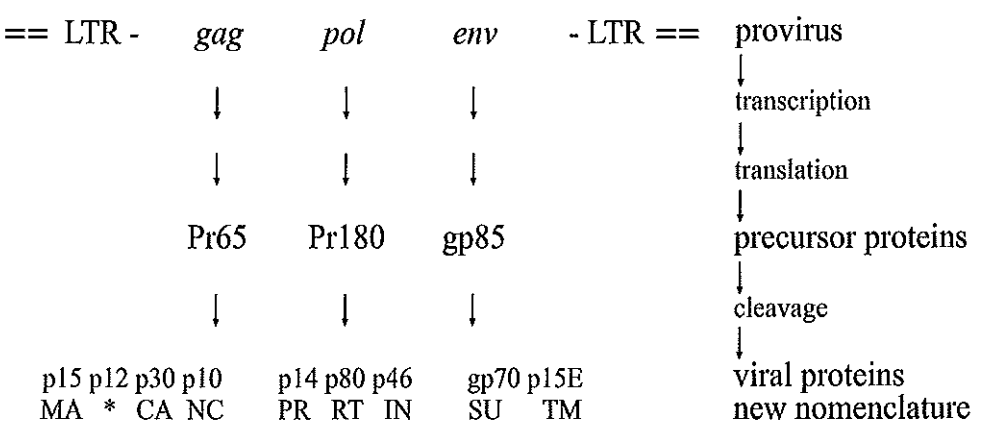

Fig. 1. Structure of the retroviral provirus genome and origin of the retroviral proteins. The nomenclature is based on protein location in the virion or protein function: MA, matrix protein; *, function unknown; CA, capsid protein; NC, nucleocapsid protein; PR, protease; RT, reverse transcriptase; IN, integration protein; SU, surface protein; TM, transmembrane protein; (104).

Many of these viral genomes are replication-defective, usually because of one or more lesions in their genome. Therefore, they are not able to produce functional retroviral proteins or new infectious viruses (106,107).

Retroviruses can be classified according to their mode of transmission. Infectious (exogenous) retroviruses are transmitted from one individual to another. Endogenous retroviruses are transmitted from parent to offspring.
Retroviruses can also be classified according to their ability to infect host cells: ecotropic viruses (*eco* = house) replicate only in cells of the host and closely related species, xenotropic viruses (*xeno* = foreign) replicate only in cells of heterologous species and amphotropic viruses (*ampho* = double) replicate in cells of both host and heterologous species.
Finally, retroviruses are classified in three subfamilies of unequal size: lentiviruses (causing slow developing viral infections as AIDS and probably causing rheumatoid arthritis (108) and encephalitis); spumaviruses or foamy viruses of which the biological significance remains uncertain; and the large group of oncogenic viruses, subdivided according to their morphology in types A, B, C and D (109).
Certain oncoviruses carry genes that are very closely related to highly conserved cellular genes. These genes are not required for viral replication, but can induce transformation of host cells. Therefore, these genes have been called viral oncogenes (v-*onc*). Their cellular counterparts are designated c-*onc*. Aberrant expression of v-*onc* or c-*onc* genes can lead to uncontrolled cell growth, resulting in the formation

of tumors. However, most oncoviruses do not possess viral oncogenes. Their oncogenic potential lies in their ability to integrate randomly into the cellular genome, inducing mutagenesis of host genes. This may or may not result in malignant transformation of cells (109).

Immunosuppression associated with retroviral infection

Latent and/or persistent infections are part of the lifestyle of many viruses. Virally encoded proteins from adenoviruses, herpesviruses (e.g. EBV) and retroviruses have been shown to help the virus to escape host immune surveillance (110,111).

Retrovirus induced immunosuppression can be caused by the depletion of virus infected immune effector cells, or by the dysregulaion of immunological functions. Many retroviruses preferentially infect immune effector cells like lymphocytes, monocytes and macrophages. If viral replication takes place, the infected cell may be killed. Also, when viral proteins are expressed at the cell membrane, this may result in T-cell or antibody recognition and destruction of the infected cell.
For example, human immunodeficiency virus (HIV) can infect human cells after binding of the envelope glycoprotein gp160 to CD4, which is expressed mainly on T-helper cells, but also on macrophages and dendritic cells. Destruction of infected cells results in the immunosuppression accompanying HIV infection (112,113). In addition, antibodies against HIV proteins can cross-react with human proteins, like MHC class I and class II antigens. This results in antibody binding to MHC class I and class II proteins at the cell surface of immune effector cells, followed by the destruction of these cells by phagocytosis and cytotoxic activities of macrophages (114,115).
It has also been reported that retroviruses dysregulate processes which are important for cellular activity. Infections with human T-lymphotropic virus (HTLV) or HIV are accompanied by polyclonal B- and T-cell activation, which may prevent the host from adequate immune responses to specific antigens, such as other infective agents (112,116,117).

Immunosuppression associated with inactivated retroviral particles

Apart from immunosuppression caused by viral infection of immune cells, it appeared that inactivated viral particles were also immunosuppressive.
In vitro experiments showed that blastogenic responses to concavalin A (ConA) were reduced by 65% by UV-inactivated Feline Leukemia Virus (FeLV) (118). UV-inactivated FeLV also showed a depression of T- and B-lymphocyte proliferation, accompanied by a decreased production of antibodies (119,120) and cytokines such as IL-2, IFN-α and IFN-γ (120-122), suppression of T-cell cytotoxic responses

(123), suppression of neutrophil function (124,125) and inhibition of erythroid colony-forming cells (126). Similar immunosuppressive activities have been found for inactivated Murine Leukemia Virus (MuLV) (116,127,128) (Table 1).

P15E-mediated immunosuppression

Mathes and coworkers showed in 1978 that the feline envelope protein p15E was responsible for retrovirus-related immunosuppression (134,135). Next to p15E, other retroviral proteins have immunosuppressive properties. These immunosuppressive proteins are mainly HIV-proteins, like glycoprotein gp120, glycoprotein gp41, TAR and Nef (152,162-167). However, p15E is considered the main retroviral immunosuppressive protein.

Retroviral p15E

In vivo experiments provided further evidence for the importance of retroviral p15E in retrovirus-related immunosuppression. Immunization of kittens with UV-inactivated FeLV abrogated tumor immunity and increased tumor incidence after challenge with the oncogenic Feline Sarcoma Virus (FSV) (130,157). In 1980, Cianciolo showed that macrophage accumulation at sites of delayed inflammatory reactions in mice was inhibited by low molecular weight extracts from oncogenic Friend, Molony and Rauscher MuLV (158). As macrophages are important effector cells in the immune response against tumor cells (168,169) suppression of macrophage function by p15E proved to be an important issue, resulting in a variety of publications describing the suppressive effects of p15E and p15E-related factors on the immune response against tumor cells (95,96,153,170-175).

Murine "p15E"

It appeared that murine tumor cell lines could produce p15E-related proteins of non-viral origin. A series of six murine tumor cell lines, including a methyl-cholanthrene-induced hepatocarcinoma and fibrosarcoma, a radiation induced lymphoma and three variants of the B16 melanoma were examined for the synthesis of p15E using immunoprecipitation and monoclonal antibodies directed against the viral p15E protein. All tumor cell lines examined produced p15E-related proteins, whereas normal fibroblast cells as well as unstimulated and mitogen-stimulated splenocytes did not. Viral infection of the tumor cell lines was excluded, as no viral particles were detected using electron microscopy. Also, no reactivity of the cell lines with antisera directed against retroviral reverse transcriptase, gp71 or p30 was detected. Using gel electrophoreses, it was demonstrated that the murine p15E-related protein had the same (19.000 Dalton) molecular weight as compared to feline leukemia virus p15E from Eveline cells, feline leukemia virus producing fibroblasts (170).

Table 1. Immunosuppressive properties ascribed to (inactivated) retroviruses, retroviral p15E and CKS-17. Immune functions inhibited and references.

Immune function inhibited	Retrovirus	p15E	CKS-17
formation of hemagglutinating antibodies	FLV (129,130)		
lymphocyte proliferation / lymphocyte blastogenesis (induced by ConA or IL-2)	FeLV (118,119,131) RLV (127) MuLV (128) ASV, HTLV, HIV (132,133)	119,134-138	138-142
antibody production	FeLV (120,143)		143
T-cell cytotoxic responses	FeLV (121, 123)		144
neutrophil function	FeLV (124,125)		
erythroid colony forming cells (not CFU-GM)	FeLV (126)	126	
granulocyte-macrophage CFU (not BFU-E)	HIV (133)		142
IL-2 production	FeLV (119,121)		
	FLV, ASV, HIV (132)	119	145,146
IL-12 production			146

Immune function inhibited	Retrovirus	p15E	CKS-17
IFN-α production	FeLV (120)		
IFN-γ production	FeLV (122)		146-149
TNF-α production			149,150
NK-cell activity			151
monocyte polarization	HIV (152)	153	
monocyte respiratory burst			154
IL-1 mediated killing (monocyte-mediated tumor lysis)			155,156
In vivo tumor immunity	FeLV (130,157)	134	
In vivo macrophage accumulation in mice		158	
In vivo cell mediated immunity (DTH)			159
signal transduction, protein kinase C pathway			160,161
Th1-related cytokines			146

Human "p15E"

Immunosuppressive factors related to retroviral p15E were also present in human cancerous effusions and in human malignant cell lines. Effusions from 15 different tumors (adenocarcinomas, melanoma, lymphoma and squamous cell carcinomas) were examined for immunosuppressive activity using the monocyte polarization bioassay (100,153). All malignant effusions significantly inhibited the polarization of fresh human monocytes, whereas noncancerous effusions did not. The inhibitory activity could be absorbed out by monoclonal antibodies directed against retroviral p15E (153). Human malignant cell lines contain p15E-related proteins as was demonstrated by flowcytometry. In contrast, normal human periferal blood mononuclear cells (PBMC) were negative. However, mitogen induced blast transformation of the PBMC induced reactivity with anti-p15E (171). These observations suggest that transformation, probably also malignant transformation, may induce the production of p15E-related factors in human cells.

After malignant transformation of cells, the expression of an immunosuppressive p15E-related factor may allow transformed cells to escape immune destruction. To test this hypothesis, Schmidt and Snyderman transfected moderately tumorigenic murine cell lines with a MuLV-*env*-gene. The results showed that highly tumorigenic cell lines did not always express the *env*-protein p15E and, more importantly, p15E-transfected, moderately tumorigenic cell lines, became no more tumorigenic than mock-transfectants. However, in the discussion of their results, the authors suggest that their findings do not rule out a role for p15E in promoting the growth of solid tumors. An important notion in the experiments was that the co-expressed envelope protein gp70 might be expected to be immunogenic providing a target for immune rejection of transfected cells. In general, this approach of inoculation of *in vitro* transformed cells is not a true simulation of natural tumorigenesis. Therefore, a role of p15E in any stage of tumorigenesis still seems very likely (175).

Mechamism of p15E-induced immunosuppression: CKS-17

As retroviral p15E does not only inhibit immune functions of the natural host but also of other species, it appeared that the immunosuppressive region of p15E has been evolutionary conserved among different TM proteins. A significant degree of homology (73%) between retroviral p15E and gp21 of HTLV-I and HTLV-II has been demonstrated in a 26 amino acid sequence of p15E (176). Homologies in *env*-sequences have also been detected in HIV gp41 (35%) (163). Furthermore, a high degree of homology (81%) was observed in the *nef* gene sequence of primate lentiviruses (Table 2) (167). Nef is expressed early in the viral life cycle and is critical to the maintenance of a high viral load and for the development of AIDS (177,178). Several studies have indicated that *nef* affects T-cell functions and behaves as a potent immunosuppressor (167,179).

Taken together, these results suggest that a conserved epitope could be held responsible for the immunosuppression accompanying retroviral infections.

Table 2. Amino acid sequences (one letter amino acid codes) for the conserved regions of retrovirus transmembrane evelope proteins and the synthetic peptide CKS-17.

Virus	protein	amino acid sequence
MuLV	p15E	Q N R R G L D L L F L K E G G L C A A L K E E C C F
FeLV	p15E	Q N R R G L D I L F L Q E G G L C A A L K E E C C F
HTLV-I	gp21	Q N R R G L D L L F W E Q G G L C K A L Q E Q C R F
HTLV-II	gp21	Q N R R G L D L L F W E Q G G L C K A I Q E Q C C F
HIV	gp41	Q A R I L A V E R Y L K D Q Q L
lentivirus	Nef	T Y K A A I D L S H L K E G G L
synthetic peptide		
CKS-17		L Q N R R G L D L L F L K E G G L

A 17 amino acid peptide (CKS-17) was synthesized, corresponding to this region of homology. This peptide was examined for its effects in immune functions in several studies. It appeared that CKS-17, when conjugated to a carrier protein, showed immunosuppressive properties, similar to the native retroviral protein p15E (see Table 1). Further investigations showed that the immunosuppressive effect of CKS-17 could be ascribed to 2 sequences within the peptide, of respectively 7 (LDLLFL) (180) and 8 (LQNRRGLD) (138,160) amino acids. Both these sequences inhibited lymphocyte proliferation, as do CKS-17 and p15E. Interestingly, unconjugated LDLLFL also inhibited monocyte polarization (181), as do p15E and HIV gp41. CKS-17 was used in studies to elucidate the mechanism of p15E-induced immunosuppression.

The suppressive effect of CKS-17 could never be ascribed to a direct cytotoxic effect. It appeared that several direct or indirect mechanisms were involved in CKS-17 mediated immunosuppression.
A direct effect on the early effector phase of monocytes (morphologic changes and respiratory burst) was observed. However, IFN-α or LPS-induced monocyte activiation was not inhibited by CKS-17 (154,155). The suppression of monocyte-mediated tumor lysis was not due to a direct effect of CKS-17 on monocytes, as IL-1 production was not inhibited. It appeared that IL-1 function was suppressed (155). CKS-17 interfered with IL-1 signal transduction, as was demonstrated by Gottlieb *et al.* in 1989 (156).

This interference with IL-1 signal transduction also results in a suppression of IL-1 induced T-cell proliferation and interleukin production. The suppressive effect of CKS-17 on T-cell cytotoxicity is therefore mediated through suppression of production of cytokines which stimulate the cellular immune response (T-helper 1 cytokines: IL-2, IL-12, IFN-γ, TNF-α,β) (144,146).
IL-2 production has also been shown to be inhibited in an IL-1 independent system (145). As IL-2 also stimulates macrophages and NK-cells, suppression of IL-2 production results in a diminished activation of these cells.
Moreover, NK-cell function was also shown to be directly inhibited by CKS-17. NK-cell binding to target cells was not affected, but CKS-17 did inhibit NK-cell cytolytic capacity (151).
In 1995, Haraguchi *et al.* have shown that CKS-17 not only inhibits, directly or indirectly, cytokine production, but also stimulates the production of other cytokines. *In vitro*, CKS-17 reduces levels of type 1 cytokines (IL-2, IL-12, IFN-γ), but augments the production of type 2 cytokine IL-10. It appeared that CKS-17 influenced the regulation of mRNA expression for human Th1 and Th2 cytokines, responsible for, respectively, cellular and humoral immune stimulation. When human peripheral blood mononuclear cells are stimulated, cytokine mRNA can be detected using the reverse transcription-polymerase chain reaction (RT-PCR). CKS-17 down-regulated stimulant-induced mRNA accumulation for IFN-γ, Il-2 and IL-12. IL-12 is a cytokine that mediates development of cellular Th1 responses. CKS-17 upregulated stimulant-induced mRNA accumulation of IL-10 and did not suppress other Th2 related cytokines (IL-4, IL-5, IL-6 or IL-13). It appeared that suppression of IFN-γ mRNA was dependent on augmentation of IL-10 mRNA (146). Therefore, CKS-17 may act as an immunomodulatory epitope responsible for cytokine dysregulation that leads to suppression of cellular immunity.

Two molecular mechanisms have been identified which play a role in CKS-17 mediated regulation of protein transcription. First, CKS-17 induces a dramatic rise in intracellular cAMP. cAMP is an intracellular second messenger, which plays an important role in regulation of cytokine biosynthesis. Elevation of intracellular cAMP levels selectively inhibits type I cytokine production but has no effect or enhances type 2 cytokine production (182). Secondly, CKS-17 inactivates protein kinase C (PKC). PKC has been established as an important receptor for the second messenger diacylglycerol (DAG) in many cell systems. Increasing evidence supports a role for PKC in a variety of immune activation events, including responses to IL-1, IL-2 mediated proliferation, and B-cell and NK-cell activation (160,161). PKC and cAMP play a role in the regulation of transcription factors, responsible for the transcription of cytokine genes. CKS-17 induced alterations in transcription factor activity will lead to dysregulated gene transcription and altered cytokine production resulting in the immunosuppression accompanying retroviral infections and malignancies (183).

In conclusion, the synthetic peptide CKS-17 is considered the main immunosuppressive epitope of p15E. CKS-17 is able to dysregulate the production of cytokines: inhibition of the production of cytokines responsible for the cellular immune response and stimulation of the production of the immunosuppressive cytokine IL-10.
Because of the importance of the CKS-17 epitope, we used CKS-17 in our experiments investigating the role of p15E-related immunosuppressive proteins in head and neck cancer.

Aim of the study

Head and neck cancer is accompanied by immunosuppression. Retroviral p15E-related factors are candidates to be responsible for (part of) this immunosuppression. In **chapter 5** we report on the production of a new set of monoclonal antibodies, directed against the immunosuppressive epitope of p15E, the synthetic peptide CKS-17. These antibodies were used to study the relationship between retroviral p15E (and CKS-17) and the p15E-related immunosuppressive factor associated with head and neck cancer. The antibodies directed against CKS-17 react with the human immunosuppressive p15E-related factor as was shown using flowcytometry, Western blot and immunohistology. In addition, our monoclonal antibodies are biologically active, since they neutralize the immunosuppressive low molecular weight factors present in sera of patients with head and neck cancer.

In order to investigate the distribution of p15E-related immunosuppressive factors in human malignancies in further detail, we used our new monoclonal antibodies in an extended immunohistochemical study. In **chapter 6** we present the results of this large study. It appeared that p15E-related proteins could be detected in squamous cell carcinoma of the head and neck, esophagus, lung, cervix and skin, indicating that different types of tumors secrete p15E, hence a p15E induced immunosuppressive mechanism seems generally used by tumor cells. Also, p15E-related proteins were detected in adenocarcinoma of the breast, colon, lung and ovary and in malignant melanoma. P15E-related proteins could be considered tumor "associated" proteins, but not tumor specific, as we also detected a weak expression of p15E in a minority of normal tissues.

As p15E-related proteins were highly expressed in squamous cell carcinoma of the head and neck, we investigated whether p15E-related proteins could be detected in a sensitive and quantitative way in sera of patients with head and neck cancer. The presence of p15E-related proteins appeared to be related to the malignant state (chapter 6). Therefore, the detection of these proteins might have prognositc or diagnositc value. In **chapter 7** we present the results of our attempts to develop a sandwich enzyme-linked immunosorbent assay for the detection of p15E-related

proteins in human sera. Unfortunately, using the sandwich-ELISA, we could not reach sufficient sensitivity to detect this cytokine-like protein in sera of patients with head and neck cancer. Our studies furthermore indicate that the detection of p15E-related proteins in human sera was hampered by blocking factors present in the serum. The nature of these blocking factors remains to be solved.

Since we found that p15E-related proteins were associated with different types of human cancer, we were very interested to investigate a possible immunotherapeutic potential of our monoclonal antibodies.In **chapter 8** we describe a murine model to study the immunotherapeutic potential of antibodies directed against p15E. Immunotherapy was performed in mice inoculated with a Rauscher virus-transformed myeloid cell line, expressing p15E antigens. Using our antibody ER-IS5, we could demonstrate an inhibition of tumor growth, resulting in a significant longer survival of antibody treated mice. Using a monoclonal antibody, directed against retroviral p15E (19F8), we even cured 8 of 23 mice. These results indicate an important immunotherapeutical potential of anti-p15E antibodies.

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5

New monoclonal antibodies against the putative immunosuppressive site of retroviral p15E

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New monoclonal antibodies against the putative immunosuppressive site of retroviral p15E

Both retroviral infections, as well as human tumors may cause immunosuppression. One of the factors involved in immunosuppression in patients with squamous cell carcinoma of the head and neck (SCC-HN) is a protein related to the retroviral protein p15E. A conserved 17 amino acid sequence represents the immunosuppressive epitope of retroviral p15E.
In order to study the relationship between SCC-HN associated immunosuppression and retroviral p15E we produced three new monoclonal antibodies (MAbs: ER-IS1, ER-IS2 and ER-IS5) directed against the immunosuppressive synthetic CKS-17 peptide. These MAbs react with the immunosuppressive peptide (in enzyme-linked immunosorbent assay), with human tumor cell lines (in FACScan analysis) with retroviral p15E (on Western blot) and with cryostat sections of SCC-HN tumor tissue. In addition, the MAbs neutralize the immunosuppressive low molecular weight factors (LMWFs) present in sera of patients with SCC-HN.
These results show that retroviral p15E and the immunosuppressive factors associated with SCC-HN share a conserved immunosuppressive epitope and that MAbs against this epitope can be used for detection and neutralization of the tumor associated immunosuppressive protein(s).

Introduction

Retroviral infections and virus-induced tumors in both humans and animals are often accompanied by the development of immunosuppressive effects (1,2). The env gene-encoded surface and transmembrane proteins of retroviruses are responsible for these effects (3).
P15E is a hydrophobic transmembrane protein of approximately 19 kDa, present in all transforming type C retroviruses (4).
The immunosuppressive activities of retroviral p15E have been thoroughly studied *in vivo* and *in vitro*. Isolated p15E of feline leukemia virus inhibits *in vivo* tumor immunity and murine leukemia virus p15E inhibits macrophage accumulation at inflammatory foci in mice. *In vitro*, p15E causes inhibition of lymphocyte proliferation, inhibition of IL-2 secretion and monocyte chemotaxis. (for reviews, see refs. 3 and 5).
The antiinflammatory activities of p15E may, in general, contribute to the pathogenicity of retroviruses. P15E might also be responsible for part of the immunosuppression associated with retroviral infections (5) or virus induced tumors.
Proteins physiochemically related to retroviral p15E have been identified in (virus-free) murine malignant cell lines as well as in human malignant cell lines, malignancies and cancerous effusions. These p15E-related proteins also have immunosuppressive properties (6-8).

