CHAPTER *1*

Introduction

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Response of colon carcinoma cells to pro-inflammatory cytokines

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INTRODUCTION

*Recurrences in gastro-intestinal malignancies*

Cancer of the gastro-intestinal tract represents a major health problem. In the western world, there are 300.000 new cases of colorectal cancer with 200.000 deaths each year[1]. Pancreatic cancer is one of the most fatal malignant diseases nowadays ranking fifth in cancer mortality in the western world[2].

Surgical resection for limited stages of disease is the only treatment which can provide long-term disease-free survival in gastro-intestinal malignancies. Unfortunately, the majority of patients present with either microscopic metastatic disease in distant sites or advanced tumour growth exceeding the limits of the resection. As for pancreatic cancer, resectability rates are extremely low ranging from 5 to 22% in reviews of multicenter studies[3-5].

In the patients eligible for surgery, tumour recurrence after intentionally curative surgery remains a major problem. For colon cancer recurrence rates up to 40% have been reported after surgery with curative intent[6], most frequently involving the liver, lung and loco-regional sites[7-10]. For pancreatic cancer reported recurrence rates are even up to 80%[11]. Local recurrences, liver metastases and peritoneal carcinosis comprise the majority of recurrences. Most of these recurrences occur within one year after surgery and therefore a relation between surgery and tumour recurrence was suggested. This thesis will investigate this relation with the focus on colon carcinoma and pancreatic carcinoma.

Metastasis

Metastases, rather than primary tumours, are responsible for most cancer deaths. These haematogenous and lymphogenous metastases arise following the spread of tumour cells from a primary site and the formation of new tumours in distant organs. Unfortunately, research regarding the development of metastasis is still in its infancy because metastasis formation is difficult to observe. Studies using *in vivo* video microscopy and quantitative approaches that follow the fate of cancer cells in the body are shedding light on this occult process[12]. In this way it became apparent that the development of hematogenous metastasis consists of a cascade tumour cells have to go through [13-15] (Figure 1). The first step of this cascade is the detachment of tumour cells from the primary tumour and involves angiogenesis of the primary tumour, adhesion of the tumour cell to the subendothelial basement membrane, degradation of and migration through this membrane and subsequent intravasation into the tumoral vascular bed and further into the body's circulatory blood

Figure 1. Metastasis model
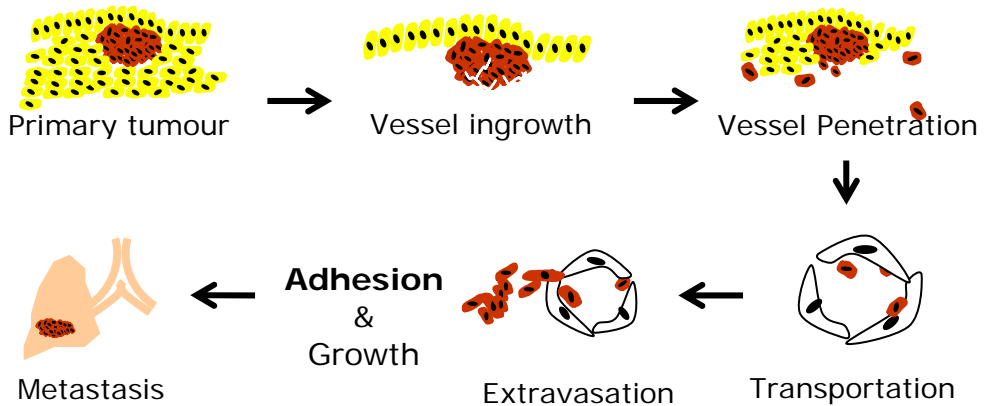
system. The second phase comprises the vascular phase in which one of the strongest selection pressures is applied to the tumour cells. Here, circulating tumour cells have to overcome defense mechanisms consisting of humoral and cellular constituents of blood and haemodynamic forces due to blood pressure and contact with both the vessel wall and the circulating blood cells, resulting in the death of 99.0 – 99.9% of the circulating tumour cell population[15].

Furthermore, life span of circulating tumour cells is limited. Adhesion of the tumour cells is a necessity to survive, so if tumour cells do not adhere within a certain time, they will die. Longer survival of circulating tumour cells is assessed by clustering with platelets and fibrin. The minority of cells surviving the vascular phase need to adhere to the endothelial cells lining the blood vessels of distant organs. For successful adhesion, adhesion molecules on the tumour cells have to bind to their ligands on endothelial cells.

