SURGERY AND PERITONEAL TUMOUR RECURRENCE

PATHWAYS STUDIED *IN VITRO*

W.M.U. van Grevenstein

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Pathways studied *in vitro*

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Het mechanisme bestudeerd *in vitro*

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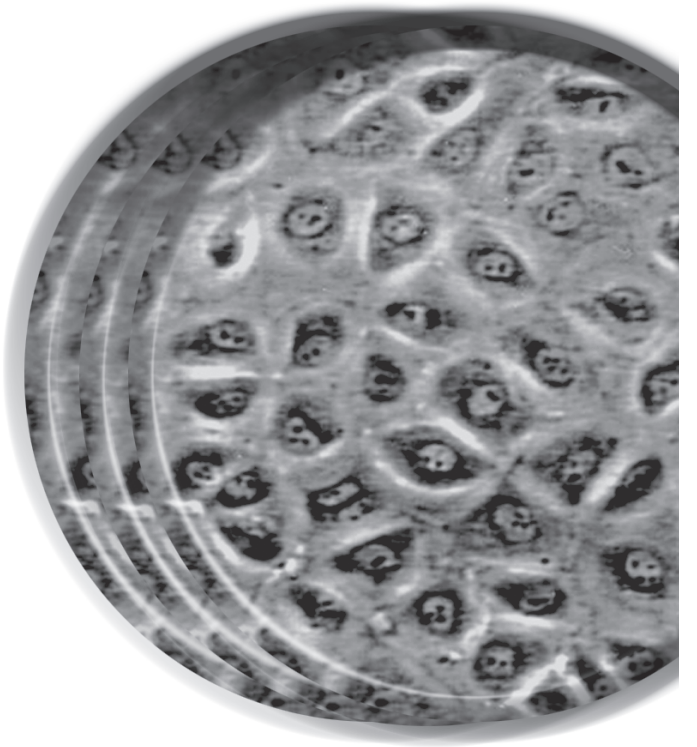
CONTENTS

To my parents,

In a world of fiction, you are a truth I can believe in.

CHAPTER 1

General introduction



INTRODUCTION

This prologue focuses on the fundamentals of peritoneal tumour recurrence after surgical resection of gastro-intestinal tumours. This will be the rationale on which the investigations described in this thesis are founded. For the studies outlined in this thesis only human derived cancer and mesothelial cells were used.

Gastro-intestinal malignancies

The incidence of gastro-intestinal malignancies is increasing in the western world and accounts for more than 50% of all malignancies. Colorectal cancer is a leading cause of morbidity and mortality with about 400 000 new cases and 200 000 deaths in Europe each year.[1, 2] According to an estimation of the International Agency for Research on Cancer, colorectal cancer was in the year 2004 the most common form of cancer diagnosed in the European Union, both sexes combined.[1] The overall 5 year survival rate for colon cancer is 60-70%. For patients with local recurrence the 5 year survival rate is approximately 35%. For patients with distant metastasis the 5 year survival is 25%.[3]
An more fatal disease is pancreatic cancer. Pancreatic ductal adenocarcinoma is a major cause of cancer death in the Western world and is responsible for nearly 65 000 deaths per year in Europe.[4, 5] Pancreatic cancer is a disease with a very poor prognosis and is often in an advanced state of disease. Less than 15% of the patients survive the first year and the 5-year survival is approximately 3%. As with colorectal cancer the most common site of local recurrence is the resection site. The intra-abdominal recurrence rate after curative resection ranges between 38% and 86%.[6-9]
For both colon and pancreatic cancer the locoregional recurrence rate is high. Therefore, knowledge of the pathophysiology of recurrences is of the utmost importance in order to develop more specific tools to attack the initial process of implantation of tumour cells in the peritoneal cavity.

Local tumour recurrence

Local tumour recurrence after curative surgery is a persistent problem in the treatment of gastro-intestinal cancer. Local recurrence is denoted as regrowth of tumour in and around the resection site, including the pericolic fat, the adjoining mesentery and lymph nodes, the peritoneum or the suture or staple line of the bowel anastomosis.
The incidence of local recurrence following colorectal cancer varies between 3 and 32%.[10-16] Although time to recur varies, 55-80% of local recurrences occur during the first two years after curative resection.[10] There are ample speculations on the evolution of local recurrence. The first and most feasible explanation, is that local recurrence may occur after resection of a locally advanced colorectal carcinoma, which already penetrates the peritoneal surface or adjacent organs. Local peritoneal involvement has a detrimental

influence on the prognosis after planned curative resection.[17, 18] Secondly, the presence of cancer cells in the abdominal cavity prior or during surgery has a significantly deteriorating influence on overall survival. Recent studies revealed that 20-30% of the patients with colorectal cancer have a positive immunocytology of the peritoneal washings before handling of the tumour. According to the results of these recent investigations, peritoneal cytology after curative resection might serve as a prognostic tool in colorectal cancer management.[19-23]