Especially patients with squamous cell carcinoma of the head and neck (SCC-HN) manifest defects in their cell-mediated and humoral immune function (9,10). Retroviral p15E-related immunosuppressive factors were identified in serum obtained from patients with SCC-HN (11) and can be detected in tumor tissue of SCC-HN (12,13).
The exact molecular nature of these p15E-related immunosuppressive factors has not yet been clarified. Immunosuppressive low molecular weight factors (LMWFs) isolated from human tumors show cross-reactivity with monoclonal antibodies directed against retroviral p15E (14).
In retroviral p15E the putative immunosuppressive site is located in a highly conserved 26-amino-acid region of the protein (15). A synthetic peptide, CKS-17, corresponding to 17 amino acids selected from this region (conjugated to a carrier protein; ref. 16), has similar immunosuppressive actions as the intact retroviral p15E protein (17-20).
In this study, we investigated whether p15E-related immunosuppressive proteins expressed in or on human tumor cell lines, or in tissue specimen of human SCC-HN, contain an epitope similar to the conserved CKS-17 epitope. For this purpose we applied peptide specific monoclonal antibodies (MAbs) generated against the immunosuppressive CKS-17 epitope.
Furthermore we investigated whether immunosuppression by LMWFs in sera from patients with tumors expected not to be virally induced *e.g.,* SCC-HN, is caused by proteins containing this immunosuppressive CKS-17 epitope.
We found that our MAbs react with human tumor cell lines and with antigens from cells of human SCC-HN. Of special interest is the fact that the antibodies block the immunosuppressive activity of LMWFs, present in serum of patients with SCC-HN.

Materials and methods

Cell lines

U937 is a human histiocytic lymphoma cell line (21), which produces p15E related immunosuppressive factors (8). Cells were cultured in RPMI 1640 medium containing 5% FCS, 100 μg/ml penicillin and 60 μg/ml streptomycin (P/S).
P2 is a human squamous cell carcinoma cell line, isolated from a patient with lung carcinoma, (cells were obtained from dr. L. de Ley, University Hospital, Groningen, The Netherlands). Cells were cultured in RPMI 1640 medium containing 15% FCS, 0,05 mM β-mercapto-ethanol, 2 mM glutamine, 1 mM sodium-pyruvate and P/S.
JLS-V5, a Rauscher Murine Leukemia Virus (MuLV) producing cell line (22), was used for isolation of MuLV for control experiments. The cells were cultured in DMEM containing 7% FCS and P/S. Isolation of MuLV was performed according to Duesberg and Robinson (23).

Peptide synthesis and conjugation

CKS-17 peptides were synthesized using solid phase synthesis as described originally by Merrifield in 1963 (24). Purification of peptides was performed by gel filtration and reverse phase high performance liquid chromatography. A cysteine residue was added to the amino-terminus during synthesis.

Peptide-conjugates for immunizations were prepared by coupling peptides to KLH by means of *m*-maleimidobenzoyl-N-hydroxysuccinimide esther as described in detail by Van Denderen *et al.* (25). Peptide-conjugates for ELISA experiments were produced by coupling to BSA by means of EDC.

The synthesized peptide consisting of the CKS-17 amino-acid sequence (LQNRRG-LDLLFLKEGGL) (16) is hereafter called SP124. A shorter peptide, lacking the first 6 amino-acids (LDLLFLKEGGL), is named SP125.

SP124 coupled to KLH via of *m*-maleimidobenzoyl-N-hydroxysuccinimide esther is referred to in this report as SP124-KLH, peptides coupled to BSA via EDC are referred to as SP124-BSA and SP125-BSA.

Production and purification of monoclonal antibodies

Female, 12 wk old, Balb/c mice were immunized s.c. on the upper side of both hind feet with 50 µg of SP124-KLH in complete Freund's adjuvant (26). An i.p. booster injection was given four weeks later with 50 µg SP124-KLH in incomplete Freund's adjuvant. Popliteal lymph node cells were isolated three days later for fusion with mouse SP2/0-Ag14 myeloma cells using polyethylene glycol (PEG 4000, 72%). Cell fusion and subcloning of positive hybridomas was performed under standard conditions. Selection was performed with 1 µg/ml azaserine (27) in the presence of 40 units/ml interleukin 6.

For large scale production of MAbs 1 x 10^8 hybridoma cells were cultured in 1.5 liters RPMI medium containing 5% FCS, add P/S for 10 days in gas permeable Fenwal Lifecell bags (Baxter). MAbs were purified using a protein A-Sepharose column. IgM antibodies were purified using rat-anti-mouse-κ antibodies coupled to Sepharose beads (28).

ELISA experiments

Micro-ELISA experiments were performed using Terasaki microtiter trays and β-galactosidase labeled sheep-anti-mouse antibodies (Amersham; ref. 29). For the determination of immunoglobulin production wells were coated overnight at 4 °C with 10 µl of 10 µg/ml 226-α-κ antibodies (28). Specific binding of MAbs to synthetic peptides was determined using wells coated overnight at 4 °C with peptide-BSA conjugates (0.1 µg per well) or BSA alone (0.1 µg per well) as a control. Binding was expressed as arbitrary fluorescence units.

For blocking experiments, antibodies were incubated overnight at 4 °C with free synthetic peptide (SP124) in various concentrations, before testing the mixture in ELISA. Binding is expressed as percentage of the binding of the unabsorbed antibody.

Flow cytometry

For FACScan analysis, U937 and P2 cells were washed in medium without serum, and fixed in 2% paraformaldehyde (Merck) in PBS for 10 min at 4 °C. Next, the cells were washed three times in PBS and permeabilized with 100% methanol for 20 min at 4 °C. After washing the cells twice, the cells were blocked for at least 30 min in PBS-0.1% BSA. Cells (0.5-1 x10^6) in a volume of 10 µl of washing buffer (PBS-0.05% Tween 20 and 0.1% BSA) were incubated with 50 µl of relevant MAb containing culture supernatant for 10 min at room temperature. After washing the cells four times, a fluorescein isothiocyanate-labeled secondary antibody (rabbit-anti-mouse fluorescein isothiocyanate; DAKO) was added in an optimal dilution. Incubation was allowed for 10 min at room temperature. Cells were then washed and stored in the dark at 4 °C until analysis. MAbs directed against irrelevant synthetic peptides (25) were used as control antibodies. Culture supernatant of SP2/0 cells was used to determine background fluorescence. A Beckton and Dickinson FACScan was used for flow cytometric analysis.

Gel electrophoresis and immunoblotting

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting, 150 µg of lysed MuLV particles were electrophorated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred from the gel onto a 0.2 µm nitrocellulose membrane. After transfer, the nitrocellulose sheet was washed in PBS and submerged in a solution of nonfat milk for at least 30 min to block remaining binding sites. The sheet was cut into strips for individual staining with antibodies. Detection of bound antibody was performed with alkaline phosphatase conjugate antibodies, using 5-bromo-4-chloroindoxyl phosphate (BCIP) and nitro blue tetrazolium (30). Culture supernatant of the MAb 19F8, directed against MuLV p15E (IgG2b; ref. 31) was used as a positive control; antibodies directed against irrelevant synthetic peptides (25) were used as negative control antibodies.

Monocyte polarization

The monocyte polarization assay, originally described by Cianciolo *et al.* (6) was performed with slight modifications (11). After induction of polarization with *N*-formyl-methionyl, monocytes were counted with a hemocytometer using a light microscope. Two hundred cells were counted and judged individually by two persons for "polarization" as described by Tas *et al.* (32). The capability of LMWFs of patient sera to inhibit the polarization of healthy donor monocytes was determined as described (32).

Adsorption of patient sera with monoclonal antibodies.

Sera of patients with SCC-HN, which inhibited the above described polarization assay, were incubated with the new MAb supernatant at 4 °C for 16h, folllowed by Amicon ultrafiltration to remove immune-complexes formed. Control adsorption experiments were carried out with a mixture of the p15E-specific antibodies 19F8 and 4F5 (IgG2a; ref. 7) in a final concentration of 25 µg/ml each or with the negative

control antibody MPC11.UoA (IgG2b; ATCC, Rockville, MD). The adsorbed serum fractions were tested in the monocyte polarization assay.

Immunohistochemistry
Immunohistochemistry was carried out on cryostat sections of SCC of the larynx. Sections (6 µm) were fixed in acetone for 15 min at 4 °C. Sections were incubated with MAb culture supernatant. Bound antibodies were detected with horseradish-peroxidase-conjugated goat-anti-mouse Ig antibodies (Miles). The diaminobenzidine staining procedure was carried out as described in detail elsewhere (33). Sections were weakly counterstained with hematoxylin.

Results

Monoclonal antibodies react with CKS-17 peptide and with human cell lines expressing p15E-related factors
Fusion experiments resulted in the generation of several hybridomas, primarily selected on the basis of Ig production and reactivity with peptide conjugates.
Fig. 1, *A-C* shows the reactivity of three monoclonal antibodies, ER-IS1 (IgM), ER-IS2 (IgM), and ER-IS5 (IgG2b), with the peptide conjugates BSA-SP124 and BSA-SP125. The antibodies ER-IS1 and ER-IS5 react with SP124, no cross reactivity with BSA was detected. The antibody ER-IS2 reacts with the shorter peptide SP125, in addition, a very weak background signal against BSA was detected.
Free SP124 peptide was able to block the reactivity of ER-IS1, ER-IS2 and ER-IS5 with the relevant peptide conjugate, in a concentration dependent manner (Fig. 1*D*). The reactivity in peptide-ELISA of suboptimal concentrations (10 µg/ml) of ER-IS1 and ER-IS2 antibodies could be blocked up to 80%, whereas the reactivity of ER-IS5 could be blocked up to 50% of the maximum signal.

To study the reactivity of the antibodies with the human p15E-related protein present in malignant cell lines we performed FACScan analysis. Cells were fixed and permeabilized to be able to detect antigens located in the cytoplasm of the cells. All three antibodies recognized the immunosuppressive epitope in U937 (histiocytic lymphoma) and P2 (squamous cell carcinoma) cells (Fig. 2). Control antibodies showed fluorescence at background levels (data not shown). No clear positive staining was shown without fixation, indicating the cytoplasmic localization of the immunosuppressive protein(s), similar to p15E-related proteins detected with the MAb 19F8 (6).

Retroviral p15E is recognized by the MAbs ER-IS1, ER-IS2 and ER-IS5
Reactivity of the new MAbs with MuLV-p15E was determined by immunoblotting. The MAb 19F8, directed against MuLV p15E, was used as a positive control. Two distinct bands, corresponding to p15E (19 KDa) and its degradation product p12E

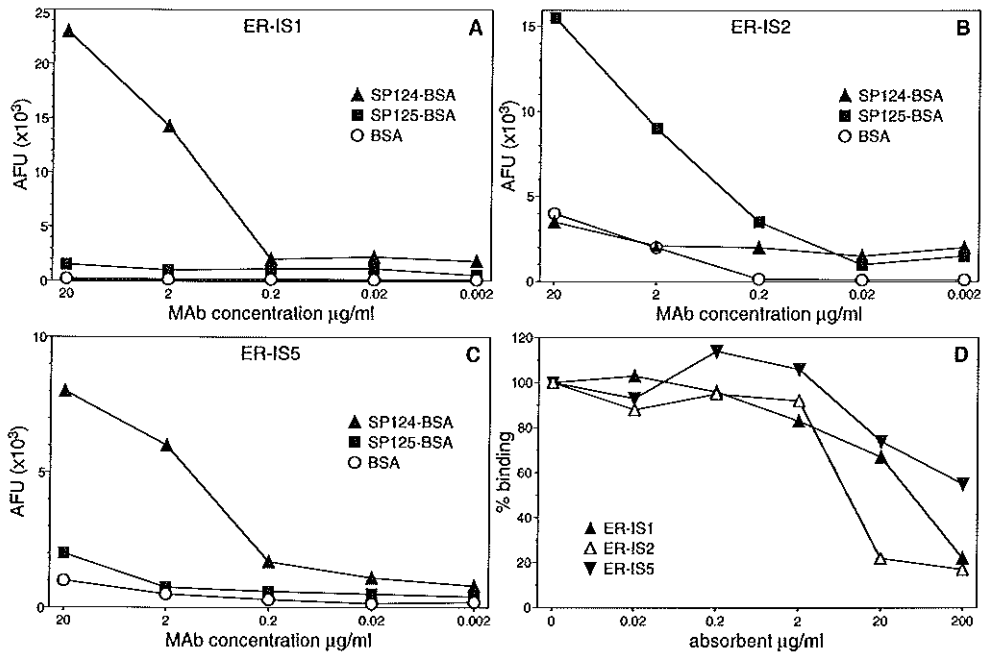

Fig. 1. Binding characteristics of MAbs to SP124-BSA, SP125-BSA and BSA alone in ELISA (*A-C*). Serial dilutions of hybridoma culture supernatant with known antibody concentrations were tested for binding to peptide conjugates. Binding is expressed as arbitrary fluorescence units (AFU). *D*, inhibition of binding after preadsorption with SP124. Antibodies were incubated with various concentrations of SP124 and tested in ELISA for reactivity with SP124-BSA (ER-IS1 and ER-IS5) or SP125-BSA (ER-IS2). Binding is expressed as percentage of the binding of suboptimal concentrations of unadsorbed MAb.

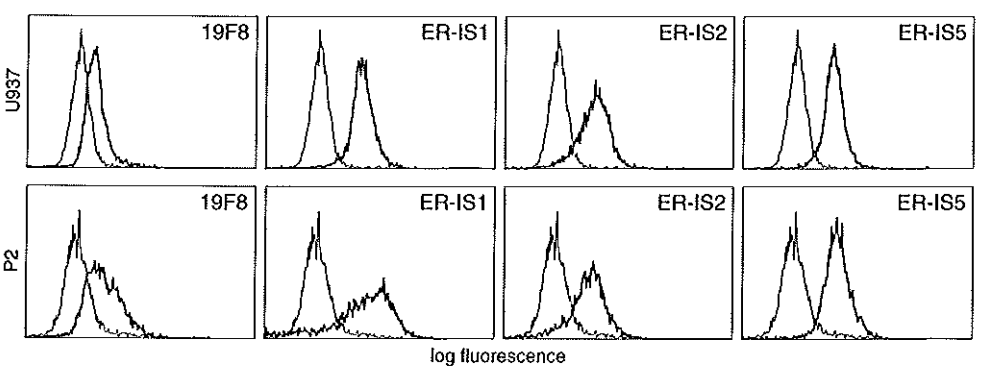

Fig. 2. FACScan analysis of human tumor cell lines (U937 and P2). Cells were fixed, incubated with MAbs against retroviral p15E (19F8) or against the immunosuppressive epitope (ER-IS1, ER-IS2 and ER-IS5), and analysed. *Thin line*, background fluorescence.

(17 Kda; ref. 7) were detected by 19F8. Fig. 3 shows that the monoclonal antibodies ER-IS1, ER-IS2 and ER-IS5 reacted specifically with the MuLV-p15E protein; these three antibodies detect the dominant p12E band. Control antibodies against irrelevant synthetic peptides showed no reactivity with retroviral proteins.

Fig. 3. Western blot of MuLV proteins detected with MAbs against retroviral p15E (19F8), irrelevant control antibody or antibodies against the immunosuppressive epitope of p15E (ER-IS1, ER-IS2, and ER-IS5).

Antibodies reduce LMWF-induced inhibition of monocyte polarization

To investigate whether the immunosuppressive epitope was detectable on p15E-related LMWFs in sera of patients with SCC-HN, we performed polarization experiments. We selected patient sera with a high inhibition percentage in the polarization assay (LMWF, 40-60% inhibition) for adsorption experiments with our panel of MAbs. Three experiments were carried out (Table 1). After overnight incubation of sera with the MAbs directed against CKS-17, polarization experiments were performed. Inhibition in the polarization assay by serum LMWFs was significantly reduced after preincubation with ER-IS1, ER-IS2 and ER-IS5 at 4 °C. The mixture of 19F8/4F5 directed against retroviral p15E causes a comparable reduction of inhibition. Incubation with control antibody MPC11 did not reduce inhibition of polarization (Table 1).

Not all the inhibitory activity in the sera could be adsorbed out by incubation with the

antibodies. Furthermore, combining the anti-p15E antibodies with the three anti-CKS-17 antibodies could not further reduce the inhibition as compared to the individual antibodies (data not shown). Taken together, these results indicate that the immunosuppressive epitope represented by the CKS-17 peptide is expressed on human p15E-related factors.

Table 1. Relative inhibition of the fMLP-induced polarization of human monocytes by low molecular weight factors (LMWFs) in sera of patients with SCC-HN, before and after adsorption with monoclonal antibodies.

			Adsorption with			
expt. (n)	LMWF	contrAb	19F8/4F5	ER-IS1	ER-IS2	ER-IS5
1 (8)	39±6[a)]	37±4	14±8[b)]	ND	ND	23±8[b)]
2 (5)	55±5	50±12	28±10[b)]	20±14[b)]	ND	ND
3 (1)	42	ND	ND	12	21	ND

[a)] Values (±SD) represent the *mean inhibition percentage* of the fMLP-induced monocyte polarization in the presence of LMWFs. To investigate whether the MAbs against the immunosuppressive epitope bind to these LMWFs, adsorption experiments were performed using different monoclonal antibodies.
[b)] $p<0.005$ (paired Student's *t* test). ND, not done.

Staining of SCC-tumor tissue with ER-IS1, ER-IS2 and ER-IS5

In order to investigate the presence of the CKS-17 epitope in human SCC-tumor tissue, we performed immunohistology. Cryostat tissue sections were made from SCC larynx-biopsies and stained with monoclonal antibodies.

The MAbs ER-IS1, ER-IS2 and ER-IS5 showed reactivity with human squamous cell carcinoma tissue. The control antibody 19F8 directed against retroviral p15E showed only a weak staining of tumor cells. The antibody 4F5, directed against a different epitope of retroviral p15E (7), showed strong reactivity with the corneal layers of well-differentiated areas of the tumor, as was shown before (13).

Antibody ER-IS1 showed a very strong reactivity with well-differentiated areas of the tumor (Fig. 4*A*). A cytoplasmic staining pattern was observed. However, cells in the basal layer of the epithelium did not react with ER-IS1. No cells in the surrounding connective tissue (fibroblasts, endothelial cells, or lymphocyte infiltrate) were stained, as was shown by comparison with the staining pattern of the control antibody (MPC 11; Fig. 4*D*).

The antibodies ER-IS2 and ER-IS5 detected all layers of infiltrating tumor in the tissue sections (Fig. 4, *B* and *C*). These antibodies also detected antigens in the cytoplasm of the tumor cells, but their staining was less strong than that of ER-IS1. In contrast to the staining with anti-p15E antibodies 19F8 and 4F5 and ER-IS1, with ER-IS2 and ER-IS5 a few cells in the surrounding tissue were stained. These cells could

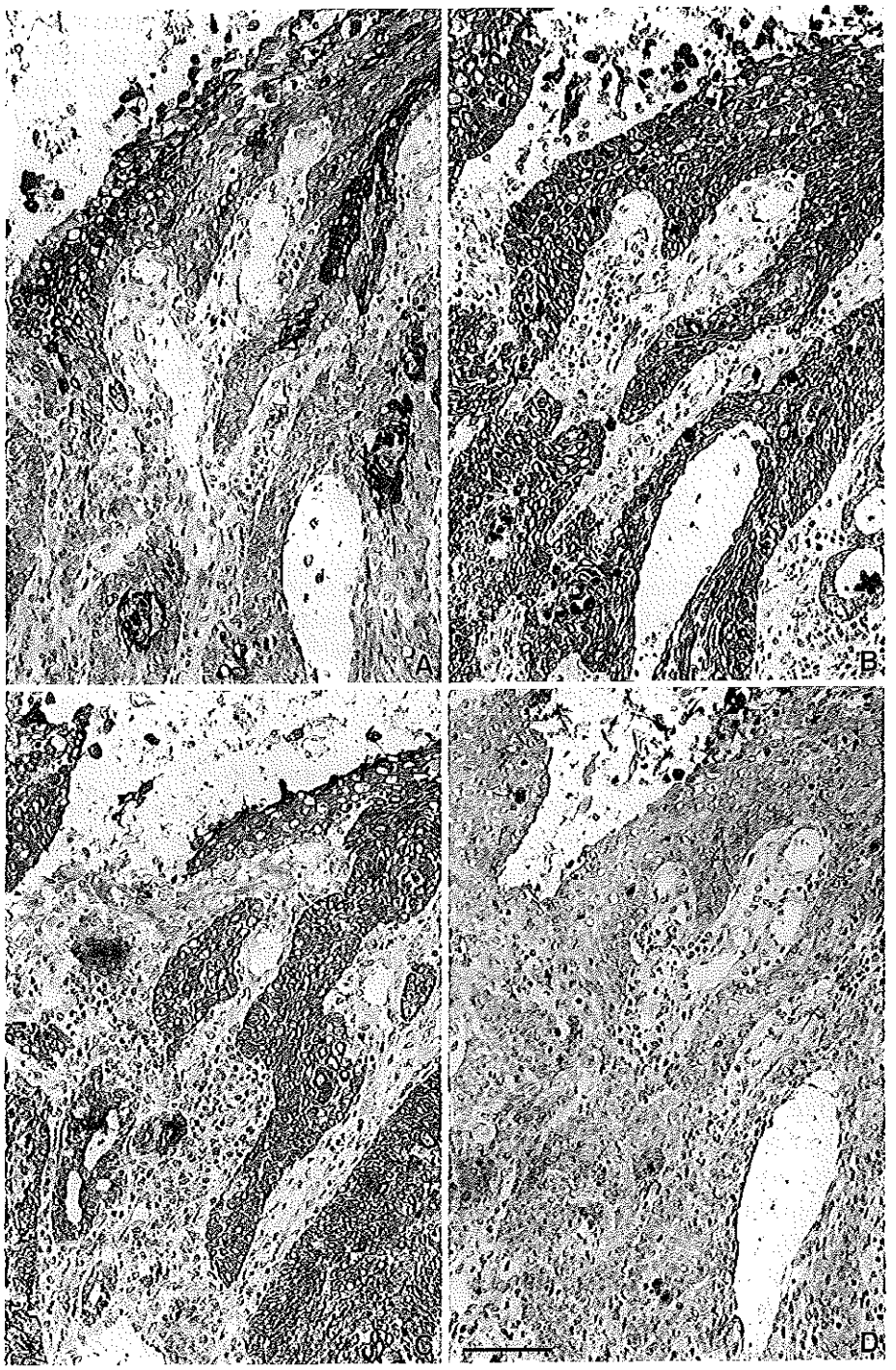

Fig. 4. Immunohistochemical staining of serial cryostat sections of infiltrating SCC of the larynx with monoclonal antibodies. (*A*, ER-IS1; *B*, ER-IS2; *C*, ER-IS5; *D*, control antibody). *Bar*, 100 µm.

be single epithelial tumor cells or macrophages involved in the inflammatory processes accompanying tumor growth.

Discussion

Identification and quantitative detection of immunosuppressive proteins associated with human cancer is necessary for a better understanding of the immunological processes involved in human cancer. Knowledge about immunosuppression is of special importance regarding immunomodulatory therapy. The limited succes of immunomodulatory therapy could well be explained by the presence of immunosuppressive proteins interfering with therapy. Blocking of these proteins could be helpful in improving the succes of immunotherapy, especially for patients with head and neck cancer.