To question in which organ tumour cells preferential adhere, two theories regarding organ selective metastasis development exist. First Paget's seed and soil theory[16], implicating that both the unique phenotype of the tumour cells and the unique phenotype of the host organ together are responsible for the organ selective metastasis. The theory of Ewing[17] is contradictory, suggesting that mechanical filtering is of greater importance. Both hypotheses do have merit, although the theory of Paget is likely to influence the distribution of metastasis more, since metastasis has a non-random pattern with certain tumour cells having specific affinity for the milieu of certain organs[18,19]. Considering the theory of

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mechanical filtering with the arrest of tumour cells in capillaries, the diameter of capillaries in relation to the size of the tumour cell does not predict the outcome, since tumour cells are capable of passing through capillaries with a diameter narrower than the diameter of the tumour cells themselves by stretching out[20].

Following adhesion to the endothelium, tumour cells have to invade this layer and subsequently adhere to the underlying extracellular matrix (Figure 2). By the production of proteases like the matrix metalloproteases the extracellular matrix has to be digested before the tumour cells can finally enter the tissue. In this last phase, the tumour cells have to initiate and maintain growth to form micrometastases. The successful formation of macroscopic metastasis only occurs if the tumour cells are able to develop new blood vessels, called neoangiogenesis, to supply themselves. All of the preceding steps have to be taken successfully by the tumour cell in order to form a metastasis, rendering this process to be highly inefficient[21]. So, under normal conditions tumor cell arrest in distant organs is negligible, but trauma or inflammation may provide better conditions for tumour cells to succeed in the formation of metastases, since clinical and experimental observations indicate preferential tumor cell arrest at sites of injury, healing and inflammation[22-25].

Inflammation

Wound healing comprises of 4 phases, being bleeding, inflammation, proliferation and remodelling[26-28]. Inflammation is a normal and necessary prerequisite to healing. When inflammation is complicated by microbes like bacteria it is called infection. The inflammatory phase has a rapid onset (few hours) and increases in magnitude to its maximal reaction (2-3 days) before gradually resolving. The acute phase of inflammation is characterized by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes and inflammatory mediators. The chronic phase is characterized by the development of specific humoral and cellular immune responses to pathogens present at the site of tissue injury. The cellular components of the inflammatory response include polymorphonuclear leukocytes (granulocytes, PMN) mostly comprising of neutrophils next to eosinophils and basophils. Mononuclear cells are the other cellular components and consist of monocytes, macrophages, lymphocytes, plasma cells and platelets.

In inflammation, there is an early migration (within minutes) of neutrophils to the site of injury to phagocytose microbes or inflammatory debris and eliminate pathogens by the release of lysosomal enzymes and reactive oxygen species (ROS)[29]. These ROS will be produced by activation of the enzyme NADPH oxidase, which catalyzes the following reaction: $NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$. The superoxide anion ($O_2^-$) is rapidly dismutated to hydrogen peroxide (H_2O_2) and other toxic species that are responsible for injury to pathogens or

surrounding tissue[30]. In the presence of iron O_2^- and H_2O_2 can react to release the highly toxic hydroxyl radical through the Haber-Weiss reaction.

The early migration precedes the attraction of other cells like monocytes, lymphocytes, eosiniphils, basophils and platelets to the site of injury[31]. Monocytes, once in the tissue become macrophages with antimicrobial and phagocytic qualities[32]. These monocytes and macrophages are the predominant producers of cytokines.

Cytokines, which are cell-derived polypeptides, orchestrate to a large extent the inflammatory response. They are major determinants of the make-up of the cellular infiltrate, the state of cellular activation and the systemic responses to inflammation. Most cytokines are multifunctional and elicit their effects locally as well as systemically in an autocrine or paracrine manner. The heterogeneity in cytokines exhibiting both negative and positive regulatory effects on various target cells renders a differentiation between pro-inflammatory cytokines and anti-inflammatory cytokines. Among the pro-inflammatory cytokines, interleukine-1β (IL-1β), tumour necrosis factor-α (TNF-α) and IL-6 are the most potent[33].