In this paper we describe three new MAbs which react with the putative immunosuppressive site of retroviral p15E. These MAbs were generated after immunization of mice with an immunosuppressive peptide, corresponding to the conserved immunosuppressive site of p15E. This CKS-17 peptide has similar immunosuppressive actions as the retroviral p15E-protein. CKS-17-carrier inhibits human mitogen and alloantigen stimulated lymphocyte proliferation (16,34) human NK-cell activity (35), interleukin-1 mediated monocyte tumor killing (17,36), interleukin-2 production (19), immunoglobulin synthesis (37), TNF-α mRNA expression (38) and protein kinase C activity (39,40). Monocyte chemotactic responsiveness was inhibited by free CKS-17 peptide (20).

Although ER-IS1, ER-IS2 and ER-IS5 all detect p15E-related proteins, their reactivity pattern is not identical. The observed difference in reactivity could be explained by the fact that these MAbs are not directed against exactly the same peptide epitope. In ELISA, antibodies ER-IS1 and ER-IS5 reacted with the 17-aa peptide SP124 coupled to BSA; ER-IS2 reacted with SP125 coupled to BSA. Conjugation of SP124 and SP125 to BSA was performed through their COOH-groups, using EDC as an coupling agent. SP124 exposes three COOH-groups; consequently, EDC-coupling may result in a mixture of three types of peptide-BSA conjugates, each of which would be capable to form a loop-like conformation in which NH_2- and COOH-terminal ends are in close association. For the 11-aa SP125 conjugate different loop-like conformations are formed. Therefore, the MAbs could react with one of these different peptide conjugates of SP124 and SP125. Inhibition experiments with free SP124 showed that reactivity of ER-IS1, ER-IS2 and ER-IS5 against peptide conjugates could be blocked for 50%-80%. This result proves their specificity for the immunosuppressive epitope, but the fact that the reactivity could not totally be blocked with the linear peptide indicates that only specific conformational epitopes are recognized by the antibodies. The specificity of ER-IS1, ER-IS2 and ER-IS5 for the immunosuppressive epitope of

p15E was further revealed by the detection of MuLV-p15E on Western blot. These MAbs detected the same protein-band as the MAb 19F8. However, the reactivity of our new MAbs against retroviral-p15E was less strong than the reactivity of 19F8 against p15E. Therefore, only the p12E-degradation band was detected with ER-IS1, ER-IS2 and ER-IS5.

MAbs ER-IS1, ER-IS2 and ER-IS5 recognized cytoplasmic p15E-like proteins present in human tumor cell lines, as determined by FACScan analysis. Although not all human tumors contain the p15E-related epitope (11), the tumor cell lines presently investigated contain p15E-related proteins as determined with antibodies against retroviral p15E (8). FACScan analysis of cell lines with our MAbs reveales the presence of the CKS-17 epitope in the cytoplasm of human tumor cell lines.
In human tumor tissue, the presence of proteins containing the immunosuppressive epitope was shown with immunohistology. However, the staining pattern with the three antibodies was not identical. ER-IS1 recognized more differentiated areas of the tumor, whereas ER-IS2 and ER-IS5 stained all tumor cells. The existence of different proteins expressing the same immunosuppressive epitope in different tumor areas or differentiation stages could be an explanation for these staining patterns. Until now, it is not clear whether there is one p15E-like protein or whether there are more immunosuppressive proteins, all containing (parts of) the immunosuppressive epitope represented by the CKS-17 peptide. Some homologies of CKS-17 with other (immunosuppressive) proteins have already been described. These comprise homology with TGF-β (41) and interferon-α (40).
However, our preliminary experiments show that anti-CKS-17 MAbs have a different reaction pattern with cell lines compared to anti-TGF-β MAb, indicating that these MAbs do not recognize the same protein
Monocyte polarization experiments confirmed the presence of the immunosuppressive epitope on low molecular weight proteins in sera of patients with SCC-HN. Adsorption experiments with the MAbs against the immunosuppressive epitope showed a significant reduction of the inhibition in the polarization assay. However, only about 50% of the inhibition could be blocked; combining the anti-p15E with the anti-CKS-17 antibodies did not result in a higher blocking percentage, indicating the presence of other polarization inhibitory molecules of low molecular weight. Observations made in our laboratory show that interferon-α could possibly be one of the polarization inhibitory cytokines present in serum of patients with SCC-HN (42). Another polarization inhibitory cytokine present could well be TGF-β, a cytokine which was identified in several tumors associated with immunosuppression (10). Immunoprecipitation and purification experiments are presently being performed in order to reveal the exact nature of the immunosuppressive p15E-related protein(s).
We are planning to use our antibodies directed against a functional immunosuppressive epitope for the quantitative detection of immunosuppressive proteins involved in the development of human cancer. The detection of immunosuppressive molecules

could be a helpful tool in the detection of a recurrent tumor, because the production of p15E-related molecules is correlated with the growth of a tumor (43).

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Immunosuppressive p15E-related proteins are expressed in squamous cell carcinoma, adenocarcinoma and melanoma

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Immunosuppressive p15E-related proteins are expressed in squamous cell carcinoma, adenocarcinoma and melanoma

The presence of immunosuppressive, p15E-related proteins in human cancer was investigated in an extended immunohistochemical study. A total of 151 malignant and 61 normal tissues were investigated. In order to detect p15E-related proteins, we applied a monoclonal antibody, ER-IS1. This antibody is directed against the functional immunosuppressive epitope of retroviral p15E (represented by the CKS-17 peptide) and cross reacts with human p15E-related proteins. Using this monoclonal antibody, p15E-related proteins were detected in tumors associated with tumor induced immunosuppression, like squamous cell carcinoma, adenocarcinoma and malignant melanoma. P15E-related proteins were detected in 89% of the squamous cell carcinomas of the head and neck and in 47% of squamous cell carcinomas of the esophagus, lung, cervix, and skin. In adenocarcinoma of the breast, colon, lung and ovary and in malignant melanoma p15E-related antigens were found in 42% of the tissues. In only 11% of normal tissues we detected a weak expression of p15E-related proteins. From this study we conclude that p15E-related proteins are tumor associated proteins, which are particularly over-expressed in some squamous cell carcinomas, adenocarcinomas and melanomas.

Our study also indicates a relationship between the expression of p15E related proteins and patient survival. Four out of six patients suffering from esophagus carcinoma, with a high p15E expression died of metastases within a year of surgery, whereas only one out of five patients negative for p15E died as a consequence of metastatic tumors.

Taken together, these results indicate that the human p15E-related protein is a tumor associated antigen, with a possible predictive value for patient survival.

Introduction

In human tumors, tumor-derived soluble immunosuppressive factors interfere with the immune system, thereby preventing an immune response against the malignant cells (1,2). One of such tumor derived immunosuppressive factors is a protein related to the retroviral transmembrane (TM) protein p15E. Over the past years, several studies and reviews have been published describing the *in vitro* and *in vivo* immunosuppessive effects of p15E-related factors present in human tumors (3-5).

The retroviral p15E-related proteins have been detected in malignant cells, using MAbs directed against retroviral p15E (19F8 and 4F5) (6) and were expressed at high levels in squamous cell carcinoma of the head and neck (SCC-HN) (7). More recently, p15E-related immunosuppressive activity was detected in the serum of patients with head and neck cancer (8) and breast cancer (9). Besides, p15E-related mRNA was detected in tumor tissue of patients with colorectal and gastric cancer (10).

The immunosuppressive epitope of p15E is represented by the synthetic peptide CKS-17 (11). This epitope is responsible for many immunosuppressive effects caused by p15E-related factors produced by tumor cells (12). Recently, we produced a monoclonal antibody (MAb ER-IS1) directed against the immunosuppressive, CKS-17, domain of p15E (13). This MAb recognizes the p15E-related immunosuppressive protein and also neutralizes p15E-related proteins present in sera of patients with head and neck cancer.

In the present study, we investigated whether immunosuppressive proteins containing the CKS-17 epitope, as recognized by ER-IS1, were restricted to squamous cell carcinomas of the head and neck, or whether p15E-related proteins could also be detected in other tumor types associated with immunosuppression like breast cancer, colon cancer and malignant melanoma. Furthermore, cross-reactivity of ER-IS1 with normal human tissues was examined.
In our immunohistochemical analysis of 151 malignant and 61 normal tissues, we detected a strong expression of p15E-related proteins in carcinoma of the head and neck. However, the expression of p15E-related proteins was not restricted to SCC-HN. P15E-related proteins could also be detected in 47% of squamous cell carcinomas of the esophagus, lung, cervix and skin and in 42% of adenocarcinomas of the breast, colon and ovary and malignant melanomas.
Since reactivity of ER-IS1 with normal tissues was only minimal, p15E-related proteins can be considered tumor associated proteins; such proteins are over-expressed in squamous cell carcinoma, adenocarcinoma and melanoma.
Finally, the prognostic value of the expression of p15E-related proteins in squamous cell carcinoma of the esophagus and head and neck was evaluated. Our study indicates a relationship between p15E-expression and survival of patients with esophagus carcinoma.

Material and methods

Tissues

Malignant tissues used for immunohistochemistry were obtained from the head and neck (n=38, including larynx, pharynx, floor of the mouth and tongue), esophagus (18), lung (15), breast (22), colon (12), ovary (21), cervix (4), skin (3), bladder (2), melanoma (7) and basal cell carcinoma (9). Normal tissues tested were obtained from a variety of different organs. We studied at least two different samples of each normal tissue tested. Snap frozen tissues were obtained from the departments of Pathology from the University Hospital Dijkzigt, Rotterdam, the Daniel den Hoed Cancer Clinic, Rotterdam and the Free University Hospital, Amsterdam.

Monoclonal Antibodies
MAbs used in the present study were: ER-IS1 (IgM), directed against the CKS-17 epitope of p15E (13); 19F8 (IgG2b) (14) and 4F5 (IgG2a) both directed against retroviral p15E, but recognizing different epitopes (15). ER-IS1 and 19F8 were used as undiluted culture supernatant, with an antibody concentration of 25 µg/ml. Protein A purified 4F5 was a generous gift of Dr. G.J. Cianciolo, Macronex, USA.
Control MAbs used were MPC11 (IgG2b) and P1.17 (IgG2a), obtained from the ATCC (Rockville, USA) and supernatant from irrelevant IgM producing hybridoma cells, prepared in our own laboratory.

Immunohistochemistry
5 µm frozen sections were fixed in acetone for 15 min and rinsed with phosphate buffered saline, containing 0.05% Tween 20 (PBS-Tw). Endogenous peroxidase activity was blocked with H_2O_2 (0.25%) in PBS for 15 min. Slides were washed with PBS and incubated with supernatant from clones ER-IS1 and 19F8, or with purified 4F5 antibody, for 30 min at room temperature. Next, the slides were washed in PBS-Tw and subsequently incubated with biotinylated goat-anti-mouse antibodies, which was followed by a routine ABC-immunoperoxidase procedure (DAKO, code K377). 3-Amino-9-ethylcarbazole (AEC) was used as a chromogen for peroxidase. The slides were washed, slightly counterstained with Mayer's haematoxylin and mounted with a coverslip.

Tissue reactivity was scored visually for the intensity of staining (score 0-3) and for the percentage of positive staining cells (score 4-7). For intensity we scored: 0=negative, 1=very weak, 2=moderate and 3=strong. In the negative scored samples, there was no difference in reactivity, as compared with the control MAb used. In our results we presented 0 and 1 scores as negative and 2 and 3 scores as positive. The percentage of staining cells was scored: 4=all cells positive (>90%), 5=many cells positive (10-90%), 6=few cells positive (<10%) and 7=focal, some fields of positive tumor cells present.

Results

Comparison of anti-p15E MAbs for immunohistochemistry
At the initial phase of investigation we compared the different types of monoclonal antibodies reactive to p15E in immunohistology. The anti-retroviral p15E MAb 19F8, detected p15E in a minority of the head and neck and lung carcinoma tissues. Although it is known that 19F8 is directed against a sequence within the CKS-17 epitope (16), its weak staining in immunohistochemistry made this MAb not suitable for our study of the distribution of CKS-17 in human cancer. Anti-p15E MAb 4F5 has already been shown to react with SCC-HN (17). In our experiments, this MAb also showed a positive staining of larynx, colon and breast cancer tissues (data not shown).

However, because of its unknown epitope specificity we did not include this anti-p15E MAb in our complete study. The staining intensity using one of our newly developed antibodies, ER-IS1, was comparable to the intensity of 4F5. Because of the defined specificity of ER-IS1 for CKS-17, the immunosuppressive epitope of p15E, this antibody was chosen to study the distribution of p15E-related proteins in human malignancies.

Reactivity of ER-IS1 with malignant tissues

a. Squamous cell carcinoma of the head and neck (SCC-HN)

MAb ER-IS1 detected p15E-related proteins in 34 of 38 squamous cell carcinomas of the head and neck (SCC-HN) (Table 1). In this group, only two carcinomas showed a weak staining (score 1) and two were negative for ER-IS1. In 32 of the ER-IS1 positive carcinomas the large majority of tumor cells was positive (score 4 or 5). In four carcinomas only a few cells were positive (e.g. 2/15 larynx carcinomas). Noteworthy, ER-IS1 reacted primarily with the more differentiated cells of the malignant epithelium. In carcinomas where not all tumor cells stained positive, ER-IS1 did not react with the basal cell layer (larynx, Fig. 1a, negative control antibody, Fig. 1b).

b. SCC of the esophagus, lung, cervix and skin

Of the SCC of the esophagus, lung, cervix and skin, we observed a total of 17 of 36 positive for ER-IS1 (Table 1). The staining pattern was comparable to that of SCC-HN: positivity in the more differentiated tumor cell layers and in some cases no or only a weak staining of the basal cell layer. Nine of eighteen esophagus tumors we examined were positive for ER-IS1 (esophagus, Fig. 1c, negative control antibody, Fig. 1d). Among lung tumors we found only a few positive samples (3 of 11) with the majority of tumor cells showing a moderate staining intensity (score 2). Only a limited number of samples from cervix and skin were examined, however, p15E-expression could be detected in the majority of these samples (Table 1).

c. Basal cell carcinomas (BCC)

The observation that the basal cell layer in SCC was often negative for ER-IS1, led us to examine nine basal cell carcinomas. As expected, none of these carcinomas was positive for ER-IS1. A weak granular reaction was only observed in more differentiated, centrally located areas within the tumor (data not shown).

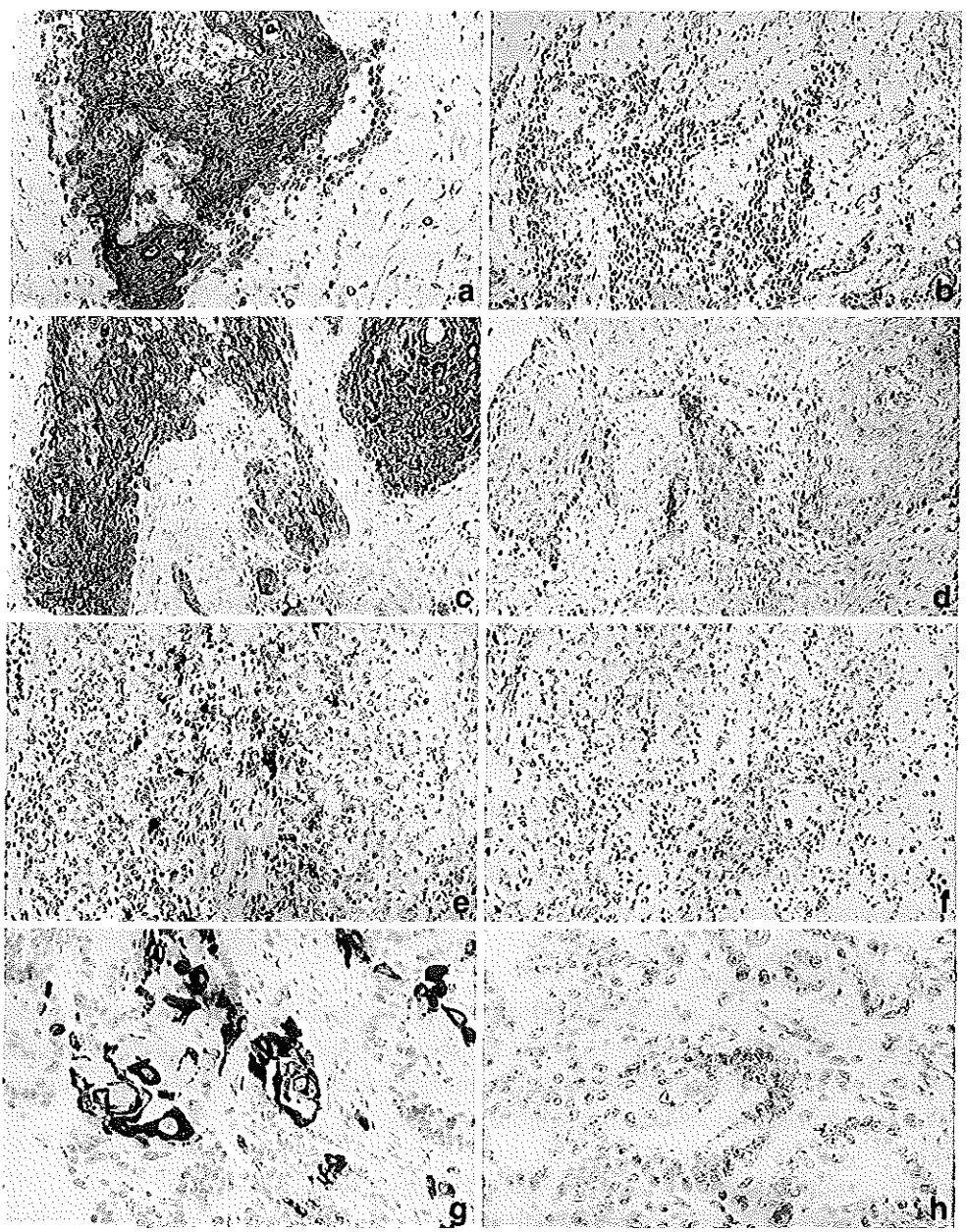
d. Malignant melanoma.

Malignant melanoma expressed p15E-related proteins in three out of seven samples (Table 1). In these tumors only a few scattered melanocytes were detected by the antibody ER-IS1, the large majority of the malignant cells was p15E-negative (malignant melanoma, Fig. 1e, negative control antibody, Fig. 1f).

e. Adenocarcinoma of the breast, colon and ovary

P15E-related antigens were detected in adenocarcinoma of the breast (10/22), colon

(6/12) and ovary (8/21). In breast cancer tissue, the staining intensity was moderate (score 2) and we found no positivity using 19F8. However, we did observe a strong reactivity of ER-IS1 and 19F8 with the glandular secretion product, whereas surrounding epithelial cells remained negative.
In colon carcinoma, p15E-related antigens were present in 6 out of 12 samples. Using 19F8, we found a moderate staining in three (ER-IS1 positive) tumor samples. Spe-



cific staining was detected at the apical site of the epithelial tumor cells. We also observed some staining of the glycocalyx overlying the epithelial cells.
Ovarian carcinomas of different origin were examined for the expression of p15E-related antigens. A moderate positive staining was detected in endometroid carcinoma, in adenocarcinoma and in androblastoma. No, or only a very weak staining was found in mucinous cystadenocarcinoma and in serous papillary cystadenoma. In two ovarian endometroid carcinomas and in one androblastoma we detected scattered positive cells, surrounded by negative tumor cells (endometroid carcinoma, Fig. 1g, negative control antibody, Fig. 1h).

Reactivity of ER-IS1 with normal tissues

A large panel of tissues derived from different organ systems was used to evaluate cross reactivity of ER-IS1 with normal human tissues (Table 2).
54 Out of 61 normal tissues showed no reactivity with ER-IS1 (89%). The positive reactivity of 7 tissues was either a weak to moderate staining (score 1 to 2) of (mature) epithelial cells, observed in the epithelium of the nasal mucosa, the tonsil and the skin; a positive reaction, observed in the internal root sheath of a hair follicle in one of the tissue samples (data not shown); or a very weak staining (score 1), observed in some epithelial cells in the stomach, the parotid and the testes. In these tissues no reactivity with connective tissue or lymphoid cells was detected. As mentioned earlier, we observed a positive reactivity with the secretion product of glandular epithelial cells in normal breast tissue and in colon tissue.

Fig. 1. Immunohistochemical staining of malignant tissues using ER-IS1 (a,c,e,g) or a negative control antibody (b,d,f,h). Slides were weakly counterstained with hematoxylin. Magnification 250x.

a (b). Squamous cell carcinoma of the larynx: cells of the basal cell layer remain negative using ER-IS1.
c (d). Squamous cell carcinoma of the esophagus: all tumor cell layers stain positive using ER-IS1.
e (f). Malignant melanoma: scattered ER-IS1 positive tumor cells.
g (h). Endometroid carcinoma of the ovary: tumor fields with ER-IS1 positive tumor cells surrounded by ER-IS1 negative tumor cells.

Table 1. Reactivity of ER-IS1 with malignant tissues.

Tumor	samples positive/tested	% +ve samples
Squamous cell carcinoma		
Head and Neck	34/38	89
Esophagus	9/18	50
Lung	3/11	27
Cervix	3/4	75
Skin	2/3	67
Total	51/74	69
Basal cell carcinoma	0/9[1]	0
Transitional cell carcinoma	0/2	0
Adenocarcinoma		
Breast	10/22	45
Colon	6/12	50
Lung	1/4	25
Ovary	8/21	38
Total	25/59	42
Malignant melanoma	3/7	42
Overall	79/151	52

1: some positivity in cells of central, more differentiated tumor areas; and in surrounding epidermal cells (score 2-5).

Relation between differentiation and ER-IS1 reactivity

Our study indicates that more differentiated epithelial cells in carcinomas react with ER-IS1. Because of this observation, we examined whether there were more positive samples among the good or moderate differentiated carcinomas of the head and neck, esophagus and lung, compared to poorly differentiated carcinomas. The differentiation stage of a limited number of tumors could be established and the number of p15E-positive tumors was scored. As shown in Table 3, 22 out of 25 good or moderate differentiated carcinomas were p15E-positive (88%). Only 6 out of 16 poorly differentiated tumors were ER-IS1 positive (38%). This indicates that p15E-related antigens are expressed at higher levels in more differentiated tumor cells. However from these data we can not conclude that p15E, as detected by ER-IS1, is a tumor differentiation marker, because almost 40% of the poorly differentiated tumors of the head and neck and esophagus are also p15E-positive (Table 3).

Table 2. Reactivity of ER-IS1 with normal tissue samples.

Organ		positive/tested	
Respiration			
	Nasal mucosa	1/3	
	Lung	0/3	
	Bronchus	0/3	
Digestive tract			
	Stomach	0/2[1]	
	Colon	2/2[2]	
	Parotid	0/2[1]	
	Liver	0/2	
	Gall bladder	0/2	
	Pancreas	0/2	
Urogenital cells			
	Kidney	0/3	
	Prostate	0/2	
	Bladder	0/2	
Reproductive system			
	Testes	0/2[1]	
	Ovary	0/2	
	Uterus	0/2	
	Mammary gland	1/3[2]	
Nervous system			
	Brain	0/2	
	Peripheral nerve	0/2	
Endocrine organs			
	Thyroid	0/2	
	Adrenal	0/2	
Lymphoid system			
	Thymus	0/3	
	Lymph node	0/2	
	Spleen	0/2	
	Tonsil	1/2[1]	
Integument			
	Striated muscle	0/2	
	Fibroblasts	0/2	
	Skin	2/3[1,3]	
Overall		7/61	11%

1: weak staining of (keratinizing) epithelial cells, lymphoid tissue stains negative.
2: some positive staining on the apical site of colon epithelial cells; positive staining in breast tissues, in the lumina surrounded by glandular epithelial cells.
3: positive staining in hair follicle and sebaceous glands.

Table 3. Relation between differentiation stage and a positive immunohistology pattern for carcinomas of the head and neck, esophagus and lung. The number of p15E-positive tissue samples is calculated as percentage of the total number of tissue samples tested per differentiation stage (good/moderate or poor).

Tumor	Differentiation			
	good/moderate		poor	
	p15E +ve	% p15E +ve	p15E +ve	% p15E +ve
Head and Neck	14/16	88%	4/6	67%
Esophagus	6/7	86%	2/7	29%
Lung	2/2	100%	0/3	0%
Overall	22/25	88%	6/16	38%

Relation between ER-IS1 positivity and prognosis

To evaluate whether the presence of p15E-related proteins in tumor tissue had any prognostic significance, we compared a positive or negative reaction of ER-IS1 with tumor stage and prognosis, for patients with esophagus carcinoma or with squamous cell carcinoma of the head and neck.

Data from 11 patients with esophagus carcinoma were evaluated and are depicted in Table 4. We detected that out of six patients with a high p15E-expression, four had died within a year after surgery as a consequence of metastases, one is still alive after three years and one died of a non tumor-related cause. Out of five patients with a low, or no p15E-expression, three p15E-negative patients were still alive 3-5 years after surgery. One patient, with a low p15E-expression, died as a consequence of metastases and one patient with a low p15E-expression died of a cause not related to the tumor.

34 Of 38 squamous cell carcinomas of the head and neck were positive for p15E (89%). No correlation could be found between p15E-expression and tumor recurrence or survival. For patients with a follow-up of at least one year (21 patients), we detected that 2 patients with a low p15E expression had died of recurrent tumor or distant metastases. Of 19 patients with a high p15E-expression, 11 were alive after 18-52 months, with a mean of 32 months. 8 Patients had died of local recurrent disease (2), distant metastases (4) or unknown causes (2).

Table 4. Clinical data of patients with squamous cell carcinoma of the esophagus and detection of p15E-related factors in tumor tissue using MAb ER-IS1.

Patient	m/f	Age	Tumor stage	P15E-score	Survival
1	f	54	T3N0M0	3	died of metastases
2	f	51	T3N1M0	3	died of metastases
3	m	65	T3N0M0	3	died of metastases
4	f	47	T3N1M0	2	died of metastases
5	f	70	T2N0M0	2	tumor free
6	m	71	T3N0M0	2	non tumor-related death
7	m	73	T3N0M0	1	died of metastases
8	m	63	T3N0M0	1	non tumor-related death
9	f	66	T3N0M0	0	tumor free
10	f	67	T2N0M0	0	tumor free
11	f	6	T2N0M0	0	tumor free

Discussion

In the present study, a large panel of malignant and normal tissues has been examined for the expression of p15E-related antigens. Using a new MAb (ER-IS1), specific for the immunosuppressive domain of p15E (CKS-17), p15E-related proteins were detected in 51 of 74 squamous cell carcinomas, 25 of 59 adenocarcinomas and 3 of 7 melanomas. Our data showed that the staining intensity was high in squamous cell carcinoma and moderate in adenocarcinoma. P15E-related antigens were expressed in most SCC-HN (34 of 38). Only 7 of the 61 normal tissues showed a weak reactivity with ER-IS1. These observations indicate that p15E is a tumor associated protein, with a high expression predominantly in squamous cell carcinoma, adenocarcinoma and melanoma. These data are in contrast to the results obtained by Scheeren *et al.*, who postulated that p15E-related antigens are exclusively restricted to malignant and inflamed epithelia of the upper airways (17). However, the observed discrepancy could be due to the fact that the staining intensity of the antibody applied in their study, MAb 19F8, is very weak in immunohistochemistry. Several authors have reported the presence of p15E-related proteins in sera or in effusions of patients with malignancies like melanoma or breast, colon, ovarian or gastric carcinoma (3,9,10). These data are in accordance with our results in tumor immunohistochemistry.

Although p15E was highly expressed in tumor tissues, we observed a low expression in normal epithelial cells from the nasal mucosa, skin and tonsils. These results could be explained by the fact that p15E-related proteins are also present in sera of patients with chronic infections of the upper airways (17,18), indicating that our p15E positive

samples were possibly derived from an infected area. Background levels of p15E activity have also been detected in normal human sera, using a bio-assay, the monocyte polarization assay (19). These observations indicate that p15E-related factors are present at low levels in healthy individuals. In the malignant state, however, these proteins are highly upregulated.

Apart from the reactivity of ER-IS1 with tumor associated p15E-related proteins, and epithelial cells, we detected cross-reactivity with the internal rooth sheath of a hair follicle and with myoepithelial cells surrounding lobular ducts in breast carcinoma tissue. This pattern, taken together with the positive reaction with epithelial cells could point to cross-reactivity of ER-IS1 with cytokeratins (CKs). Based on the CK expression pattern in epithelia, hair follicles and myoepithelial cells, ER-IS1 could cross react with cytokeratins 5 and 14. However, the strong expression in malignant epithelia as compared to the weak expression in some normal epithelia does not support this notion, as most CKs retain their level of expression during malignant transformation (20). Besides, although CK 5 and 14 are present in the internal rooth sheath and in myoepithelial cells, their expression in stratified epithelia is restricted to the basal cell layers (21). These cells were often found to be negative for ER-IS1. Finally, 2D-electrophoresis experiments using the epithelial cell line HaCat, which contains 15 of the 20 existing CKs, among which CK 5 and 14, have shown that ER-IS1 does not recognize any of the cytokines present in this epithelial cell line.

Cross-reactivity of anti-p15E MAbs with the immunosuppressive cytokines IFN-α and TGF-β has also been postulated (22,23). A 10 amino acid sequence of IFN-α with a high level of homology with CKS-17 has been identified (22). Experiments in our department have shown that MAbs directed against p15E (4F5 and 19F8) and CKS-17 (ER-IS1) did react with IFN-α (24). The immunosuppressive cytokine TGF-β also has sequence homology with CKS-17 (23). TGF-β has been detected in carcinoma (1,25). Experiments performed by one of us (Oostendorp), using antibodies against TGF-β showed a staining pattern comparable to that of the anti-p15E MAb 4F5. In these experiments, both antibodies, ER-IS1 and 19F8 showed the same pattern in most, but not all cases (data not shown).
These results indicate that MAbs directed against p15E react with other immunosuppressive proteins. Recent data from Turbeville *et al.* have demonstrated the presence of p15E-related sequences derived from endogenous retroviral sequences in human cancer (26,27). Obviously, studies on the nature of p15E-related and cross-reactive proteins and on the (molecular) characterization of these proteins are needed for further characterization of the tumor-associated p15E-related proteins

In conclusion, our present data indicate that the immunosuppressive epitope of p15E is expressed in squamous cell carcinoma, adenocarcinoma and malignant melanoma. Therefore, p15E-related proteins can be used as tumor markers. In selected groups of

patients, like patients with esophagus carcinoma, the expression of p15E may be useful in prediction of patient survival.
Moreover, p15E-related proteins could play a role in tumor associated immunosuppression. In studies using p15E as a target for immunotherapy in animal models, antibodies directed against p15E have been able to inhibit tumor growth (28-30). Today, more specific immunotherapeutic approaches, based on the presence of the functional immunousppressive epitope of p15E and the use of MAbs against this epitope, come into view (31).

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7

Sandwich Enzyme-Linked Immunosorbent Assay for the detection of retroviral p15E and head and neck cancer associated p15E-related proteins

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Sandwich Enzyme-Linked Immunosorbent Assay for the detection of retroviral p15E and head and neck cancer associated p15E-related proteins

In previous studies, using the monocyte polarization bioassay, immunosuppressive p15E-related proteins have been detected in sera of patients with head and neck cancer. In this study, monoclonal and polyclonal antibodies, directed against the retroviral immunosuppressive protein p15E and its immunosuppressive CKS-17 epitope were evaluated for the development of a p15E-specific sandwich-ELISA. We developed a quantitative sandwich-ELISA system for the detection of p15E-related proteins, using two monoclonal antibodies (MAbs 4F5 and 19F8), which were directed against different p15E-epitopes. Purified p15E from Murine Leukemia Viruses (MuLV) was used as a standard for quantification. With the monoclonal antibodies 4F5 and biotinylated 19F8, we were able to detect 10 ng/ml MuLV-p15E.
We discuss the applicability of this sandwich-ELISA for the detection of p15E-related proteins in cell culture supernatants and in the serum of patients with head and neck cancer.

Introduction

Over the past years, several studies have been published concerning immunosuppressive p15E-related proteins associated with human malignancies. The retroviral transmembrane (TM) protein p15E is an important immunosuppressive protein associated with retroviral infections (1,2). Using monoclonal antibodies directed against retroviral p15E, immunosuppressive p15E-related proteins were detected in human malignancies and tumor cell lines (3,4). Especially in tissues from squamous cell carcinoma of the head and neck (5,6). Using the MAbs in combination with a sensitive monocyte polarization bioassay for the detection of p15E-activity, p15E-related proteins were detected in the majority of sera taken from patients with SCC-HN (7). As these p15E-related proteins disappear after surgical removal of the tumor (8,9) the hypothesis was postulated that the malignant cells are the major source of production of p15E-related proteins.
In an interesting follow-up study by Tas *et al.* it was found that the reappearance of p15E-activity in the serum of patients with head and neck cancer after surgical removal of the tumor, was associated with residual or recurrent disease (10). However, this study included only 20 patients and was hampered by the use of the laborious monocyte polarization bioassay for the detection of p15E-activity.

As serum titers of p15E-related proteins might predict recurrence of the tumor, direct measurement of p15E-related proteins would be helpful for diagnosis.
To this purpose, we developed a sandwich-ELISA system for the detection of p15E. Purified retroviral p15E was used as a standard for quantification and several monoclonal and polyclonal antibodies directed against different p15E-epitopes, or against

the immunosuppressive CKS-17 epitope of p15E, were evaluated in the sandwich-ELISA.

We were able to detect purified retroviral p15E with a sensitivity of 10 ng/ml. However, detection of p15E-related proteins in human sera was limited, due to low concentrations of p15E. Besides, the detection of purified retroviral p15E titrated in human serum was hampered because of binding of p15E with a factor present in human serum.

Material and methods

Antibodies

Polyclonal antibodies and affinity purified monoclonal antibodies used in the present study are listed in Table 1. Purified 4F5 was a generous gift from Dr. G.J. Cianciolo, Macronex, USA. Purified 19F8 was conjugated to biotin (N-hydroxysuccin-imidobiotin, Boehringer Mannheim GMBH) by standard procedures.

Table 1. Monoclonal and polyclonal antibodies with specificity for retroviral p15E or the immunosuppressive epitope of p15E, CKS-17.

MAb	Isotype	Specificity	Source	Reference
19F8	IgG2b	retroviral p15E	Lostrom	(26)
372	IgG3	MuLV-p15E	ATCC 1893-CRL	(27)
4F5	IgG2a	retroviral p15E	Cianciolo	(19)
A2-J	IgG2a	FeLV-p15E	Biodesign USA	-
ER-IS1	IgM	CKS-17	Lang	(17)
ER-IS5	IgG2b	CKS-17	Lang	(17)
PAb	**Animal**	**Immunization**	**Source**	**Reference**
Z48	rabbit	R-MuLV	Bloemers	(28)
poly C	rabbit	R-MuLV p15E	Cianciolo	(19)

Cell lines

Hybridoma cell lines 19F8, 372, ER-IS1 and ER-IS5 were cultured in RPMI 1640 containing 10% FCS and 0.05 mM β-mercapto-ethanol. JLS-V5 (11) and 3T3-Cl9 (12) are established Rauscher Murine Leukemia Virus producing cell lines which were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 7% FCS, 100 ug/ml penicillin and 60 ug/ml streptomycin (P/S).

Feline Leukemia Virus, FeLV-F422 was a generous gift from Dr R.A.J. Oostendorp, Free University, Amsterdam.

Purification of retroviral p15E

Rauscher-MuLV particles were obtained from JLS-V5 and 3T3-Cl9 cells. Culture supernatant was centrifuged and virus particles were isolated using a sucrose density gradient as previously described (13). MuLV-particles were suspended in TNE-buffer (50 mM Tris-HCl, 100 mM NaCl, 1mM EDTA) containing 2 mM PMSF and 1% TX-100.

MuLV-p15E was purified by a two step procedure (14). First, hydrophobic proteins were released from the viral envelope (15) and separated from hydrophilic proteins using TNE-buffer containing TX-114. This solution is homogeneous at 0 °C, but separates in an aqueous phase and a detergent phase after incubation at 30 °C for 3 min (16). Second, hydrophobic p15E from the detergent phase was affinity purified using a 19F8-sepharose column and suspended in TN-buffer, containing 0.1% TX-100. The protein concentration of p15E in TN-buffer containing TX-100 was determined using the BCA Protein Assay (Pierce). Out of six isolation procedures (using 50 mg MuLV-particles in total) we obtained 225 µg of purified p15E.

The procedure was verified by a p15E-purification using biotinylated MuLV particles. SDS-gelelectrophoresis and Western blotting of biotinylated viral proteins were performed as described before (17). Biotinylated MuLV proteins from all successive steps and the final purified p15E were detected after incubation of the blots with alkaline phosphatase-labeled streptavidin (Southern Biotechnology Associates, 1:1000). In the purified sample we detected a single p15E-band, whereas in the MuLV-sample all biotinylated viral proteins were present.

Patient sera and monocyte polarization assay

Pre-operative sera and post operative sera (week 1 and month 1, 6 and 12 post operative) were obtained from patients with squamous cell carcinoma of the head and neck. Sera were stored in 4 ml volumes at -70 °C.

The monocyte polarization assay for the determination of suppression of monocyte polarization by p15E-related proteins in SCC-HN patient sera was performed as described before (3,9). In brief, human monocytes were obtained from healthy volunteers and incubated for 15 min with patient sera and the chemoattractant fMLP. The polarization process was stopped by adding an ice cold 10% formaldehyde buffer. The percentage of polarized cells (cells with elongated or triangular shape, broadened lamellipodia or membrane ruffling) was determined by counting 200 cells in a hemocytometer using an ordinary light microscope.

To determine the percentage of inhibition caused by p15E-related proteins, serum fractions were neutralized by overnight incubation with p15E-binding MAbs 4F5 and 19F8 before testing in the polarization assay. The percentage of inhibition caused by p15E-related proteins was calculated as the difference in inhibition with or without pre-incubation with MAbs (9).

ELISA and sandwich-ELISA

ELISA and sandwich-ELISA experiments were performed using PVC 96-well Micro Test flatbottom plates (Falcon).

For ELISA experiments, plates were coated with antigen (virus particles or purified p15E) in a twofold dilution (in PBS, 1% BSA) and incubated overnight at 4 °C. After incubation of antigen, the plates were washed three times (using PBS, 0.1% BSA, 0.05% Tween 20) and incubated with detecting antibody (0.5 µg/ml in blocking buffer, PBS, 1% BSA, 0.05% Tween 20) for one hour at room temperature. After washing the plates three times, they were incubated with the appropriate conjugate: streptavidin-peroxidase (Jackson) 1:1500 in blocking buffer, or goat-anti-mouse-peroxidase (Southern Biotechnology Associates) 1:500 for one hour at room temperature. Next, the plates were washed three times and incubated for 1-15 min at room temperature, with ABTS-substrate (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (Sigma): *x* mg ABTS + *x* ml buffer (0.2 M $Na_2HPO_4.2H_2O$: 0.1 M citric acid = 1:1) + *x*/10 µl 30% H_2O_2). The color reaction was ended using 50 µl/well of a 0.2 M citric acid solution. Finally, extinction was measured by 414 nm.

For sandwich-ELISA experiments, 100 µl of catching antibody (1 µg/ml MAb, or poly C 1:1000 in PBS) per well was coated and incubated overnight at 4 °C. Plates were washed three times and blocked for one our at room temperature. Samples to be determined were twofold diluted in RPMI+ containing 1% BSA and were incubated for 2-3 hours at room temperature. Incubation of detecting antibody, conjugate and substrate were performed as described.

Immunoblotting

156 ng of purified viral p15E was mixed with serial dilutions of normal human serum, separated on a 12.5% polyacrylamide gel under non-reducing conditions (β-mercaptoethanol and SDS were omitted from all buffers) and transferred to nitrocellulose membranes. Blots were blocked with 5% non-fatty dry milk (NFDM) (Profitar, N.V. Nutricia-Zoetermeer) in PBS, 0.05% Tween and incubated with first (19F8, 0.6 µg/ml) and second (HRP-conjugated rabbit anti-mouse Ig, 1 µg/ml) step antibodies in 1% NFDM in PBS, 0.05% Tween. Blots were developed by ECL (Amersham Life Science, Buckinghamshire, UK).

Results

Specificity and affinity of monoclonal and polyclonal antibodies

To select for high affinity binding anti-p15E antibodies, all monoclonal and polyclonal antibodies were tested for their reactivity against p15E from MuLV and FeLV in Western blots. Both polyclonal antibodies, poly C and Z48 were specific for the 19 kD p15E protein (and its degradation product p12E) of MuLV and FeLV (19,28). However, poly C had a much stronger reactivity against p15E, as it could be 100-fold more diluted than Z48, still showing the same intensity of staining (data not shown).

MAbs 19F8 and 4F5 recognized p15E and p12E with high affinity and specificity (Fig. 1). MAb 372 did recognize p15E, but its affinity was low compared to 19F8 and 4F5. MAbs ER-IS1 and ER-IS5 showed a weak affinity for p15E, see also Lang *et al.* (17). MAb A2-J only recognized FeLV-p15E and not MuLV-p15E and was therefore excluded from further studies.

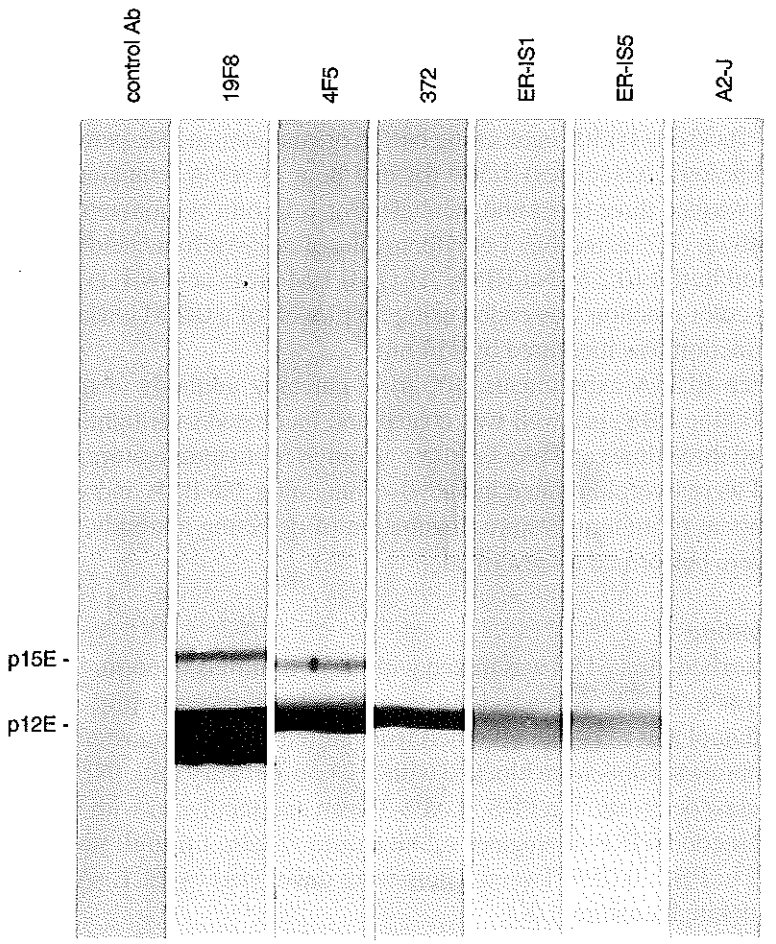

Fig. 1. Western blot of MuLV proteins detected with MAbs against p15E and CKS-17: 19F8 (supernatant, 20 µg/ml), 4F5 (10 µg/ml), 372 (supernatant, 10 µg/ml), ER-IS1 (supernatant, 25 µg/ml), ER-IS5 (supernatant, 15 µg/ml), A2-J (10 µg/ml) and irrelevant control antibody (20 µg/ml).

The detection limit of the MAbs for MuLV-p15E was evaluated in ELISA's, using direct coated purified p15E. Again, the MAbs showed differences in affinity (Fig. 2). 19F8 and 4F5 showed a detection limit of 50 ng/ml. The detection limit of 372 was 0.2 µg/ml. The anti-CKS-17 MAbs ER-IS1 and ER-IS5 did not recognize purified MuLV-p15E in ELISA.

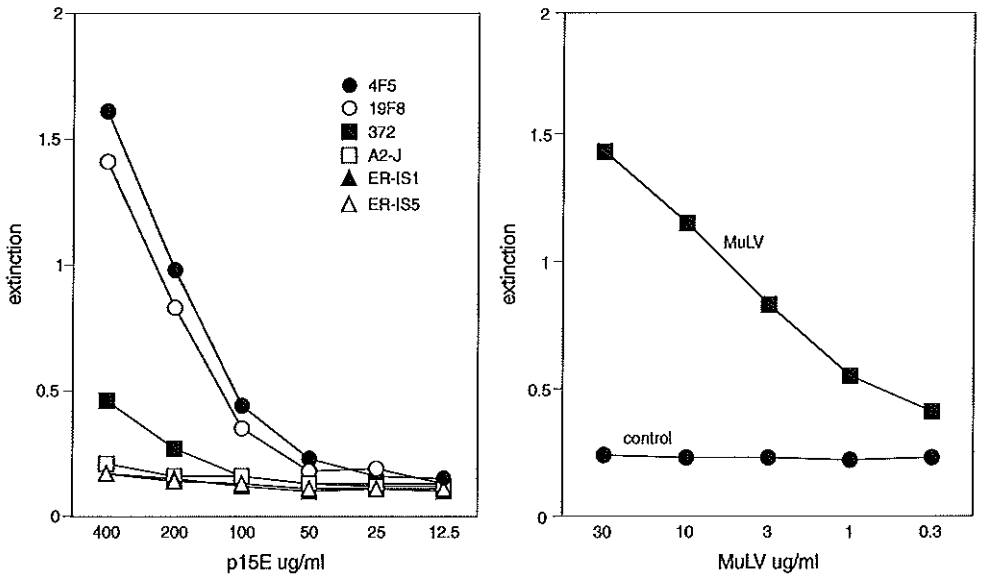

Fig. 2. (left) Titration of purified p15E in 96-wells ELISA plates and detection with MAbs 4F5 (10 µg/ml), 19F8 (supernatant, 20 µg/ml), 372 (supernatant, 10 µg/ml), A2-J (10 µg/ml), ER-IS1 (supernatant, 25 µg/ml) and ER-IS5 (supernatant, 15 µg/ml).