Inflammatory response during and after surgical trauma

Inflammation is an important phase in wound healing and therefore surgery, in which trauma to the tissue is performed, provokes an evident inflammatory response. The degree of inflammation depends on the severity and duration of the surgical trauma. After major trauma, local levels of IL-1β and TNF-α are significantly elevated, with even systemically elevated levels, though lesser than locally, since these cytokines exerts their effect close to their site of release and have short existence[34-36]. These cytokines stimulate the production of IL-6, which markedly peaks at 4-48 hours after surgery and the maximum serum IL-6 levels reflect the severity of surgical stress[35,37]. This cytokine induces an acute phase response with elevated levels of C-reactive protein (CRP) and PMN elastase[38]. Furthermore, increase in circulating PMN, whether or not mature causing leukocytosis, can be detected following surgery with increased superoxide anion release and chemotaxis[39]. Surgery activates monocytes as well, leading to further increase in cytokine release[40].

Although IL-6 is a potent pro-inflammatory cytokine, it accounts also for the up-regulation of major anti-inflammatory mediators such as prostaglandin E2, IL-10 and transforming growth factor-β[41,42]. This results in surgical, trauma-induced, immunosuppression as indicated by monocyte deactivation among other restraints[43]. This immunosuppression is associated with the development of infectious complications with higher morbidity and mortalty[44].

Microvascular endothelium

The vascular endothelium is a confluent monolayer of thin, flattened, rhomboid-shaped cells lining the intimal surface of all blood vessels. It functions as a ubiquitous organ system in the body, providing a vital and responsive infrastructure for the circulation of blood and the homeostasis of all organs. The endothelium fulfils the definition of an organ because of its physical size (circa 5.000 m^2 surface area) and its ability to synthesize and release a myriad of physiologically active products in reaction of changes in its environment[45]. Among the factors produced are prostaglandins, nitric oxide, endothelins, reactive oxygen species, anti-oxidants, and cytokines. Together with the underlining vascular basement membrane it forms a physical boundary between the intravascular and extravascular compartments that limits passive transfer of cellular and fluid elements[46]. Although originally regarded as an inert physical barrier, endothelial cells are now known to take an active part in regulating a large number of homeostatic and pathological processes including embryogenesis, regulation of vasomotor tone, (anti-)coagulation and fibrinolysis, wound healing, lymphocyte homing, atherogenesis, acute inflammation and haematogenous metastasis[47].

Depending on the diameter of the blood vessels, there is a distinction in macrovasculature and microvasculature. Much of the knowledge regarding the vasculature is obtained from studies using human umbilical vein endothelial cells (HUVEC), which are macrosvascular embryonic cells, simply because of the ease with which HUVEC are isolated and cultured. Unfortunately, observations obtained from HUVEC cannot necessarily be extrapolated to processes which involve the microcirculation[48-54]. Vessels of the microcirculation are the conduits that are responsible for the local delivery and transfer of cell substrates and metabolites. Because of their specialized function, they have different properties compared to vessels of the macrocirculation. Even between different organs with each having its own unique microenvironment, there are differences in the microvascular endothelial cells[55-58].

In haematogenous metastasis, adhesion of tumour cell to the endothelium is an important step, which occurs in the microvasculature. The heterogeneity between microvascular endothelial cells derived from different organ systems may validate Paget's seed and soil theory that metastasis follows a non-random pattern with preference of a tumour cell type for homing in a specific organ.

Extracellular matrix

The next phase in haematogenous metastasis is the adhesion to and invasion through the extracellular matrix (ECM). This layer consists of a subendothelial basement membrane and underlying parenchymal tissue[59-61]. The basement membrane is mainly build up from collagen type IV, laminin and heparin sulphate proteoglycan, whereas the parenchymal

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tissue mainly consists of collagen type I, fibronectin, vitronectin and hyaluronic acid. The ECM provides structural integrity, mechanical strength and elasticity to the blood vessels, but can also be described as a dynamic meshwork actively regulating critical cellular functions such as migration, survival, proliferation and differentiation[62,63]. Binding of the tumour cells to components of the ECM is mainly accomplished by the integrins, a subset of adhesion molecules[64,65]. Invasion through this ECM involves degradation of the layer by enzymes including the serine-protease family, the matrix metalloproteinases and the cysteine proteinases[66,67]. Successful tumour cells are able to synthesize some of these enzymes themselves. After the invasion tumour cells may enter the tissue of the distant organ.

Cell adhesion molecules

Defective interactions between adhesion molecules have a critical role in cancer. During the different phases in metastasis the presence or absence of adhesion molecules contributes to the metastatic properties. In haematogenous metastasis adhesion of the tumour cells to the endothelium lining a distant organ and next binding to components of the ECM is essential. For this binding appropriate adhesion molecules on tumour cells are necessary which can bind to ligands on the endothelium or ECM. A number of cell surface associated molecules have been characterised and classified in four groups according to their biochemical structure: integrins,immunoglobulin-like proteins, cadherins and selectins[68-70].
The integrins are membrane glycoproteins with two subunits, designated α and β[71]. A subgroup of the integrins, the very late activation integrins or VLA-integrins with variable α subunits combined with the β1 subunit, play a major role in the binding of cells to components of the ECM. Tumour cells express a scale of integrins[64].
Members of the immunoglobulin superfamily contain immunoglobulin-like structures and consist of the intracellular adhesion molecules (ICAM-1, 2 and 3), vascular cell adhesion molecules (VCAM-1 and 2) and neural cell adhesion molecules (NCAM). ICAM is expressed on virtually any cell and is a ligand for lymphocyte function associated antigen-1 LFa-1 expressed on many inflammatory cells as well as on many cancer cells. VCAM is expressed on stimulated endothelial cells and can bind to the α4β1 or α4β7 integrin found on leukocytes and on several cancer cells.
The cadherins establish molecular links between homophilic adjacent cells. Loss of E-Cadherin expression on the tumour cell accounts for the detachment of the tumour cell from the primary tumour.
Three types of selectins exist, namely P-Selectin which is stored in platelets and endothelial cells, L-Selectin which is found on leukocytes and mediates the homing to lymph nodes and E-Selectin which have low basal expression on endothelial cells. Counterparts of E-Selectin
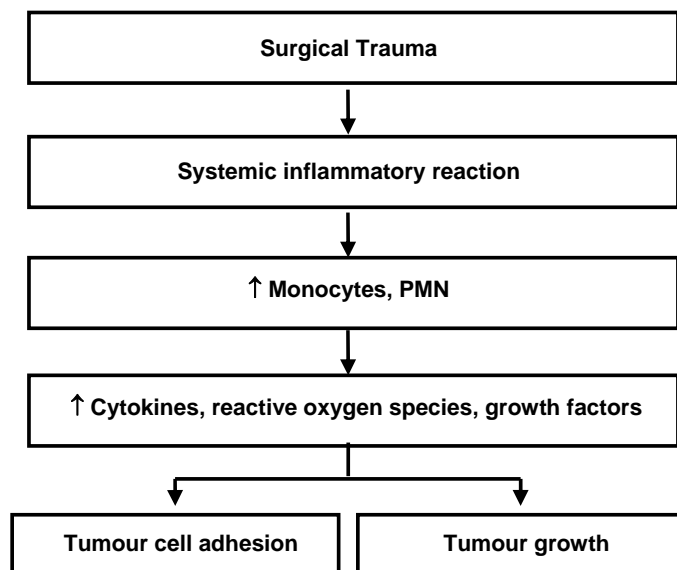
are the carbohydrate ligands sialyl Lewis a (sLe^a) and sialyl Lewis x (sLe^x) antigens, both belonging to the Lewis blood group antigens. These two antigens are involved in many gastrointestinal tumours[72].

In inflammation, mediators like the cytokines are known to induce or upregulate adhesion molecules on endothelial cells and in this way inflammation increases the prospect of cell adhesion to the stimulated endothelium[73]. Implantation of tumour cells may therefore benefit from this inflammatory state.

Surgery and loco-regional tumour recurrence

Handling of the primary tumour during resection can provoke detachment of tumour cells[74,75]. Intraperitoneal and circulating tumour cells are often found in patients with gastrointestinal cancer, not only during resection of the primary tumour[76-78]. Although the amount of circulating tumour cells is enhanced during resection of the primary tumour, it will not entirely explain the high recurrence rate found after intentionally curative surgery. Implantation of circulating tumour cells appears to be highly inefficient and most circulating tumour cells are rapidly destroyed[21,79].