Fig. 3. (right) Sensitivity of the sandwich-ELISA with poly C and 19F8-bio, for MuLV-particles. MuLV was titrated and could still be detected at a concentration of 0.3 µg/ml. The control line represents background extinction from buffer without MuLV in the sandwich-ELISA.

Polyclonal antibody poly C was selected for further experimental use, based on its strong reactivity against p15E. Monoclonal antibodies 19F8 and 4F5 were selected based on the results from Western blot and ELISA. Besides, 19F8 and 4F5 were selected, based on their reactivity with human immunosuppressive p15E-related proteins (5,7). The polyclonal and monoclonal antibodies were used in a sandwich-ELISA for the quantitative detection of tumor-associated p15E-related proteins.

Sandwich-ELISA using poly C and 19F8

The combination of a polyclonal antibody with a high affinity for p15E (poly C) as catching antibody, with a detecting monoclonal antibody (19F8) was first evaluated. Using poly C (1:1000 in PBS) and 19F8 (1 µg/ml), we could detected MuLV in a concentration-dependent manner, with a sensitivity of 0.3 µg/ml (Fig. 3).
Subsequently, sera from SCC-HN patients, were evaluated for the presence of p15E-related proteins. Three sera with a high percentage of p15E-dependent inhibition of monocyte polarization (30-50%) were considered p15E-positive and three sera with a low, or no p15E-dependent inhibition (0-8%) were considered p15E-negative (Table 2). The mean signal in the sandwich-ELISA of the positive sera was 0.1 AFU higher as compared to the mean of the negative sera. Although there is a detectable differ-

ence, this combination of antibodies cannot be used in a sandwich-ELISA for reliable p15E-detection in sera of patients with head and neck cancer, because the extinction is just above background level (0.2 AFU) and because the difference between positive and negative sera is very small.

Table 2. Extinction in sandwich-ELISA of 6 patient sera with a high or a low percentage of p15E-dependent inhibition of the monocyte polarization assay.

		Extinction in Sandwich ELISA		
Patient	Inhibition in %	Serum dilution 1:10	1:100	1:1000
A	32	0.37	0.3	0.26
B	49	0.41	0.36	0.34
C	31	0.41	0.32	0.30
mean±SD	37.3±10.1	0.4±0.02	0.33±0.04	0.3±0.03
D	6	0.3	0.26	0.23
E	8	0.3	0.28	0.23
F	8	0.3	0.27	0.23
mean±SD	7.3±1.1	0.3±0	0.27±0.01	0.23±0

Sandwich-ELISA using 4F5 and 19F8

Because the combination of PAb and MAb did not result in a sandwich-ELISA with the desired sensitivity, a combination of two monoclonal antibodies (4F5 and 19F8) with a high affinity for p15E, was evaluated. Purified p15E was now used as a standard to be able to quantitate the results of the sandwich-ELISA. Using 4F5 (1 µg/ml) as catching antibody together with biotinylated 19F8 (19F8-bio, 0.5 µg/ml) as detecting antibody, the standard protein, purified MuLV-p15E, was detected in a concentration dependent manner, with a sensitivity of 10 ng/ml (Fig. 4). Using this sandwich-ELISA, we could detect retroviral p15E in culture supernatant of the virus producing cell lines JLS-V5 and 3T3-Cl9, as shown in Fig. 5.

P15E-binding proteins in human sera

Because we could detect purified MuLV-p15E in a concentration dependent manner, we investigated whether, using the sandwich-ELISA, purified p15E could be detected in the presence of human serum. Twofold dilutions of purified retroviral p15E were prepared in normal human serum (NHS, 1% and 10% in PBS). To our surprise, the detection limit of the sandwich-ELISA was 10-fold reduced when p15E was diluted in 1% NHS. When p15E was diluted in 10% NHS, we completely lost the detection of p15E (Fig. 6).

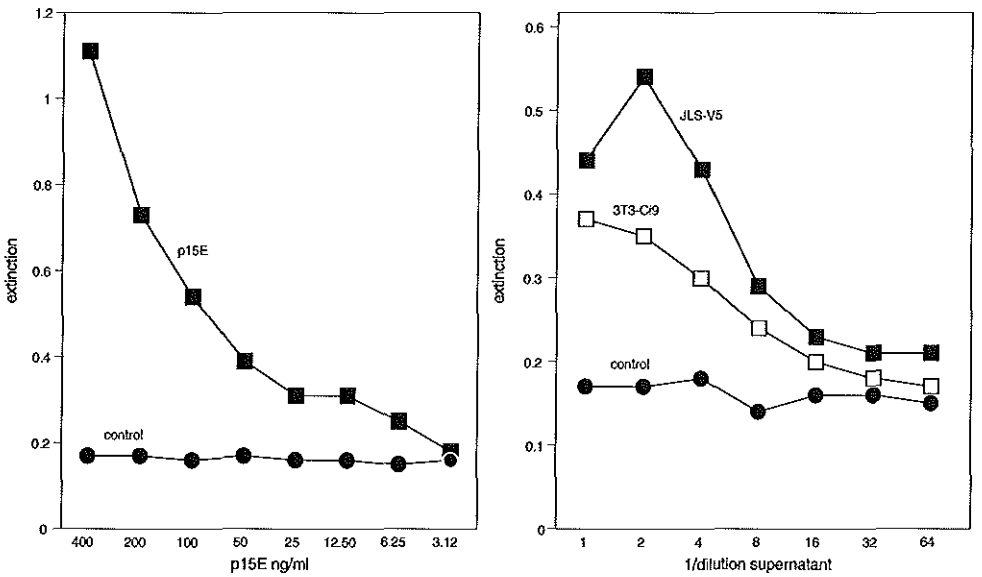

Fig. 4. (left) Detection of purified MuLV-p15E using the sandwich-ELISA with 4F5 and 19F8-bio. Purified p15E was tirated in RPMI + 1% BSA. RPMI + 1% BSA was used as a negative control.

Fig. 5. (right) Detection of p15E and p15E-related factors in cell culture supernatant using the sandwich-ELISA with 4F5 and 19F8. Supernatants were twofold diluted in RPMI + 1% BSA. RPMI + 1% BSA was used as negative control.

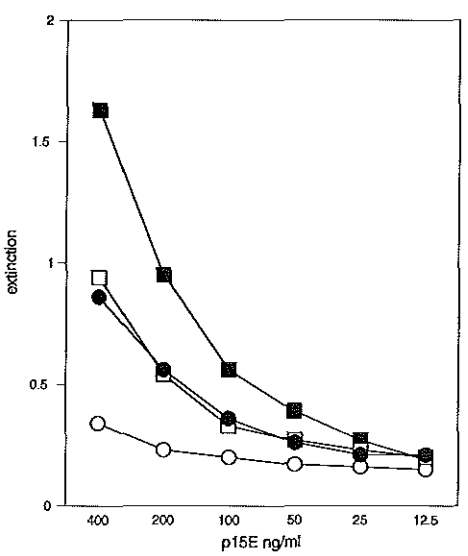

Fig. 6. Detection of MuLV-p15E using the sandwich-ELISA after titration of MuLV-p15E in 1% NHS (●), in 10% NHS (○), in RPMI + 1% BSA (■) and in 1% IgG-depleted HNS (❑).

To exclude a masking effect of p15E-blocking IgG-antibodies, we diluted purified p15E in IgG-depleted normal human serum. This serum was obtained by purification using a protein-A-sepharose column. Our ELISA-experiments indicated that 1% of this IgG-free human serum still caused a strong reduction of sensitivity (Fig. 6).

The loss of signal was also not due to the digestion of p15E by proteolytic enzymes present in human serum, since the addition of protease inhibitors like PMSF, trypsin inhibitor, leupeptin, pepstatin or bestatin did not increase the p15E signal (data not shown). Subsequently we tested whether factors present in NHS can bind to purified p15E, thereby blocking the epitopes for the anti-p15E antibodies. To this purpose, 156 ng of purified viral p15E was mixed with serial dilutions of NHS, separated on a non-reducing polyacrylamide gel and analyzed by Western-blotting using MAb 19F8 (Fig. 7). MAb 19F8 specifically recognized a single protein in the sample containing purified viral p15E in PBS (lane A). When p15E was mixed with serial dilutions of normal human serum, a second band specifically reacting with MAb 19F8 could be detected (lane B-E). This (p15E related) molecule was of viral origin as no bands reacting with MAb 19F8 could be identified (data not shown) in dilutions of normal human serum alone. These results clearly demonstrate that purified viral p15E can form a complex with a factor present in normal human serum which can be separated from unbound p15E by non-reducing polyacrylamide gelelectrophoresis.

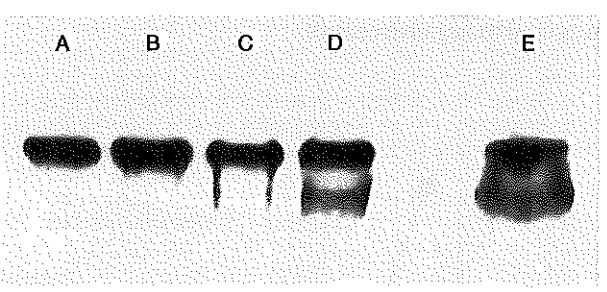

Fig. 7. Western-blot analysis of purified p15E mixed with serial dilutions of NHS. 156 ng of purified p15E was diluted in PBS (lane A), 0.1 % NHS (lane B), 1% NHS (lane C), 10% NHS (lane D) or 50% NHS (lane E). Proteins were separated on a non-reducing polyacrylamide gel and analyzed by immuno-blotting using MAb 19F8.

Discussion

In this study, the presently available monoclonal and polyclonal antibodies, directed against p15E or CKS-17 were evaluated for application in a sandwich-ELISA for the quantitative detection of retroviral p15E and p15E-related immunosuppressive pro-

teins. Quantitative detection of tumor associated p15E-related proteins could be a helpful tool in diagnosis of head and neck cancer recurrence.
Using the polyclonal antiserum poly C, and monoclonal antibody 19F8, p15E-related proteins could be detected in sera of patients with head and neck cancer. However, the observed difference between the p15E-positive sera and p15E-negative sera was only very small and very close to the detection limit of the sandwich-ELISA. Therefore, this combination of PAb and MAb was not suitable for the detection of p15E-related proteins in follow-up studies for patients with head and neck cancer.
Using the two MAbs, 4F5 and 19F8, in the sandwich-ELISA, we were able to detect 100 ng/ml p15E in the supernatant of the virus producing cell line 3T3-Cl9 and 10 ng/ml of purified p15E.
Using these data, we could calculate whether the sandwich-ELISA could be used for the reliable detection of p15E. From our virus isolation procedure, it was known that 3T3-Cl9 culture supernatant contained 20 µg/ml MuLV. From the molecular structure of MuLV we concluded that 1 mg of MuLV contained approximately 50 µg (5%) p15E protein. Therefore, in MuLV containing 3T3-Cl9 supernatant, 1 µg/ml p15E could be expected, either still present in the viral envelope, or present as free detectable p15E from disrupted viral particles. The presence of 10% p15E (100 ng) in a detectable form is an accurate estimate, indicating that the sandwich-ELISA, using purified p15E as a standard can indeed be used for the quantitative detection of p15E in cell culture supernatant. Unfortunately, this detection limit is not sufficient for the detection of biologically active levels of p15E or p15E-related proteins in serum. Cianciolo *et al.* reported that as little as 2 ng of disrupted Rauscher Leukemia Virus (RLV) particles caused a significant inhibition of macrophage accumulation at sites of delayed inflammatory reactions in normal mice (18). In another study, using a competition-ELISA for the detection of p15E, the presence of the equivalent of more p15E antigen than that derived from 100 ng of RLV per mg murine tumor tissue was reported (19). Because these authors also reported that 40 µg tumor tissue still inhibited macrophage accumulation in normal mice by >80%, an estimation could be made of the required detection limit for a p15E-specific sandwich-ELISA. Since 1 mg tumor tissue is equivalent to 100 ng RLV, this equals 5 ng p15E protein (5%). 40 µg tumor tissue still causes >80% inhibition of macrophage accumulation, meaning that 200 pg p15E-related protein is the biologically active amount. P15E detection, therefore, should be in the pg range, as could be expected for cytokine-like factors.

The other major obstacle in the detection of p15E-related proteins in human sera is the presence of blocking factors. We could demonstrate that these factors can bind to purified viral p15E and form a complex which can be physically separated from unbound p15E. As this complex can still be detected by Western-blotting using MAb 19F8, the epitopes for the α-p15E antibodies are not blocked completely. At present the nature of such factors is unclear, the blocking factors could either be IgM antibodies, complement factor C1 (20,21) or p15E-receptor molecules, although until now

only one study which reported the existence of p15E-receptors on human cells has been published (22).
As our sandwich-ELISA was hampered by binding of serum factors to p15E and could not detect retroviral p15E in the ng range, new sensitive techniques and possibly new MAbs should be developed for the quantitative detection of p15E and p15E-related factors.
Preliminary studies have shown that purified MuLV-p15E, as well as p15E-related serum proteins can be detected in the pg range, using one p15E-specific antibody, a DNA-conjugated anti-mouse antibody and PCR amplification of the bound DNA. Using this immuno-PCR technique we will continue our search for a sensitive p15E-detection system, especially because recent studies have proven the presence of immunosuppressive p15E-related proteins not only in SCC-HN, but also in breast tumors (23) and in colorectal and gastric cancer (24). Besides, a large immunohistochemical study performed in our laboratory has demonstrated the presence of p15E-related proteins also in SCC of the esophagus and cervix, as well as in adenocarcinoma of the ovary and in malignant melanoma (25).

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8

Immunotherapy with monoclonal antibodies directed against the immunosuppressive domain of p15E inhibits tumor growth

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Immunotherapy with monoclonal antibodies directed against the immunosuppressive domain of p15E inhibits tumor growth

Immunosuppressive retrovirus related proteins, like p15E, are involved in tumor associated immunosuppression. In the present study we investigated whether such proteins could be used as targets in tumor immunotherapy using MAbs. Immunotherapy was performed in mice inoculated with the Rauscher virus-transformed myeloid cell line RMB-1. RMB-1 cells express retrovrial antigens at their cell surface. In order to obtain constant serum titers of MAbs over a prolonged period of time during therapy, anti-p15E antibody producing hybridoma cells were encapsulated in alginate and injected i.p. in tumor bearing mice. Using this technique, serum antibody titers of 50-100 mg/ml were obtained, which remained constant over a period of at least 3 weeks.

Therapy experiments were performed using anti-p15E antibodies 19F8, which recognizes both cell surface associated as well as circulating p15E, and ER-IS5, which did not react with surface bound p15E byond background, but which neutralizes circulating p15E. Inoculation of alginates containing anti-p15E hybridoma cell lines in RMB-1 tumor bearing mice showed inhibition of tumor cell growth. In survival experiments, 19F8 cured eight of 23 tumor-bearing mice. The p15E neutralizing antibody ER-IS5 caused a significant longer survival, but therapy with this MAb alone was not sufficient to cure the animals of the RMB-1 tumor.

Introduction

Immunosuppressive factors produced by tumors are often responsible for a disturbed functioning of the patient's immune system (1). Some of the tumor associated immunosuppressive factors have a structural relationship to retroviral immunosuppressive proteins (2).

In this respect, p15E is a well known retroviral immunosuppressive protein, responsible for, at least part of, the immunosuppression accompanying retroviral infections (3). Retroviral p15E-related proteins are expressed by several virally as well as non-virally induced tumors and tumor cell lines (4). P15E and its immunosuppressive conserved domain CKS-17, exert their suppressive function on cells important in the immune response against tumor cells like macrophages, T-cells and NK-cells. Immunosuppressive p15E-related proteins are also associated with (non-retrovirally induced) human diseases, mainly tumors (reviewed by Oostendorp *et al.* (5)). Recently, studies have been published describing the presence of p15E-related proteins of low molecular weight in breast cancer (6), RNA corresponding to the highly conserved immunosuppressive CKS-17 epitope of p15E has been detected in colorectal and gastric cancer (7). Especially in patients with squamous cell carcinoma of the head and neck (SCC-HN), p15E-related proteins are thought to play a role in the depression of the cellular and humoral immune response (8). In this context,

retroviral p15E-related immunosuppressive factors were identified in serum obtained from patients with SCC-HN (9) and could be detected in tumor tissue of SCC-HN (8,10).

As it becomes more and more evident that immunosuppressive p15E-related proteins are associated with several types of human cancer it is of great importance to study the potential of p15E as a target for immunotherapy. In the present study we investigated whether p15E could be used as a target for immunotherapy in a syngeneic mouse leukemia model. Fast growing, disseminated tumors were induced in mice by i.v. injection of the Rauscher virus transformed myeloid cell line RMB-1. RMB-1 tumors express viral antigens, as was demonstrated by Berends *et al.* (11), and RMB-1 cells produce immunosuppressive factors which could interfere with the immune response against the tumor cells.
We used MAbs directed against retroviral p15E (antibody 19F8) and against the immunosuppressive (CKS-17) domain of p15E (antibody ER-IS5) to investigate the potential of p15E as a target in immunotherapy. Both 19F8 and ER-IS5 cross-react with and neutralize the circulating immunosuppressive p15E-related protein (12). However, only 19F8 binds to retroviral p15E expressed at the cell surface of RMB-1 cells. Both antibodies were compared for their effectiveness in tumor therapy. Therapy experiments were performed using alginate encapsulated hybridoma cells. Encapsulated hybridoma cells serve as a continuous source of monoclonal antibody production, after i.p. injection in (tumor bearing) mice. The present study shows that (i) monoclonal antibodies directed against the immunosuppressive protein p15E have immunotherapeutic potential in our mouse leukemia model and (ii) alginate encapsulation of hybridoma cells producing antibodies directed against tumor associated antigens can be succesfully applied in tumor therapy models in mice.

Material and methods

Cell lines

RMB-1 is a Rauscher virus transformed myeloid cell line of Balb/c origin (H-2^d) (13). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS).
The hybridoma cell lines used were 19F8 (IgG2b), producing MAbs directed against the retroviral protein p15E, which originated from a 129 mouse x NS1 fusion (14) and ER-IS5 (IgG2b) of Balb/c origin, producing MAbs directed against the immunosuppressive CKS-17 domain of p15E (12). Hybridoma 19F8 was a generous gift from Dr. RAJ Oostendorp, Free University Amsterdam. Hybridoma cell lines were cultured in RPMI 1640 containing 10% FCS, 0.05 mM β-mercaptoethanol and 40 units/ml IL-6.
The non-antibody producing myeloma cell line SP2/0 was used as a negative control cell line to exclude a possible effect of i.p. injection of alginate encapsulated

hybridoma cells on tumor growth. SP2/0 cells were cultured in RPMI 1640 containing 10% FCS. 1C5F5 (IgG2a), a hybridoma cell line of Balb/c origin (11), producing monoclonal antibodies directed against viral 50- and 29-kD (glyco)proteins present on the surface of RMB-1 cells, was used as a positive control to evaluate our method of immunotherapy using alginate encapsulated hybridoma cells.

Mice and tumor

Male and female Balb/c mice (H-2^d) were bred and kept in our own colony and used at 12-14 weeks of age. The microbial status of the mice fulfilled the standard of 'specific pathogen free V' according to the criteria of the Dutch Veterinary Inspection, as described in the law on animal experiments.

In all tumor experiments, mice were were injected i.v. with $1x10^7$ RMB-1 tumor cells, causing the rapid growth of disseminated tumors in liver and hemopoietic organs. This tumor kills the mice in 10-15 days, with a Mean Survival Time (MST) of 12 days.

Flow cytometry

Flow cytometry was performed to determine the reactivity of the MAbs with retroviral antigens on the surface of RMB-1 cells and to determine the presence of specific MAbs in mouse serum after alginate injection.

RMB-1 cells were washed twice with cold serum-free medium and were incubated with culture supernatant of hybridoma cell lines or with diluted mouse serum (1:100), both containing 20 mM NaN_3, for 10 min at room temperature. After washing the cells three times with cold buffer (PBS, 0,5% BSA, NaN_3) a fluorescein isothyocianate-labeled secundary antibody (rabbit-anti-mouse-FITC, Dako, Glostrup, Denmark) was allowed to incubate for 10 min at room temperature. Finally the cells were washed and fluorescence was recorded using a Becton Dickinson FACScan. Antibody binding to cell surfaces was analysed and mean fluorescence units were used as a measure of antibody binding. Normal mouse serum or antibody MPC11 (IgG2b, ATCC, Rockville, MD) were used as negative control antibodies.

To determine the number of RMB-1 tumor cells in mice at several time points after tumor inoculation, spleen cell populations were analysed. To obtain spleen cell preparations, mice were killed by carbon dioxide exposure and spleens were removed. Cell suspensions were prepared and cells were washed three times with cold medium containing 5% serum and counted using a Coulter counter. Two-color analysis had to be performed to be able to distinguish 1C5F5 positive RMB-1 cells from mouse B-cells, which were also stained positive by the secondary antibody rabbit anti-mouse-FITC. In these experiments we used rat anti-B-220 MAb, directed against mouse B-cells (clone RA3 6B2 (15)), followed by incubation with a PE-labeled secondary antibody (goat anti-rat-PE, Calltag S. San Francisco, CA) and 1C5F5, followed by rabbit anti-mouse-FITC. In this way FITC-labelled tumor cells could be distinguished from FITC and PE double-labelled murine B-cells.

Gel electrophoresis and immunoblotting

Gel electrophoresis and immunoblotting were performed to detect retroviral p15E reactive antibodies in mouse serum after i.p. injection of alginate encapsulated hybridoma cells. Experiments were carried out as described earlier (12) with slight modifications. Lysed MuLV particles (150 µg) were electrophoresed using a preparative comb with one reference well in the mini-PROTEAN II slab cell system (Bio-Rad, Hercules, CA). The nitrocellulose sheet was incubated overnight at 4 °C with purified control MAb (19F8 0.1 µg/ml) or with diluted mouse serum (1:100) using a Miniblotter system (Immunetics, Cambridge, MA).