Figure 2. Hypothesis

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│              Surgical Trauma                 │
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                      ▼
┌─────────────────────────────────────────────┐
│       Systemic inflammatory reaction         │
└─────────────────────────────────────────────┘
                      │
                      ▼
┌─────────────────────────────────────────────┐
│             ↑ Monocytes, PMN                 │
└─────────────────────────────────────────────┘
                      │
                      ▼
┌─────────────────────────────────────────────┐
│ ↑ Cytokines, reactive oxygen species, growth factors │
└─────────────────────────────────────────────┘
            │                       │
            ▼                       ▼
┌──────────────────────┐  ┌──────────────────────┐
│ Tumour cell adhesion │  │    Tumour growth     │
└──────────────────────┘  └──────────────────────┘
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The hypothesis that operative trauma in itself may favour the development of locoregional tumour recurrence was object of previous *in vivo* and *in vitro* studies. First, Busch *et al.*[80] found that blood transfusions decrease survival after surgery for colorectal cancer. However, not the blood transfusions themselves but rather the circumstances necessitating them, namely the degree of surgical trauma, turned out to be the real predictors of prognosis. Indeed, van den Tol *et al.*[81] demonstrated that surgical trauma enhanced loco-regional tumour load in a rat model and that the severity of surgical trauma influenced the degree of tumour load. This is reflected in the finding that laparoscopy, which causes minor trauma, gave significantly less loco-regional tumour load compared to laparotomy[82].

Subsequent *in vivo* experiments demonstrated that one of the cellular components of blood, i.e. the erythrocytes, once introduced in the abdominal cavity after abdominal surgical trauma, in fact effectively inhibited loco-regional recurrences[83]. The responsible beneficial components of the red cells turned out to be the antioxidant enzymes superoxide dismutase (SOD) and catalase[84]. These enzymes neutralize the reactive oxygen species O_2^- and H_2O_2. On the other hand, passive transfer experiments demonstrated that the cellular fraction, mainly consisting of PMN, obtained from abdominal lavages in rats with abdominal surgical trauma enhanced tumour load in naïve recipients. Since abdominal surgical trauma provokes a local inflammatory reaction with an exsudate consisting of more than 75% PMN that are the main producers of ROS, a condition is created that promotes loco-regional tumour recurrence.

In the passive transfer experiments described above not only the cellular fraction enhanced loco-regional tumour recurrence, but the fluid component as well. This fluid component contains the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, mainly produced by monocytes and macrophages.

Summarizing, the inflammatory sequelae provoked by surgical trauma promotes the development of loco-regional tumour recurrences. Tackling of the products released by inflammatory cells seems a feasible way of preventing loco-regional tumour recurrences.

Furthermore, surgery induces not only an inflammatory reaction, but is accompanied by immunosuppression as well. By the phagocytic dysfunction of monocytes and macrophages and suppressed activity of the natural killer cells and lymphokine activated killer cells the defence against tumour cells is diminished. In this way survival of circulating tumour cells is favoured and may therefore lead to more successful metastasis establishment

Surgery and distant tumour recurrence

The inflammatory reaction does not confine locally but induces a systemic reaction as well, although to a lesser extent[35,40,85-87]. Therefore, surgical trauma may not only promote loco-regional recurrences, but spread to distant sites as well. Indeed, in mouse models it was

found that surgical trauma enhanced the development of melanoma pulmonary metastases, which could be inhibited by the addition of antibodies against pro-inflammatory cytokines[88]. In rat models it was found that laparotomy enhances abdominal metastases as well as that thoracotomy enhances abdominal metastases, emphasizing the systemic effect[89]. Furthermore, laparotomy in mouse models caused more tumour load in the flank or dorsal skin compared to laparoscopy[90,91]. There are also reports of enhanced pulmonary metastasis formation after partial liver resection, although it is not clear whether this involves increased tumour cell implantation in the lung or that the regenerating liver produces growth factors enhancing the growth of successfully implanted tumour cells in the lung[92].

These studies clearly show that surgical trauma not only affects loco-regional tumour recurrence, but affects tumour recurrence to distant sites as well.

Figure 3. In vitro adhesion assay
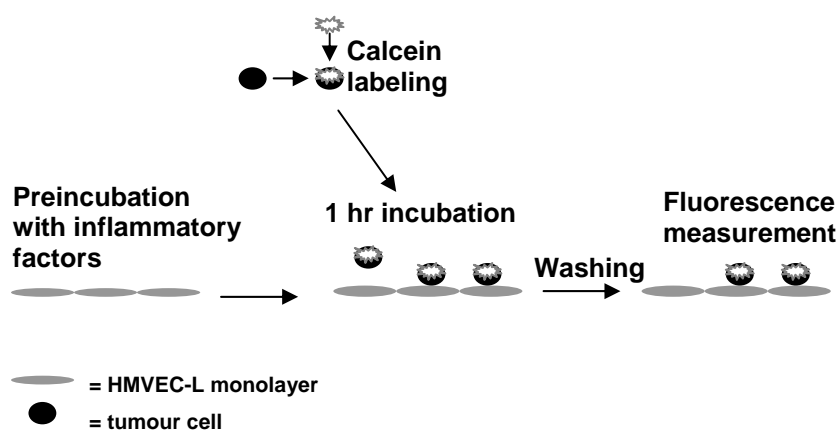
AIM OF THE THESIS

Previous *in vivo* and *in vitro* studies performed in animal models clearly demonstrated the promoting effect surgery has on the establishment of loco-regional tumour recurrence as well as distant tumour recurrence. In loco-regional tumour recurrence, the inflammatory reaction provoked by surgery activated polymorphonuclear leukocytes and monocytes/macrophages with the release of ROS and pro-inflammatory cytokines. These mediators seem to be responsible for the observed enhancement in tumour recurrence. This thesis focuses on the influence of surgical trauma on the development of distant metastasis. The mechanisms by which surgical trauma influence distant tumour recurrences are not fully understood. It may act on tumour cell implantation by increasing the amount of adherent cells to endothelium, but another mechanism might be the influence on tumour growth, enhancing the growth of successfully implanted tumour cells (Figure 2). Especially in partial liver resection, the regeneration of the liver provokes a plethora of growth factors that may act on tumour growth. In **chapter 2** therefore, a rat model was developed in which the effect of partial liver resection on remote tumour recurrence was evaluated. This study clearly demonstrated a distant tumour enhancing effect of partial liver resection as well as of surgical trauma in general. The main mechanism seems not to be enhanced tumour growth and so we hypothesized that the responsible mechanism is enhanced tumour cell implantation. To evaluate the effect of surgery-derived inflammatory factors on tumour cell – endothelial cell interactions, a reproducible human *in vitro* model was developed in which the adhesion of human tumour cell lines to human microvascular endothelial cells could be investigated (Figure 3). **Chapter 3** describes the influence of the pro-inflammatory cytokines on the adhesion of colon carcinoma cells to microvascular endothelium. We studied whether exposure of microvascular endothelial cells and / or tumour cells to these cytokines modulated the adhesion. The binding mechanism in this interaction involving adhesion molecules was further studied. In **chapter 4** we studied the effect of these pro-inflammatory cytokines on the adhesion of three pancreas carcinoma cell lines to this endothelium. Once more the role of the involved adhesion molecules was examined.

In **chapter 5** we evaluated the effect of activated PMN on the adhesion of both colon and pancreas carcinoma cells to microvascular endothelium. The role of ROS in PMN modulation was verified by the use of antioxidant enzymes in inhibition assays. Since ROS turned out to be the main perpetrators in the PMN-modulated enhanced tumour cell adhesion, we investigated in **chapter 6** the effect of ROS more detailed by using the ROS superoxide anion directly, produced by the combination of xanthine and xanthine oxidase. In this way more profound knowledge of the effect of ROS on cellular level was obtained.

After adhesion to and invasion through the microvascular endothelial layer, the next step in metastasis is adhesion to the extracellular matrix. Not much is known regarding the

establishment of binding between tumour cells and the varying components of this matrix, although a role for tumoral integrins is suggested. In **chapter 7** we present the results regarding the adhesion of two colon carcinoma cell lines to different components of the extracellular matrix.

Finally, in **chapter 8** the results of all chapters are discussed and in **chapter 9** a summary of this thesis followed by the conclusions is given in English and Dutch.

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