Alginate encapsulation of hybridoma cells

Cultured hybridoma cells were washed four times in cold sterile saline and mixed with two parts of a sterile 1.2% alginate solution at room temperature (FMC, Vallensbaek Strand, Denmark). The mixture was transferred into a syringe equipped with a 25 G needle and squirted into a fresh prepared solution of 80 mM $CaCl_2$ (in water) at room temperature under continuous gentle mixing. Newly formed alginate capsules were washed four times with cold saline and mice were injected i.p. with a volume of 1 ml, containing $2x10^6$ hybridoma cells, using a 22 G needle. A few alginate capsules containing hybridoma cells, were cultured for several days in medium te serve as a control for sterility and antibody production.

In all our experiments, not one of the mice developed peritoneal tumors accompanied by the formation of ascites fluid, caused by outgrowth of hybridoma cells, due to breaking of the alginate capsules as was described earlier (16).

Therapy experiments

The ability of the MAbs 19F8, ER-IS5 and 1C5F5 to delay tumor growth, or cure tumor bearing animals was evaluated in several therapy experiments.

The delay in tumor cell growth in the spleen of treated *versus* control mice was determined using two-color FACScan analysis of spleen cell populations as described above. Mice were injected with encapsulated hybridoma cells two days before tumor inoculation. They were killed and spleens were taken out and analysed at 5 or 10 days after tumor inoculation.

For survival experiments, groups of 7 or 8 mice were inoculated with RMB-1 cells. In these experiments, antibody treatment with alginate encapsulated hybridoma cells was started at different time points, either 2 days before tumor inoculation, or 2 days after tumor inoculation. Also, the effect of two injections with alginate encapsulated hybridoma cells, three days before and four days after tumor inoculation, on survival was determined.

In each experiment, non-tumor-bearing control mice were injected with alginate encapsulated hybridoma cells in order to monitor the antibody titers present in the serum at consecutive days.

19F8 antibody titers were measured with Western blot, using its specificity for MuLV-

p15E. 1C5F5 antibody titers in mouse serum were measured with FACScan analysis, using its strong reactivity for RMB-1 cells.
In therapy experiments, animals were monitored daily to score their mortality. The results are expressed graphically as survivors against time and the MST was scored. Survival curves, as observed at the 60th day after inoculation of tumor cells, obtained from different groups were tested for similarity using the Mann-Witney test. Values of $p<0.05$ were considered significant.

Results

The main goal of our experiments was to investigate whether neutralization of immunosuppressive p15E could be a succesful approach in immunotherapy in cancer. To achieve this, p15E neutralizing monoclonal antibodies were used which did (19F8) or did not (ER-IS5) recognize cell surface p15E on RMB-1 tumor cells. Both antibodies allowed us to study the immunotherapeutic potential of anti-p15E antibodies by neutralization and cytotoxicity.
In our experiments we applied the monoclonal antibody 1C5F5 as a control antibody to evaluate the efficacy of the method of alginate encapsulation of hybridoma cells in immunotherapy against cancer in mice. Purified 1C5F5 has succesfully been used before in immunotherapy of mice bearing disseminated RMB-1 tumors (17). 1C5F5 has a very strong reactivity for retroviral antigens at the surface of RMB-1 cells, as is shown in Fig.1.

Reactivity of 19F8 and ER-IS5 with RMB-1 tumor target cells

To determine the reactivity of the anti-p15E MAbs with surface proteins on the tumor target cell line RMB-1, indirect immunofluorescence using FACScan analysis was performed. Culture supernatant of 19F8, reacted with surface p15E on viable RMB-1 cells (Fig. 1). This observation indicates that RMB-1 cells are in principle a suitable target for antibody mediated cytotoxicity using 19F8. ER-IS5 showed no reactivity for surface proteins on the RMB-1 cells byond the negative control MPC11 (IgG2b) (Fig. 1). Because ER-IS5 does neutralize p15E in serum (like 19F8 (12)), this antibody was included in the immunotherapy studies, to evaluate the effect of serum-p15E neutralization on tumor growth and survival.

Technical aspects of hybridoma encapsulation for immunotherapy

Encapsulation of hybridomas in alginate was performed according to the method described by Savelkoul *et al.* and Hashimoto & Shirai (16,18). Encapsulated hybridoma

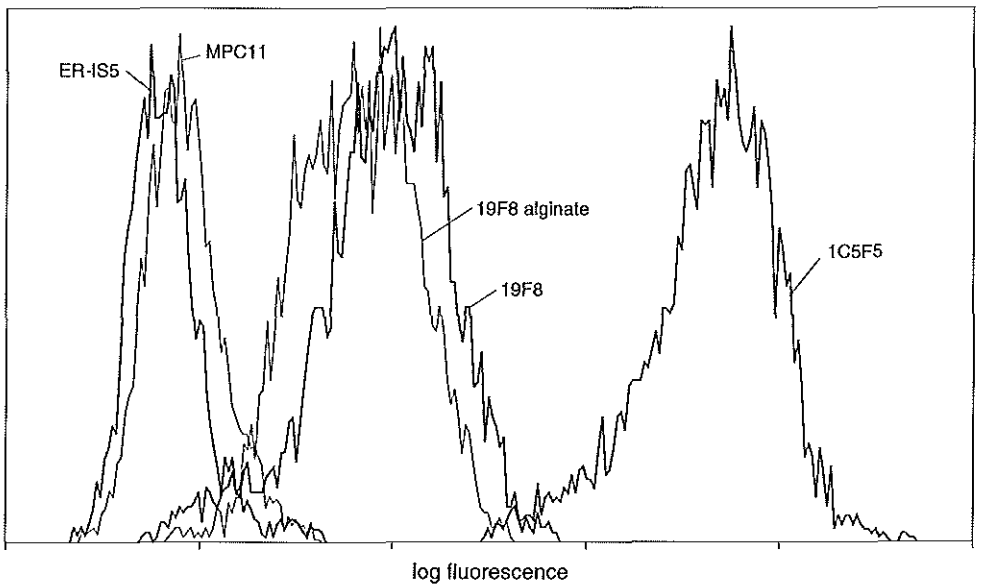

Fig. 1. Cell surface expression of 19F8, ER-IS5 and 1C5F5 antigens on RMB-1 cells. Cells were labeled with 19F8 cell culture supernatant (25 µg/ml) or with culture supernatant of 19F8 encapsulated in alginate, with ER-IS5 cell culture supernatant (20 µg/ml) or with 1C5F5 cell culture supernatant (12.5 µg/ml). Fluorescence was determined using a FACScan. *Thin line,* isotype control MPC11 supernatant.

ma cells produced MAbs *in vitro* as expected: supernatant derived from cultures of encapsulated hybridoma cells showed the same reactivity for RMB-1 cells as did supernatant from hybridoma tissue cultures. This is shown for 19F8 alginate and tissue culture supernatants in Fig. 1.

In the present study, we applied alginate encapsulation to distribute MAbs for immunotherapy in our mouse tumor model. Alginate-encapsulated antibody producing cells caused high *in vivo* antibody titers which remained constant over a period of several weeks.

To determine the rate at which specific antibodies appeared in the serum after i.p. injection of encapsulated hybridoma cells, kinetic experiments were performed. Mouse serum, taken before 19F8 alginate injection at day 0, showed no reactivity for retroviral proteins on Western blots, but already two days after alginate inoculation, 19F8 antibodies which detected p15E (19 kD) were present in the serum of alginate injected mice (Fig. 2A). The level of MAb in the serum peaked between day 5 and day 19 and then slowly declined, disappearing after 3-4 weeks. The concentration of 19F8 antibody in mouse serum was determined by comparison with the reactivity of purified MAb in a titration series. The intensity of staining of diluted (1:100) mouse serum taken 2 weeks after alginate injection was comparable to the intensity of 1 µg/ml of the purified antibody, indicating that we were able to reach serum concentrations for 19F8 of at least 100 µg/ml.

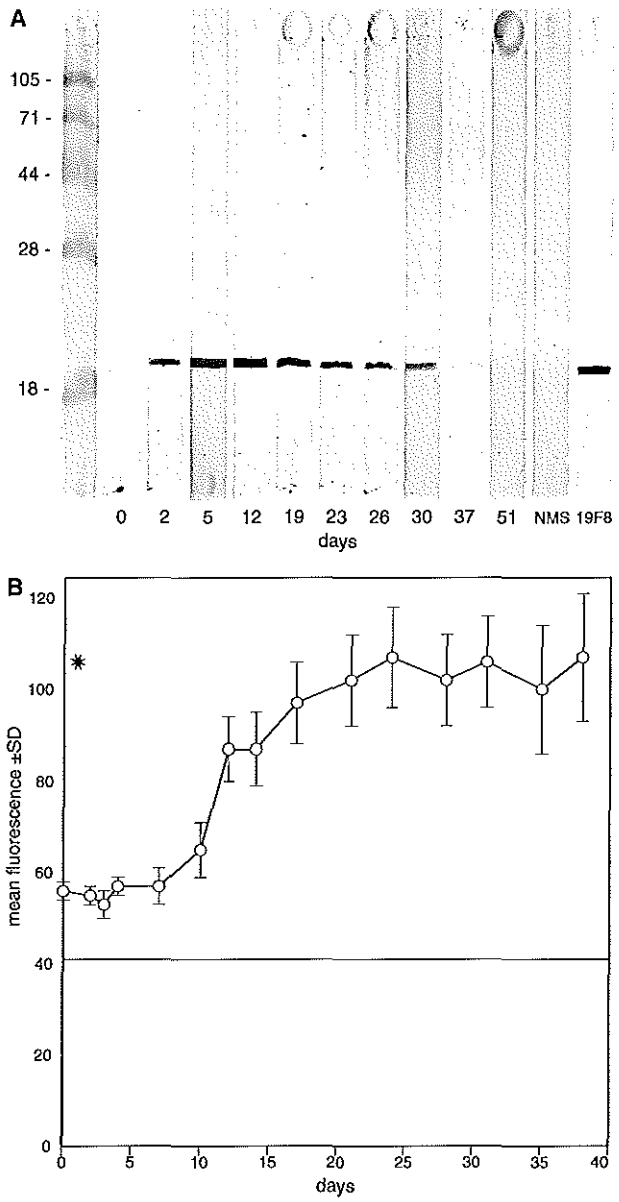

Fig. 2. Kinetics of 19F8 and 1C5F5 appearence in mouse serum after i.p. injection of $2x10^6$ hybridoma cells encapsulated in alginate. A. P15E specific 19F8 in mouse serum was detected on MuLV blot. Normal mouse serum (NMS) was used as negative control, purified 19F8 (1 µg/ml) was used as positive control. Results are from one representative mouse out of four. B. Levels of 1C5F5 expressed as mean fluorescence of RMB-1 cells incubated with diluted mouse serum (1:100). Each point represents the mean of 4 mice ± SD. *Horizontal line,* background fluorescence. *: Mean fluorescence of purified 1C5F5 (0.5 µg/ml).

Serum samples from mice injected with 1C5F5 cells encapsulated in alginate were taken and analysed for their reactivity with RMB-1 cells using flow cytometry. Control serum, taken at day 0, showed only background fluorescence (Fig. 2B). Seven days after injection of alginate, 1C5F5 titers raised above background fluorescence. After 2 weeks a maximum concentration was reached, remaining stable for at least 5 weeks. As is depicted in figure 2B, this maximum concentration corresponds to 0.5 µg/ml of the purified MAb, indicating that the concentration of 1C5F5 in serum reached values of 50 µg/ml.

Although the alginate-encapsulated hybridoma cells show different kinetics, we were able, after a single alginate injection, to detect the specific mouse MAb in mouse serum for at least three weeks, indicating that alginate encapsulation and i.p. injection is a suitable method for the administration of antibodies for our immunotherapy studies in mice.

Immunotherapy (1): comparison of tumor load in spleens of alginate-treated mice

To investigate whether treatment with alginate-encapsulated hybridoma cells interfered with tumor cell growth, we analysed the number of tumor cells in the spleens of mice treated with alginates containing antibody producing cells or non-producing SP2/0 cells.

As is shown in Fig. 3, in control mice, treated with SP2/0 cells in alginate, we observed a rapid metastasis of RMB-1 cells to the spleen: already 5 days after i.v. inoculation of $1x10^7$ RMB-1 cells, $6.5x10^7$ RMB-1 cells were detectable in the spleen. Ten days after tumor cell inoculation, the tumor cell load comprised over 65% of the total cell number in the spleen.

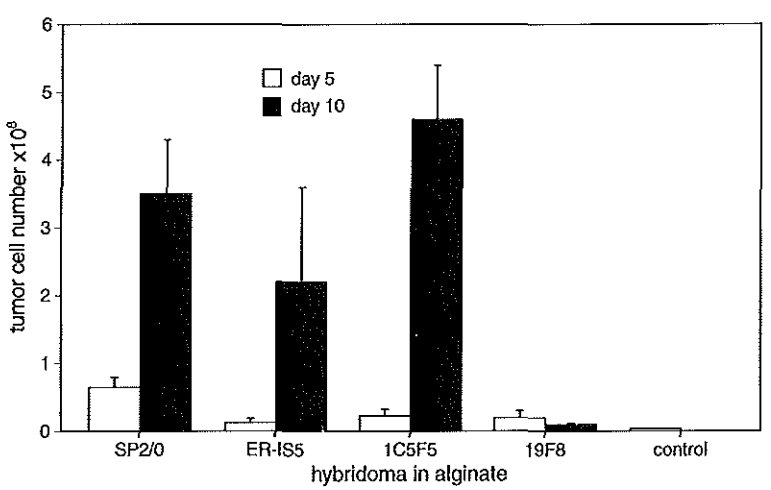

Fig. 3. RMB-1 tumor cell load in the spleen of tumor bearing mice 5 and 10 days after tumor cell inoculation. Mice were injected with alginate encapsulated hybridoma cells two days before tumor cell inoculation. Spleen cell suspensions were incubated with 1C5F5 and the number of tumor cells was determined using FACScan analysis. A non tumor bearing control mouse was used to obtain background levels. Each bar represents the mean of 4 mice ± SD.

Alginate-encapsulated hybridoma cells, producing 19F8 and ER-IS5 antibodies, both inhibited the outgrowth of tumor cells in the spleen at day 5 (Fig. 3). After 10 days, however, a difference in the effectiveness of the therapy with different hybridomas was observed. In mice treated with 19F8 we still observed a decrease in cell number as compared to control SP2/0 treated mice, but the efficacy of the therapy varied between individual animals. Whereas in some animals this therapy caused a reduction in tumor cell number, 19F8 treatment was not sufficient in others to prevent the tumor from outgrowth. In ER-IS5 treated mice, the number of tumor cells after 10 days is comparable to that of the control SP2/0 treated mice, over 65%.
Using alginate encapsulated 1C5F5 hybridoma cells, tumor cell growth was strongly inhibited, and, after 10 days only very low numbers of tumor cells could be detected. This reduction in tumor cell number illustrated the capacity of this antibody to kill RMB-1 cells, as was already shown by Berends *et al.* (17) and it proved that the method of alginate encapsulation is a reliable alternative for the repeated i.p. injections with purified MAbs.

Table 1. Effect of antibody treatment on host survival

Hybridoma	Form	Survivors	MST days	Average survival time ± SD	*p* Value treated vs control
SP2/0	alginate	0/7	12.5	12.6 ± 1.4	-
19F8	alginate	2/7	21.0	32.4 ± 19.3	0.018
19F8	purified	5/7	60.0	54.4 ± 14.7	0.007
ER-IS5	alginate	0/7	20.0	19.6 ± 3.0	0.026
1C5F5	alginate	6/7	60.0	53.6 ± 17.0	0.007

Groups of 7 mice were inoculated with RMB-1 tumor cells (at day 0) and the immunotherapeutic effect of hybridoma cells encapsulated in alginate (injected at day -3 and 4) and of purified 19F8 (injected at day 1, 4, 8 and 11) was determined. Median and average survival time were scored after 60 days.

Immunotherapy (2): survival of alginate-treated mice

Because a tumor cell growth-inhibiting effect of the MAbs *in vivo* was observed, we investigated whether the alginate encapsulated antibodies directed against p15E were able to cause a prolonged survival or even cure tumor bearing animals in survival experiments.
Alginate treatment in itself had no effect on tumor growth and survival of the animals. The survival curve of animals treated with SP2/0 cells encapsulated in alginate was similar to the survival curve of animals that received no treatment, with all mice dying within 10-15 days (Fig. 4), with a MST of 12.5 days (Table 1).
Treatment with encapsulated 19F8 hybridoma cells showed a significant prolonged survival of tumor bearing mice with a MST of 21 days (Table 1). In three combined experiments this monoclonal anti-p15E antibody was able to cure eight out of 23

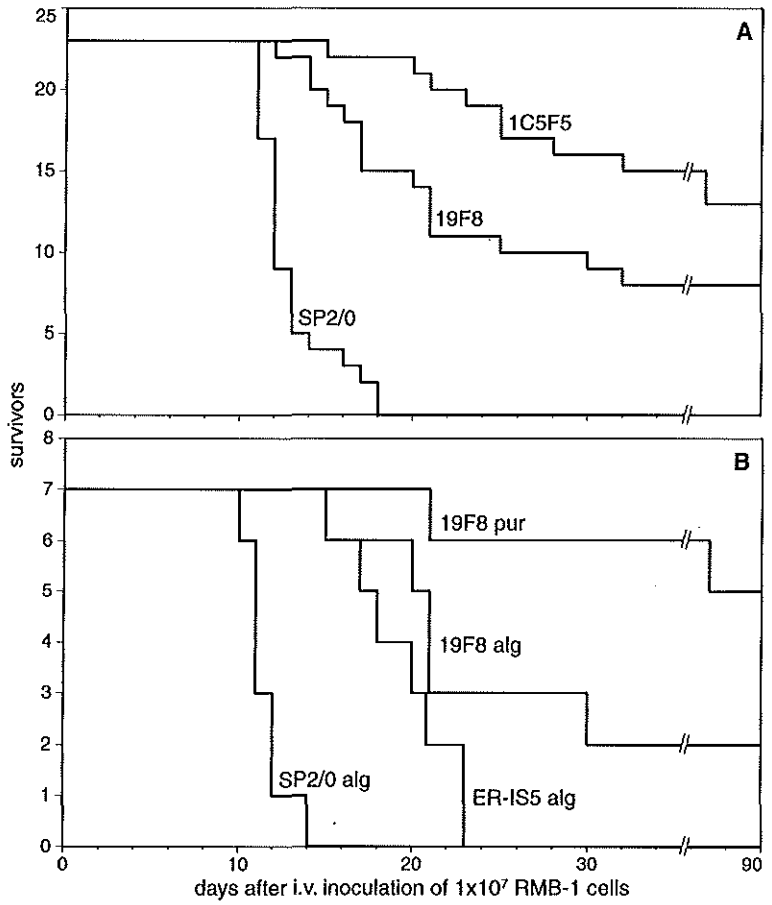

Fig. 4. Survival of RMB-1 tumor bearing mice after immunotherapy with alginate encapsulated hybridoma cells. **A.** Comparison between treatment with control alginate containing SP2/0 cells and alginate containing 19F8 or 1C5F5 hybridoma cells. Data are from three experiments. **B.** Comparison between control (SP2/0) alginate treatment and anti-p15E treatment with alginate containing 19F8 or ER-IS5 hybridoma cells and treatment with purified 19F8 antibodies (4 injections with 200 µg/ml on day 1, 4, 8 and 11 after tumor cell inoculation).

animals, with an average survival time of 30.1 ± 18.7 days ($p<0.001$) (Fig. 4A). Withthe p15E-neutralizing antibody ER-IS5, we detected a significantly prolonged survival, with a MST of 20 days (Table 1). But therapy with this MAb alone was not enough to cure any of the seven treated animals (Fig. 4B).

Treatment with a single injection of encapsulated 1C5F5 producing cells showed a significant better survival ($p<0.001$) (Fig. 4A), with a MST of 60 days (Table 1). Out of 23 animals, 13 were cured. These results are comparable to those obtained by Berends after multiple i.p. injections of 1C5F5 ascites (17).

Immunotherapy (3): comparison of alginate and purified antibody treatment
In order to compare the efficacy of alginate treatment with multiple injections of purified antibody in immunotherapy directed against p15E, we treated a group of seven mice with 19F8 hybridoma in alginate and a group of seven mice and two control mice with purified 19F8 antibody. The mice received i.p. injections with 0.2 mg of protein A purified MAb on days 1, 4, 8 and 11 after i.v. tumor inoculation. This treatment resulted in immediate high serum titers of 19F8 in the non-tumor bearing control mice, from day 1 until day 17, thereafter declining. This kinetics is comparable to the kinetics we found after one single injection with alginate encapsulated hybridoma cells.
Repeated injections of purified MAb until day 11 after tumor inoculation cured 5 out of 7 mice, with a MST of 60 days (Fig. 4B). In the alginate treated group in the same experiment two out of seven mice were cured. This difference was not significant ($p>0.05$).

Immunotherapy (4): effect of timing of alginate injection and survival
Since encapsulated antibody producing cells require several days in order to produce detectable and effective serum levels of antibody (see also Fig. 2), we investigated the effect of the timing of alginate injection in tumor-bearing mice. To this purpose, groups of mice were treated with alginate containing either 19F8 or ERIS5 2 days before or 2 days after tumor inoculation. No significant differences were found ($p>0.1$). We also found no significant improvement in the effectiveness of the therapy when we injected alginate twice, i.e. 3 days before and 4 days after tumor cell inoculation.
In contrast to anti-p15E antibodies, the variation in time of administration of 1C5F5 resulted in different MST per group. We observed that when treatment with 1C5F5 was started 2 days after tumor cell inoculation the MST of the tumor bearing animals was only 30 days, as compared to the MST of 60 days when we started alginate treatment 2 days before tumor inoculation. This could be attributed to the fact that the encapsulated 1C5F5 hybridoma cells needed a period of at least one week to give raise to detectable serum levels of antibody (see also Fig. 2).

Together, our data indicate that anti-p15E antibodies can be applied for immunotherapy in tumor-bearing mice when injected in the form of alginate-encapsulated hybridoma cells. However, permanent cure was only observed in mice treated with anti-p15E antibodies which detected p15E both at the tumor cell surface and in the serum.

Discussion

Tumors are often accompanied by immunosuppression (19). One of the tumor associated immunosuppressive factors is a retroviral p15E-related protein (4). As early as 1979, Mathes *et al.* reported a role for p15E in feline leukemia virus induced immunosuppression (3). Nelson *et al.* (1985) were the first to apply antibodies specific for p15E in immunotherapy studies. They were able to restore the depressed DTH in mice after injection with immunosuppressive products from bovine ocular squamous cell carcinoma (BOSCC) by using MAbs against p15E (20). In 1987, Thiel *et al.* demonstrated that the incidence of retrovirally induced AKR leukemia in mice could be reduced by treatment of mice with a combination of MAbs directed to the viral proteins gp70 and p15E. Where anti-gp70 was virus-neutralizing, anti-p15E antibodies were required to inactivate the immunosuppressive peptide (21). Also, the growth of a p15E expressing rat yolk sac tumor could be inhibited after therapy with the purified MAb 19F8, directed against p15E (22).

In the present study we investigated whether antibodies directed against the immunosuppressive domain of p15E and neutralizing serum p15E, could be applied in immunotherapy in a syngeneic mouse model. I.p. injected alginate encapsulated hybridoma cells were used as the source of *in vivo* antibody production. The retrovirus transformed mouse myeloid cell line RMB-1 was injected in mice to induce a fast growing (MST of 12 days), p15E-positive tumor in mice.

Our data indicate that MAbs directed against the immunosuppressive protein p15E (19F8 and ER-IS5), inhibit tumor cell growth and that a single injection of alginate encapsulated hybridoma cells is an efficient method to study the immunotherapeutic potential of MAbs in our mouse model.

The method of alginate encapsulation of secretory cells has recently been applied succesfully for long term administration of rat MAbs which neutralize IL-6 in mice (23) or for the administration of cytokines produced by cytokine-transfected cells which were encaspsulated in alginate and injected in mice (24,25). Our results confirm the notion that alginate encapsulation of hybridoma cells is a powerful technique to maintain constant high antibody titers over a period of several weeks. After i.p. injection of our different hybridoma cells encapsulated in alginate, antibody concentrations of 50-100 µg/ml could be detected in mouse serum. 19F8 antibodies appeared in mouse serum already 2 days after alginate injection, therefore, immunotherapy with this antibody could be started either before or after tumor cell inoculation. We found no beneficial effect of a second injection with hybridoma cells in alginate, indicating that antibody titers after a single injection are optimal for tumor immunotherapy in our model.

We also found no statistical difference between the treatment with 19F8 in alginate or with purified antibodies. However, the survival curves are not identical (see Fig. 4B). We feel that the lack of significance could be a consequence of the rather small groups (n=7). The immediate high serum titers at day one after injection of purified

19F8 antibodies caused an optimal immunotherapeutic effect. After injection of 19F8 in alginates, high serum titers were only obtained after 2 days. At this time point, the tumor load is already larger and therefore, therapy was not succesful in all treated mice.
The alginate encapsulated hybridoma 1C5F5 needed at least a week to raise detectable antibody titers in mouse serum. For the most optimal therapy results, 1C5F5 in alginate had to be injected in mice before tumor cell inoculation.
Serum levels of ER-IS5 could not be monitored, because this antibody did not react with antigens on RMB-1 cells and it has only a weak reactivity for retroviral p15E on Western blot. However, we know form *in vitro* alginate cultures that the antibody production of encapsulated hybridoma cells is comparable to the production in conventional hybridoma cultures (20 µg/ml).

Treatment of tumor bearing mice with a single injection of alginate-encapsulated hybridoma cells directed against retroviral p15E (19F8) or against the immunosuppressive domain of p15E (anti-CKS-17, ER-IS5), caused a significant delay in the outgrowth of tumor cells in the spleen of tumor-bearing mice. RMB-1 cells express retroviral p15E antigens on their surface, indicating that 19F8 could exert its therapeutic effect through antibody mediated cytotoxicity. In this respect, Lindvall & Sjogren, using 19F8 (IgG2b), showed that ADCC was the effector mechanism in their rat yolk sac tumor model (22).
In survival experiments encapsulated 19F8 hybridoma cells cured eight of 23 tumor bearing animals (survival over 90 days). Immunotherapy with 19F8 did not result in cure of all tumor-bearing mice. This could be explained by the observation that the number of 19F8 antigens expressed at the cell surface of RMB-1 cells is low compared to the expression of 1C5F5 antigens, and that some cells even are p15E negative (see also Fig. 1). Since 19F8 also binds circulating p15E, the amount of free antibody required for tumor cell binding and cytotoxicity may be reduced, resulting in inefficient cellular cytotoxicity.
ER-IS5 does not recognize p15E at the surface of RMB-1 tumor cells. Still, treatment with ER-IS5 caused a significant delay in the onset of disease. This effect is probably caused by the ability of ER-IS5 to neutralize p15E-related immunosuppressive factors in the circulation, thereby improving the immune system of the treated mice.
In this respect, recently performed experiments showed that neutralization of p15E in tumor bearing mice caused an improvement of the humoral IgE immune response of these tumor bearing mice against TNP-KLH: the suppressed secundary immune-response against TNP-KLH in tumor bearing mice was improved after treatment with anti-p15E antibodies (Lang & Savelkoul, unpublished results).

The importance of CKS-17 as target epitope for immunotherapy, directed against the immunosuppressive protein p15E, is based on the next observations. First, Nelson *et al.* described that the CKS-17 epitope could be held responsible for the immuno-

suppressive effects of p15E-related tumor products (26). And second, in a study from our group in we have shown that 19F8 is directed against a linear epitope within the immunosuppressive CKS-17 domain (27). Our results using CKS-17 specific and neutralizing antibodies provide more evidence for the importance of CKS-17 as a functional epitope in tumor associated immunosuppression.

We feel that immunotherapy directed against the immunosuppressive domain of p15E could be used in addition to conventional therapy, like surgery or radiotherapy, in the therapy of early detected, small recurrent tumors, or to improve the results of immunotherapy protocols, which, until now have had limited succes, possibly because of the immunosuppressed state of the patient.
A combination of mMAbs blocking the immunosuppressive epitope of proteins like p15E and recognizing tumor cell surface antigens should be used in order to obtain maximal immunotherapy. In this respect, squamous cell carcinoma of the head and neck offers an interesting field of research, because these tumors are associated with p15E-mediated immunosuppression (5,9). Besides, for this group of patients, several functional monoclonal antibodies directed against tumor associated surface antigens have already been developed (12,28,29). Studies in relevant animal models (nude, SCID or RAG -\-) using a combination of neutralizing and tumor cell specific antibodies together with relevant human tumor cell lines, could provide more insight into more succesfull immunotherapy protocols for patients with (head and neck) cancer.

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9

Discussion

Discussion

Malignant tumors, especially those of the head and neck are associated with immunosuppression. P15E-related proteins have been shown to play a role in this tumor-associated immunosuppression.
In this thesis I have described the production of a new panel of monoclonal antibodies (MAbs: ER-IS1, ER-IS2 and ER-IS5), directed against the putative immunosuppressive region of p15E (CKS-17). Using these antibodies we were able to demonstrate the presence of proteins related to the putative immunosuppressive region of p15E in sera of patients with head and neck cancer, and in the tumor tissues of these patients. Moreover, we have studied the distribution of these p15E-related proteins in a large panel of malignant tissues. Our studies indicate that the majority of these tumors contain p15E-related proteins. We detected only a weak expression of p15E-related proteins in a minority of normal tissues. Therefore, we conclude that p15E-related proteins, containing the immunosuppressive CKS-17 epitope are expressed in malignant tissues, especially those of squamous cell carcinoma of the head and neck.
Furthermore, our monoclonal antibodies were used in an attempt to develop a sensitive sandwich ELISA for the detection of p15E-related proteins in human sera. Unfortunately, we were not able to reach a sensitivity in the pg range, a sensitivity required for accurate detection of p15E-related proteins. Moreover, we found that serum blocking factors also hampered the use of the sandwich ELISA.
Finally, the immunotherapeutic potential of monoclonal antibody ER-IS5 was tested in an animal model. Mice inoculated with a p15E-expressing tumor cell line were treated with either 19F8, directed against retroviral p15E, or with ER-IS5, directed against CKS-17. Immunotherapy of tumor bearing mice with these antibodies resulted in a significant longer survival. These experiments indicate that immunotherapy directed against p15E-related proteins could be a promising new strategy in the battle against human cancer.

In this discussion, the nature and origin of the p15E-related proteins is reviewed, as well as their presence in human tissues and sera. Furthermore, we discuss the use of the new monoclonal antibodies directed against the immunosuppressive domain of p15E in further characterization of the protein. Finally, we discuss the clinical applicability of p15E and MAbs against p15E in patients with head and neck cancer.

What is the exact nature of p15E-related proteins?

The relationship of p15E-related proteins to the retroviral protein p15E, as well as the relationship to other immunosuppressive proteins is at present still not clear. In our experimental work we have produced a set of three new monoclonal antibodies, directed against the immunosuppressive site of p15E, represented by the synthetic

peptide CKS-17 (see Chapter 5). These antibodies did recognize the retroviral immunosuppressive protein p15E. In immunocytochemical studies with human tumor cell lines and in immunohistology of squamous cell carcinoma tissues, human p15E-related proteins were also recognized. The functional reactivity of the monoclonal antibodies with the immunosuppressive epitope of the p15E-related proteins was illustrated by the ability of the antibodies to decrease the inhibition of monocyte polarization by sera from patients with head and neck cancer. In this assay, patient sera inhibit the polarization of healthy monocytes. Antibodies directed against the immunosuppressive epitope of p15E neutralize this inhibitory potential. Altogether, our data have proven the presence of a conserved immunosuppressive site of retroviral p15E, represented by CKS-17, on immunosuppressive proteins associated with head and neck cancer.

However, our experiments did not solve the exact nature of the p15E-related proteins. Purification of the p15E-related proteins from patient sera or head and neck tumor tissue is required for further biochemical characterization. Unfortunately, although tried by several people in our group, this approach has not yet been successful. The low concentration of the protein in head and neck cancer patient sera is a major obstacle in such studies (see also Chapter 7). Affinity purification of p15E-related proteins from human tumor tissue should be possible, using the now available panel of antibodies and is an absolute requirement to elucidate the nature of p15E-related proteins.

Interestingly, regions of homology between CKS-17 and TGFβ (1) and IFNα (2) have been reported. Synthetic peptides, corresponding to these p15E-homologous regions of TGFβ and IFNα also inhibit lymphocyte proliferation. These results show that several immunosuppressive proteins contain regions with homology to the highly conserved CKS-17 epitope. However, experiments using our monoclonal antibodies directed against CKS-17 in combination with antibodies directed against IFNα have demonstrated that the head and neck cancer associated p15E-related factor and IFNα do not represent the same protein. Where the anti CKS-17 antibody ER-IS1 cross-reacted with recombinant human IFNα, the anti IFNα antibodies did not cross react with murine p15E. Both types of antibodies were able to adsorb the inhibitory effect of low molecular weight factors in sera of patients with SCC-HN. However, no correlation could be found between the adsorption capabilities of the antibodies. Therefore it is possible that the low molecular weight factors contain both p15E- and IFNα-related factors. Moreover, immunohistochemistry of head and neck squamous cell carcinoma specimens showed a different staining pattern for anti IFNα antibodies when compared to anti p15E or anti CKS-17 antibodies (3). From these results it can be concluded that the conserved CKS-17 epitope is present on several immunosuppressive proteins. And, more importantly, that the SCC-HN associated p15E-related factor is an immunosuppressive factor, not identical to presently known immunosuppressive proteins.

What is the origin of p15E-related proteins?

Since p15E-related proteins have been detected in tumor cell lines, which do not contain any other viral proteins, the origin of p15E-related proteins could well be from integrated retroviral sequences. Several human endogenous retroviral (HERV) sequences have been described (4-6). Because most of these sequences contain multiple stop codons, point deletions or frameshifts it was generally thought to be unlikely that these sequences could be expressed as proteins. However, retroviral *env*-gene mRNA is transcribed abundantly in human placenta and in U937 cell lines, as was demonstrated for the ERV3 provirus (HERV-R) (7-9). Also, mRNA from the provirus clone 4-1 (HERV-E), is expressed in placenta and in mamma and colon carcinoma cell lines (10). Several proviral ERV9 related sequences were transcribed in normal human brain and kidney tissues as well as in lymphocytes, brain tumor tissue, cultured embryonic lung cells and rhabdomyosarcoma cells. A number of these sequences lack the ERV9 stop codon present in the published ERV9 sequence (6). Therefore, the authors suggest that these sequences might encode functional immunosuppressive proteins, related to p15E (11).
In 1995, Venables *et al.* have performed experiments demonstrating that the ERV3 protein is expressed in human placenta. After cloning and expression of the transmembrane region of the gene, specific polyclonal antibodies were generated. These antibodies recognized a 65-kDa protein in syncytiotrophoblast cells. This proteins was present at high concentration (about 0.1% of the cellular protein). The similarity of the transmembrane region of ERV3 to the immunosuppressive epitope of p15E and to CKS-17 (see Table 1) suggests an immunosuppressive role for the ERV3 protein, probably protecting the fetus from immune attack by the mother, although this idea remains speculative (12).
A similar approach of developing polyclonal antibodies to an endogenous retroviral sequence was used by Turbeville *et al.* in 1996. A rabbit polyclonal antibody was developed against the most conserved sequence of the transmembrane region of clone 4-1 (HERV-E). This sequence shows 72% homology with the same region of ERV3 (HERV-R) (Table 1). The polyclonal antibody was tested in immunohistochemistry. Specific staining was observed in colon and prostate adenocarcinoma and in breast cancer. Also, a positive signal was seen in keratinocytes and in cells around the germinal center of tonsil. On Western blots of colon cell line lysates, a 58-kDa protein was recognized (13).

The 65-kDa ERV3-protein and the 58 kDa protein from clone 4-1 provirus are speculated to be unprocessed *env* proteins, containing the SU and TM sequence. Although this was questioned by Turbeville *et al.*, as 58-kDa is smaller than would be expected for the combined *env* products (14). In 1985, Jacquemin *et al.* reported the presence of a p15E-related glycoprotein of 74-kDa in sera from patients with hematopoietic disorders (15).

Table 1. Amino acid sequences (one letter amino acid codes) for the conserved regions of retrovirus transmembrane envelope proteins, the synthetic peptide CKS-17 and the homologous *env*-region of endogenous retroviruses (HERV-E = clone 4-1, HERV-R = ERV3).

Virus	Protein	Amino acid sequence
MuLV	p15E	L Q N R R G L D L L F L K E G G L C A A L K E E C C F
FeLV	p15E	L Q N R R G L D I L F L Q E G G L C A A L K E E C C F
HTLV-I	gp21	L Q N R R G L D L L F W E Q G G L C K A L Q E Q C R F
HTLV-II	gp21	A Q N R R G L D L L F W E Q G G L C K A I Q E Q C C F
HIV	gp41	A Q A R I L A V E R Y L K D Q Q L
HIV	nef	M T Y K A A I D L S H L K E G G L
synthetic peptide		
CKS-17		L Q N R R G L D L L F L K E G G L
endogenous retroviruses		
HERV-E		Y Q N R L A L D Y L L A A E G G V C G K F N L T N Y C
HERV-R		Y Q N R L A L D Y L L A Q E E G V C G K F S L T N C C

Taken together these proteins (58-kDa, 65-kDa and 74-kDa) could all represent unprocessed endogenous retroviral sequences, containing the putative immunosuppressive domain of p15E.
These experiments support the idea that proviral sequences can give rise to functional immunosuppressive proteins. Such proteins regulate the normal immune homeostasis and are responsible for the dysfunction of the immune system in patients with cancer.

Which types of tissue contain p15E-related proteins?

The presence of p15E-related proteins in head and neck cancer tissue, patient sera (16) and urine and the presence of p15E-related proteins in effusions of patients with melanoma, colon carcinoma or ovarian carcinoma (17) and in sera of patients with breast cancer (18), combined with the presence of p15E-mRNA in colon and gastric cancer (19) and the presence of human envelope proteins derived from HERV sequences in colon, prostate and breast cancer (13,14) indicates a wide distribution of p15E-related proteins in different types of human cancer. This was confirmed by our immunohistology results (see Chapter 6) , showing the presence of p15E-related proteins not only in SCC of the esophagus, lung, cervix and skin, but also in several adenocarcinomas (of breast, colon, lung and ovary).
Interestingly, p15E-related immunosuppressive factors have also been detected in sera of patients with chronic upper airway infections (20,21), Grave's thyreoiditis

(22), type 1 Insulin-Dependent Diabetes Mellitus and Sjögren's syndrome (23). These diseases are associated with immune hyperresponsiveness or autoimmune reactions. Whether the retroviral p15E-related proteins, associated with these diseases reflect a retroviral infection or an endogenously encoded negative response to immune reactivity still has to be determined. Evidence exists that retroviruses are involved in the pathogenesis of these diseases (24). On the other hand, p15E-related proteins have also been detected in normal nasal mucosa (25) and in sera of healthy controls (23). Although the level of p15E was not significant, its presence strongly suggests a physiologic role of p15E-related factors in the regulation of the immune response. A role for endogenous retroviral p15E-related sequences in the normal immune homeostasis in mice was illustrated by Fiegl *et al.* (26,27). The authors investigated glucocorticoid-mediated immunosuppression. They showed that hydrocortisone enhances the expression of p15E in mouse immune cells. Thus, it was suggested that up-regulation of immunosuppressive endogenous retroviral elements could contribute to glucocorticoid-mediated immunosuppression. As mitogen stimulation of human mononuclear cells, leukocytes and cell lines also results in expression of p15E-related antigens (28,29), a role for p15E-related sequences in the normal immune homeostasis seems likely.
In the malignant situation, significantly higher concentrations of immunosuppressive p15E-related proteins are produced by tumor cells, giving the tumor the advantage of a local suppression of the immune system. As a consequence, the expression of p15E-related proteins may allow transformed cells to escape immune destruction (30,31).

Can p15E-related proteins be used as biomarker for head and neck cancer?

Although the exact nature of p15E-related proteins is still unclear, the wide distribution and high expression in different types of cancer and especially head and neck cancer, could make these proteins important biomarkers for early tumor detection.
In this respect, the results obtained by Tas *et al.* and others, showing the disappearance of p15E-related proteins in patients with head and neck cancer after surgical removal of the tumor and their re-appearance in patients with recurrent tumor are of interest (32,33). Early detection of (recurrent) tumors is of paramount importance for a successful treatment of these patients (see also chapter 3).
Preliminary results of our immunohistology study have shown that p15E in esophagus cancer specimens was indicative for a worse prognosis. Although the relationship between the presence of p15E-related proteins and tumor prognosis has to be studied in larger patient groups, our preliminary results again emphasize the importance of p15E as a prognostic tool.
As a consequence, a sensitive and simple diagnostic test to detect the protein in human sera is required. We attempted to developed such a test (ELISA, see Chapter 7).

Unfortunately, using the available monoclonal antibodies and the standard sandwich ELISA technique, we have not been able to develop a test with the required sensitivity in the pg range. Another obstacle in the detection of p15E-related proteins in human sera appeared to be binding of p15E to blocking factors. The nature of these blocking factors is at present unclear, but they could be complement factor C1 (34) or p15E-receptor molecules (35). As the complex could still be detected by Western-blotting, using the antibody 19F8, detection of p15E or p15E-complex is still possible. Preliminary experiments of our group have shown that p15E can be detected in the pg range with a single antibody, using an immuno-PCR technique. Therefore, the detection of p15E-related factors in patient sera, using new sensitive techniques like immuno-PCR should be further investigated.

Since standard therapeutic approaches (surgery, radiotherapy and chemotherapy) have not lead to a significant improvement of survival for head and neck cancer patients in the last years, research tends to focus on markers to predict therapy responses. Using these biomarkers, patients can be monitored and the best therapeutic approach per patient can be chosen. This strategy becomes important with increasing numbers of possible therapies or combination therapies and the knowledge that individual patients show different responses to different therapeutic approaches. Valuable time can be lost if patients do not respond to a certain therapy. Predictors of response can be helpful in selecting the therapy. Whether there is a role for p15E-related proteins in this area needs to be further explored. To this purpose, an easy and sensitive detection system of the protein is a prerequisite. This again illustrates the necessity of new detection systems for p15E-related proteins.

Do antibodies directed against p15E have immunotherapeutic potential?

Animal studies have shown the possibilities of p15E as target in immunotherapy (36-39). Linvall *et al.* demonstrated that the anti-p15E antibody 19F8 was able to inhibit the growth of rat yolk sac tumors in a rat model. Interestingly, T-cells, specific for yolk sac tumor antigens appeared in draining lymph nodes of tumor free animals after treatment. The presence of these T-cells indicates that growth inhibition was not only due to a direct effect of the antibody on the tumor cells (through ADCC), but also to an activation of T-cells, directed against tumor-associated antigens.
The therapeutic potential of antibodies directed against p15E and more specific, against CKS-17, has been illustrated in our animal model using a p15E-positive tumor and the monoclonal antibodies 19F8 and ER-IS5 (see Chapter 8). Our results demonstrate that immunotherapy using antibody 19F8 resulted in a tumor free survival of 8 out of 23 treated mice. MAb 19F8 neutralizes circulating p15E and recognizes p15E expressed on the tumor cells. Our antibody ER-IS5 only neutralizes circulating p15E, hence immunotherapy using ER-IS5 did not result in complete cure of tumor bearing mice. However, mice treated with ER-IS5 survived significantly

longer after treatment with this antibody Therefore, we could conclude that immunotherapy, directed at neutralization of immunosuppressive p15E-related proteins can result in a significant inhibition of tumor growth. The different results using 19F8 and ER-IS5 are illustrative for the possibilities of p15E as target in immunotherapy. Recognition of tumor cells expressing p15E results in ADCC. Neutralization of serum p15E restores the immune system, resulting in a tumor specific immune response.

Future directions for head and neck cancer research

Head and neck cancer research has lead to a better understanding of molecular carcinogenesis and the biology of head and neck cancers. Tumor staging according to size and location (TNM-classification) can now be expanded using tumor biology, resulting in the biologic staging of head and neck cancer (40) (Table 2).

Table 2. Biologic staging of head and neck cancer (40)

Biologic stage	Definition	Stage duration	TNM-equivalent
I	intraepithelial neoplasia	30 years[1]	no equivalent
II	basement membrane invasion (without metastases)	6-18 months	stage I-III
III	metastases	6-24 months	stage III, IV
IV	therapeutically refractory disease	6 months	no equivalent

[1]Stage durations represent crude estimates and do not reflect probable marked intertumor variability. Duration estimates are made to reinforce the notion that stage I represents the greatest part of the natural history of head and neck cancer.

In the first stage, carcinogenic events take place, resulting in the expression of mutated proteins like p53-protein and over-expression of cellular proteins, possibly also p15E-related proteins. Mutations of the tumor suppressor gene *p53* have been identified in about 60% of patients with SCC-HN (41-43). Immunohistology using antibodies directed against the mutated protein can be used in the detection of premalignant lesions (44). Furthermore, serum antibodies directed against mutated p53 are reported to be prognostic indicators in head and neck cancer (45-46). Detection of biomarkers like mutated p53 protein and possibly p15E-related proteins will become useful tools in predicting tumor prognosis, in early tumor detection and in predicting responses to therapy (47-48).

In the second and third stage, immunosuppression is a major problem. P15E-related proteins, prostaglandins and IL-10 cause immunosuppression in patients with head and neck cancer (49-51). The immune system is no longer able to recognize the tumor and to destruct the neoplastic cells. Immunotherapy using IL-2 has been applied in patients with inoperable disease or unresponsiveness to conventional treatments (52-55). However, few clinical responses have been observed. As head and neck cancer associated antigens have been detected (56,57) immunotherapy, aimed at recognition of head and neck cancer cells, combined with immunostimulation should be investigated. The results of our animal model using 19F8, directed against cell surface bound p15E, and neutralizing circulating p15E have shown that this combined approach can be successful.
As mortality from head and neck cancer today is governed by uncontrolled metastatic disease, an important role of immunotherapy may well be as an adjuvant to standard treatment (surgery, radiation and chemotherapy). In this respect, the experiments performed by van Dongen *et al.* are of great interest. Radiolabelled monoclonal antibodies directed against SCC-HN antigens (MAbs E48 and U36) have been used in phase I/II trials in patients with SCC-HN. The antibodies are selectively targeted to local minimal residual disease or distant metastases. The potential of immunotherapy using these radiolabelled MAbs in patients at high risk of developing distant metastases or locoregional recurrences is presently under investigation (58).

Stage four disease is characterized by the outgrowth of a distinct population of malignant cells, resistant to therapy (59). These can be cells with a high number of mutations in oncogenes or tumor suppressor genes. For these tumors, the possibilities of gene therapy, aimed at transmission of the non-mutated wild-type gene are now being investigated (60).
Therapy resistant stage four disease can also be characterized using the available prognostic markers like prostaglandins, or growth factors like the epidermal growth factor (EGF) or transforming growth factors (TGF). The detection of p15E-related immunosuppressive proteins can also provide information about the biologic behavior of the tumor and should be applied when a sensitive detection system becomes available.

In conclusion, the ability of squamous cell carcinomas of the head and neck to produce p15E-related factors influencing the immune system is one of the most intriguing phenomena in tumor biology and needs to be further explored in the future.

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Summary

Summary

Cancer is the second cause of death in the Western World. If cancer is diagnosed at an early stage, the patient will have the most benefit of therapy and may even be cured. Cancer therapy consists at present of surgery, radiation therapy or chemotherapy. The therapy of choice depends on the type of tumor and the tumor stage. New, more effective combinations of these therapies are constantly being investigated in clinical research.
Moreover, new therapies come into view as we gain more knowledge of the molecular mechanisms responsible for the transformation of a normal cell into a malignant cell and of the biological behavior of tumor cells.

In **Chapters 1 to 4** of this thesis, malignant tumors and their effect on the immune system are introduced. In particular, head and neck cancer and immunosuppression caused by p15E related proteins, produced by this type of tumor are discussed.

In **Chapter 2**, interactions between cancer and the immune system are described. Cells that have been transformed into malignant cells may be recognized by cells from the immune system and may be killed after activation of immune cells. However, if transformed cells are not recognized by the immune system, or if the immune system cannot be properly activated to kill the malignant cells, these transformed cells will grow out to give rise to a malignant tumor. In addition, transformed cells are able to suppress the body defense systems. Tumor cells are able to produce a variety of factors which either suppress lymphocyte, macrophage or NK-cell functions.

In **Chapter 3**, the etiology and therapy of head and neck cancer is introduced. Moreover, in this chapter, interactions of head and neck cancer with the immune system are further specified.
Head and neck cancer represent malignancies of the upper aerodigestive tract (mouth, throat and voice box). Significant etiologic factors are heavy smoking and excessive alcohol drinking. At present, surgery and radiation therapy are mostly applied as therapy. Chemotherapy is applied for patients with recurrent cancer or distant metastases. Although combinations of these therapies have lead to new therapeutic strategies, the chances of survival for patients with head and neck cancer have not really improved over the past years.
Head and neck cancer is well known for its immunosuppressive influence. T-cell-, macrophage- and NK-cell proliferation and activation are suppressed in patients with head and neck cancer. Several immunosuppressive proteins play a key role in this tumor induced immunosuppression. One of the major immunosuppressive factors in head and neck cancer is the immunosuppressive protein p15E.

In **Chapter 4**, p15E and its role in immunosuppression are introduced. P15E is a retroviral immunosuppressive protein. P15E-induced immunosuppression consists mainly of suppression of B- and T-lymphocyte proliferation, a decreased production of immunostimulatory molecules like IL-2, IFN-α and IFN-γ and a suppression of NK-cell and macrophage activation. As these cells of the immune system play a central defensive role against tumor cells, suppression of these cells will result in a growth advantage for tumor cells.
P15E-related proteins, that is proteins of low molecular weight, sharing the immunosuppressive properties of viral p15E and homologous to viral p15E have been detected in sera of patients with head and neck cancer and in tumor tissue.
Studies on the retroviral p15E protein revealed a conserved domain of 26 amino acids, responsible for the immunosuppressive activities. A synthetic peptide, consisting of 17 amino acids of this conserved domain, CKS-17, was also immunosuppressive. Therefore, this peptide has been used by several groups to study p15E-mediated immunosuppression. In our experiments, aimed at studying head and neck cancer related immunosuppression, we also applied this peptide.

In **Chapter 5**, we describe the production of a new panel of monoclonal antibodies, directed against the synthetic peptide CKS-17. Mice were immunized with the peptide and monoclonal antibody producing hybridoma cells were produced after fusion of mouse cells with an immortalized cell line. The three monoclonal antibodies that were generated (ER-IS1, ER-IS2, ER-IS5), recognize the synthetic peptide and the retroviral p15E. Moreover, the antibodies react with human tumor cell lines and with head and neck cancer tissues. Furthermore, the antibodies block the functional immunosuppressive activity of p15E-related factors in sera from patients with head and neck cancer. Therefore, we could conclude that the CKS-17 epitope of viral p15E is present and functionally active on the immunosuppressive p15E-related proteins, and that our antibodies are able to block at least part of the immunosuppression in patients with head and neck cancer.

In **Chapter 6**, the presence of immunosuppressive p15E-related proteins in several types of human cancer was investigated. Using the CKS-17 specific monoclonal antibody ER-IS5, we detected p15E-related proteins in 89% of the squamous cell carcinomas of the head and neck we studied. Interestingly, a positive reaction was also found in carcinoma of the esophagus, lung, cervix and skin, in adenocarcinoma of the breast, colon, lung and ovary and in malignant melanoma. Therefore, we conclude that immunosuppressive proteins related to the retroviral p15E protein are not restricted to head and neck cancer. The presence of retroviral p15E-related proteins in other types of carcinoma suggests a role for p15E in tumor-induced immunosuppression in other types of cancer. Since we also detected a weak expression in normal cells, it could well be that p15E-related proteins also play a role in the normal regulation of the immune response.

In **Chapter 7**, the new monoclonal antibodies were used to develop a sensitive assay for the detection of p15E-related proteins in human serum. As p15E can be used as a tumor marker, predicting the presence of a tumor, a recurrent tumor, or tumor metastasis, a sensitive and quantitative assay is required in the clinical immunology laboratory.
Using combinations of several polyclonal and monoclonal antibodies, directed against p15E or CKS-17 and the sandwich ELISA technique, we attempted to develop this assay. Unfortunately, even the best combination of antibodies (19F8 and 4F5) did not result in an assay with the required sensitivity in the pg range. Moreover, the presence of blocking proteins in human serum hampered detection of p15E. By Western blotting, we detected serum proteins which bind to p15E, thereby preventing the detection of the protein in human serum using a sandwich ELISA assay. Other techniques, like the new immuno-PCR and new tools (e.g. phage antibodies) have to be developed in order to be able to detect the tumor associated p15E-related proteins in a quantitative and sensitive way.

In **Chapter 8**, we investigated the possibilities of immunotherapy directed against CKS-17 in a mouse model. Mice were inoculated with the Rauscher virus-transformed myeloid cell line RMB-1. Without intervention, the outgrowth of the tumor cells results in the death of the mice within 10-15 days. Monoclonal antibodies 19F8 and ER-IS5 were used in immunotherapy. Encapsulation of antibody producing hybridoma cells in alginate, and intraperitoneal injection of alginates was used in our study to generate a source of antibody production within the mice. A single injection of mice with 19F8 hybridoma cells encapsulated in alginate resulted in a mean survival time of 21 days and even cured 2 out of 7 mice. A single injection of mice with ER-IS5 hybridoma cells encapsulated in alginate resulted in a mean survival time of 20 days. This difference between 19F8 and ER-IS5 can be explained by the fact that 19F8 is able to recognize p15E at the cell surface and is able to neutralize circulating immunosuppressive p15E. In contrast, ER-IS5 only neutralizes circulating p15E. Therefore, 19F8 both kills tumor cells and prevents immunosuppression, whereas ER-IS5 only prevents immunosuppression. Both mechanisms have shown to improve survival of tumor bearing mice. Therefore, we suggest that future therapeutic strategies should be aimed at using MAbs able to recognize tumor associated antigens at the cell surface, resulting in antibody dependent cellular toxicity (ADCC), combined with the use of MAbs able to neutralize immunosuppression.

In **Chapter 9**, the general discussion, we review the results of our experimental work.
The exact nature of the p15E-related protein, as well as its origin are not yet known. However, we do know that the immunosuppressive epitope of CKS-17 is conserved among p15E of several retroviruses, is present on human immunosuppressive proteins of low molecular weight (p15E-related proteins) and also on the immunosuppressive proteins IFN-α and TGF-β. The origin of p15E-related proteins could be endogenous

retroviral sequences. Recent results have shown that such proviruses indeed result in the production of proteins in human cancer cells.

Since p15E-related proteins are highly expressed in several types of human cancer, and p15E-related immunosuppression disappeared after surgical removal of the tumor, the presence of p15E-related proteins can be an indication for the presence of a tumor. A prerequisite to investigate this is a sensitive detection system, able to measure p15E concentrations under various experimental and clinical conditions. P15E-related proteins can also be targets for immunotherapy. However, further studies in various experimental animal models are required before p15E immunotherapy can be applied in the clinic.

Samenvatting

Samenvatting

Kanker is de tweede doodsoorzaak in de Westerse Wereld. Wanneer kanker in een vroeg stadium wordt gediagnostiseerd, heeft de patiënt de meeste baat bij behandeling en is de kans op genezing het grootst. De behandeling van kanker bestaat uit chirurgisch verwijderen van de tumor, bestralen of chemotherapie. De keuze van de behandeling hangt af van het type tumor en van het stadium (de grootte van de tumor en/of de aanwezigheid van uitzaaiingen). Naar nieuwe en effectievere combinaties van deze behandelingsvormen wordt constant gezocht in klinisch onderzoek. Bovendien komen nieuwe behandelingsvormen in zicht, wanneer we meer kennis verzamelen over de moleculaire mechanismen welke verantwoordelijk zijn voor de verandering van een normale cel in een kankercel en over het biologisch gedrag van tumor cellen.

In de **Hoofdstukken 1 tot en met 4** van dit proefschrift worden kwaadaardige tumoren en hun invloed op het afweersysteem geïntroduceerd. In het bijzonder worden hoofd- halstumoren beschreven en wordt de remming van het afweersysteem, veroorzaakt door p15E-achtige eiwitten, die door dit type tumoren worden geproduceerd, geïntroduceerd.

In **Hoofdstuk 2** worden de interacties tussen kanker en het afweersysteem beschreven. Cellen die zijn veranderd in kwaadaardige cellen kunen door cellen van het afweersysteem herkend worden en kunnen worden gedood door geactiveerde cellen van het afweersysteem. Echter, wanneer de veranderde cellen niet worden herkend, of wanneer het afweersysteem niet goed geactiveerd kan worden, zullen de veranderde cellen uitgroeien en tot het ontstaan van een kwaadaardige tumor leiden.
De veranderde cellen zijn bovendien in staat het afweersysteem te remmen. Tumorcellen kunnen verschillende factoren produceren, die de functie van lymfocyten, macrofagen of killer cellen (de cellen van het afweersysteem, die in staat zijn tumorcellen te herkennen en te doden) remmen.

In **Hoofdstuk 3** worden de oorzaken van het ontstaan en de behandeling van hoofdhalskanker geïntroduceerd. Bovendien worden in dit hoofdstuk de interacties tussen hoofd- halstumoren en het afweersysteem verder uitgewerkt.
Hoofd- halskanker is de omschrijving van kwaadaardige aandoeningen van de bovenste lucht- en voedingswegen (mond, keel en stembanden). Duidelijke oorzakelijke factoren zijn zwaar roken en overmatig alcohol gebruik.
Tegenwoordig zijn operatie en bestraling de meest toegepaste behandelingsvormen. Chemotherapie wordt toegepast bij patiënten met een terugkerende tumor, of bij uitzaaiingen op afstand. Ondanks het feit dat combinaties van deze behandelingsvormen geleid hebben tot nieuwe therapieën, is de overlevingskans van patiënten met hoofd- halskanker in de afgelopen jaren nauwelijks of niet verbeterd.

Hoofd- halskanker staat bekend om de remmende invloed van de tumor op het afweersysteem. Cellen van het afweersysteem (T-cellen, macrofagen en killer cellen) worden geremd in hun groei en aktivatie. Een aantal immunosuppressieve eiwitten (eiwitten die het afweersysteem remmen) speelt hierbij een belangrijke rol. Een van de belangrijkste eiwitten bij hoofd- halskanker in dit verband, is het immunosuppressieve eiwit p15E.

In **Hoofdstuk 4** worden p15E en de rol hiervan bij het onderdrukken van het afweersysteem beschreven. P15E is een retroviraal immunosuppressief eiwit. De door p15E geïnduceerde immunosuppressie bestaat vooral uit remming van de groei van B- en T-cellen, een verminderde productie van afweer stimulerende stoffen zoals Interleukine-2, Interferon-α en Interferon-γ en remming van de activiteit van killer cellen en macrofagen. Aangezien al deze cellen een belangrijke rol spelen bij de afweer tegen tumorcellen, leidt remming van de cellen tot verdere groei van de tumor.
Eiwitten die lijken op p15E, dat wil zeggen met een laag molecuulgewicht, met afweer onderdrukkende eigenschappen en verwant aan het retrovirale p15E, zijn gevonden in het serum van patiënten met hoofd- halskanker en in tumorweefsel van deze patiënten.
Onderzoek naar het retrovirale p15E geeft aangetoond dat een geconserveerd deel van het eiwit, bestaande uit 26 aminozuren, verantwoordelijk is voor de immunosuppressieve werking. Een synthetisch peptide, bestaande uit 17 van deze 26 aminozuren, CKS-17, is ook immunosuppressief. Derhalve is dit peptide door diverse onderzoekers gebruikt bij het bestuderen van de onderdrukking van de afweer door p15E. Voor ons onderzoek naar de onderdrukking van het afweersysteem bij patiënten met hoofd- halskanker is ook gebruik gemaakt van dit peptide.

In **Hoofdstuk 5** beschrijven we de ontwikkeling van een nieuwe set monoklonale antistoffen, gericht tegen het synthetisch peptide CKS-17. Hiertoe werden muizen geïmmuniseerd met het peptide. Vervolgens werden muizen cellen met een geïmmortaliseerde cellijn gefuseerd, waarna een cellijn ontstaat die de monoklonale antistoffen produceert. De drie ontwikkelde monoklonale antistoffen (ER-IS1, ER-IS2, ER-IS5), herkennen het synthetisch peptide en het retrovirale p15E. Daarnaast reageren de antistoffen met menselijke tumor cellijnen en met weefsel van hoofd-halstumoren. Bovendien blokkeren de antistoffen de immunosuppressieve activiteit van p15E-achtige eiwitten uit het serum van patiënten met hoofd- halskanker. Dit leidt tot de conclusie dat het CKS-17 deel van het retrovirale p15E ook aanwezig is en functioneel is bij patiënten met hoofd- halskanker.

In **Hoofdstuk 6** wordt de aanwezigheid van p15E-achtige eiwitten bij andere typen tumoren onderzocht. Door gebruik te maken van het monoklonale antilichaam ER-IS5, gericht tegen CKS-17, vonden we p15E-achtige eiwitten in 89% van de bestudeerde plaveiselcel tumoren van het hoofd- halsgebied.

Daarnaast vonden we een positieve reactie met bepaalde typen tumoren van de slokdarm, longen, huid (onder andere melanomen), borst, dikke darm, baarmoeder en ovarium. Hieruit kunnen we de conclusie trekken dat immunosuppressieve eiwitten, verwant aan het retrovirale p15E, niet alleen bij hoofd- halskanker voorkomen. De aanwezigheid van p15E-achtige eiwitten bij andere typen tumoren suggereert dat p15E achtige eiwitten ook bij andere vormen van kanker een rol spelen bij de onderdrukking van de afweer.
Omdat we een zwakke reactie vonden met gezonde cellen, zou het kunnen dat p15E-achtige eiwitten in de gezonde situatie ook een rol spelen.

In **Hoofdstuk 7** zijn de nieuwe monoklonale antistoffen gebruikt voor de ontwikkeling van een gevoelige test voor het detecteren van p15E-achtige eiwitten in menselijk serum. P15E kan gebruikt worden als merkstof voor kanker, waarmee de aanwezigheid van een tumor, van een uitzaaiing of van het terugkeren van een tumor na behandeling aangetoond kan worden. Derhalve is het gewenst dat een gevoelige en kwantitatieve test ontwikkeld wordt voor toepassing in het klinisch immunologisch laboratorium.
Door combineren van verschillende antistoffen, gericht tegen retroviraal p15E, of tegen CKS-17, en een "sandwich ELISA" techniek, hebben we geprobeerd deze test op te zetten. Helaas kon zelfs met de beste combinatie van antistoffen (19F8 en ER-IS5) geen test ontwikkeld worden met de vereiste gevoeligheid van enkele pico grammen (10^{-12} gram). Bovendien verstoorden blokkerende eiwitten, aanwezig in menselijk serum, de detectie van p15E. Met behulp van de methode van "Western Blotting" konden we aantonen dat serum eiwitten aan p15E binden, waardoor de detectie van p15E wordt verstoord. Andere technieken, zoals de nieuwe "immuno-PCR" techniek en nieuwe antistoffen zullen ontwikkeld moeten worden om de door tumoren geproduceerde p15E-achtige eiwitten op gevoelige en kwantitatieve wijze te kunnen aantonen.

In **Hoofdstuk 8** hebben we in een muizen model onderzocht of immunotherapie, gericht tegen CKS-17, mogelijk is. Muizen werden ingespoten met de Rauscher-virus getransformeerde myeloide tumor cellijn RMB-1. Zonder ingrijpen leidt de groei van de tumorcellen na 10 tot 15 dagen tot de dood van de muizen. De monoklonale antistoffen 19F8 en ER-IS5 werden gebruikt voor immunotherapie. De antistof producerende cellen werden ingekapseld in alginaat en ingespoten in muizen. Op deze manier worden de antistoffen door de cellen geproduceerd in de muizen. Een enkele injectie van muizen, met ingekapselde 19F8 cellen, leidde tot een gemiddelde overlevingsduur van 21 dagen waarbij 2 van de 7 muizen zelfs overleefden. Een enkele injectie van muizen, met ingekapselde ER-IS5 cellen, leidde tot een gemiddelde overlevingsduur van 20 dagen. Het verschil tussen 19F8 en ER-IS5 (genezing van 2 muizen door 19F8, niet door ER-IS5), kan verklaard worden door het feit dat 19F8 p15E in het serum neutraliseert en bovendien p15E op de tumorcellen herkent. Daarentegen is ER-IS5 alleen in staat om p15E te neutralise-

ren en herkent dit antilichaam p15E op de RMB-1 tumorcellen niet. Daarom zorgt 19F8 zowel voor remming van de immunosuppressie, als voor het doden van de tumorcellen, door eraan te binden. ER-IS5 is alleen in staat de immunosuppressie te remmen. Beide mechanismen zorgen voor een langere overleving. Hierom zouden toekomstige therapeutische strategieën erop gericht dienen te zijn antistoffen toe te passen die tumorcellen herkennen (waardoor de cellen gedood kunnen worden), en die de remming van het afweersysteem kunnen blokkeren.

In **Hoofdstuk 9**, de discussie, worden de resultaten van het onderzoek nog eens besproken.
Hoe het p15E-achtige eiwit er precies uitziet, en ook waar het exact vandaan komt, is nog niet bekend. We weten echter wel dat het immunosuppressieve deel (CKS-17) op p15E van verschillende retrovirussen voorkomt en dat dit ook aanwezig is op de menselijke immunosuppressieve eiwitten met een laag molecuulgewicht (p15E-achtige eiwitten), en bovendien op andere immunosuppressieve eiwitten. De oorsprong van de p15E-achtige eiwitten kan liggen in endogene retrovirus sequenties. Recent onderzoek heeft aangetoond dat deze zogenaamde "provirussen" inderdaad kunnen resulteren in de productie van eiwitten in menselijke tumorcellen.

Omdat p15E-achtige eiwitten veel voorkomen bij verschillende typen tumoren, en omdat p15E gerelateerde immunosuppressie verdwijnt na chirurgische verwijdering van de tumor, kan de aanwezigheid van p15E een aanwijzing zijn voor de aanwezigheid van een tumor. Om dit verder te kunnen onderzoeken is een gevoelig test systeem vereist, waarmee p15E concentraties kunnen worden gemeten in experimenteel laboratorium onderzoek en bij patiënten.
P15E-achtige eiwitten kunnen daarnaast gebruikt worden als doel voor immunotherapie. Echter, verder experimenteel onderzoek in diermodellen is vereist voordat immunotherapie gericht tegen p15E als nieuwe therapie kan worden toegepast.

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Margreet

Curriculum vitae

8 januari 1963	geboren te Amsterdam
1975-1982	Atheneum B, Spinoza Lyceum Amsterdam.
1982 -1988	Studie medische biologie, Vrije Universiteit Amsterdam.
1986	Onderzoeksstage: allergische contact dermatitis voor TTS in een cavia model, afd Pathologie, VU Amsterdam. Begeleider Prof dr R.J. Scheper.
1987	Onderzoeksstage: produktie van humane monoklonale antistoffen, afd Celbiologie/Immunologie Erasmus Universiteit Rotterdam. Begeleidster dr A.C. van Denderen.
1988	Afstudeerscriptie: Immunobiologie van humane hoofd- halstumoren.
1988-1994	Promotie onderzoek afd. Keel- Neus en Oorheelkunde, Dijkzigt Ziekenhuis / afd. Immunologie EUR. Project: "Monoklonale antistoffen voor diagnostiek en therapie van hoofd-halstumoren".
1990	Cursus introduktie tot de klinische en experimentele oncologie
1992	Cursus proefdierkunde ter verkrijging van artikel 9 bevoegdheid.
1992-1994	Begeleiding practicum histologie voor geneeskunde studenten
1992-1994	Begeleiding van HLO-studenten tijdens hun afstudeerstage
1-9-1995	Clinical Research Associate bij Sandoz Pharma B.V. / Novartis Pharma B.V. Coördinatie van klinische trials (o.a. immunosuppressie bij patiënten met een niertransplantatie en gentherapie bij patiënten met een hersentumor).
1-12-1997	Clinical Research Associate bij Schering-Plough B.V. Coördinatie van klinische trials (o.a. gebruik van inhalatie corticosteroïden bij astma, gentherapie met p53, chemotherapie van solide tumoren).

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