The hypothesis extensively propagated by Sugarbaker is the tumour cell entrapment theory. Manipulating and removing a tumour, especially a stage III adenocarcinoma, will cause leakage of tumour cells out of the transected lymphatic channels into the resection site and free peritoneal cavity. Furthermore, by extensively pulling the tumour, the interstitial tissue close to the tumour might be traumatized and this will cause a release of tumour emboli from the margins of dissection. Tumour cells can also reach the free abdominal cavity by leakage out of the transected veins.[24] The free floating tumour cells will precipitate on the raw tissue surface on which fibrin is released. Hereafter, inflammatory cells will encounter the fibrin deposition and participate in the healing process by releasing cytokines and growth factors, which is an excellent environment for tumour cells to flourish.[24, 25] In order to prevent tumour spill during surgical tumour resections, 'the no touch isolation technique' was developed. This technique implies the vascular ligation before manipulation of the tumour during surgical resections.[26] Although tumour spill might be decreased by this surgical technique, a benefit in survival has not been revealed.[27]

Peritoneum and mesothelial cells

In 1827 Bichat observed that the surface of the serous cavities was covered by flattened cells. He emphasized their similarity with cells lining the lymphatics, and therefore classified the serosal cavities as part of the lymphatic system.[28]

The surfaces of the pleural, peritoneal and pericardial cavity are covered by the mesothelium. The mesothelium covering the internal organs is known as visceral, while the parietal mesothelium surrounds the abdominal cavity. This peritoneal tissue is a monolayer and its epithelial characteristics are closely shared with those of the endothelium, for this reason mesothelium and endothelium were regarded previously as one and the same tissue.[29]

The peritoneal mesothelium is a highly specialized monolayer of polarized flat epithelial cells that covers the entire surface of the abdominal cavity. It serves as a protective anatomical barrier, as a non-adhesive friction-less interface for the movement of abdominal organs and is involved in the formation and turnover of abdominal fluid. The integrity of the mesothelial cell lining is a prerequisite for these functions. Injury to the abdominal cavity is a common phenomenon. It can be caused by trauma, surgery, infections, or various neoplasms. Both inflammatory and neoplastic processes in the abdominal cavity are as-

sociated with marked structural alterations of peritoneal mesothelium, which include loss of polarization, cellular retraction with exposure of the submesothelial extracellular matrix (ECM), and frequently detachment and suspension of mesothelial cells in peritoneal effusions.[30] The precise pathological mechanisms involved in the disruption of the mesothelial integrity are still poorly defined, but are likely to involve alterations of the expression of mesothelial adhesion molecules.[31-33]

The mesothelial cells were once considered to be passive cells, but there is now compelling evidence, which highlights their critical role in antigen presentation, inflammation, wound healing, and transport of fluids and cells across the serosal cavities.[34, 35]

The introduction of peritoneal dialysis (PD) as a treatment therapy for patients with end-stage renal disease provoked much interest in the biological and molecular aspects of the peritoneal mesothelial cell. These cells provide the first line of defence in peritoneal protection during long-term or repeated exposure to unphysiologic PD solutions, and against micro-organisms during infective peritonitis. Human peritoneal mesothelial cells can be cultured *in vitro* and provide means to investigate the effects of a PD solution or its components on cell proliferation and wound healing; synthesis of matrix proteins, cytokines, and growth factors; and cell-cell, cell-matrix, and bacteria-cell interactions.[36-40]

Mesothelial cells play an important role in peritoneal homeostasis and contribute to fluid and cell transport, initiation and resolution of inflammation, tissue repair, and prevention of adhesion formation, and are the first line of defence against foreign agents. Mesothelial cells are active cells that can synthesize a plethora of pro-, anti-, and immunomodulatory mediators that include growth factors, cytokines, hyaluronan, nitric oxide, and prostaglandins. These mediators are released in response to bacterial infection, chemical or surgical injury, with the aim of initiating the reparative processes and restoring the homeostasis in the peritoneum.[41, 42] Phagocytosis of foreign agents by mesothelial cells induces secretion of chemotactic peptides, following activation with pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) and in that way contributes to the intraperitoneal recruitment of leukocytes during peritoneal inflammation.[43, 44]

The establishment of a reproducible methodology for peritoneal mesothelial cell isolation has led to an increase in mesothelial cell research in areas that encompass wound healing, inflammation and defence of foreign material. Mesothelial cell culture also offers an invaluable tool to investigate the underlying mechanism of cell signalling and cell-cell interactions and adhesion.

Trauma , Inflammation and Cytokines

Since Virchow's studies in the mid nineteenth century, the role of inflammation and wounding as an initiator and promoter of tumour development has been implied. Virchow indicated that cancers tended to occur at sites of chronic inflammation.[45]

The investigation aiming to clarify the relationship between inflammation and cancers first led to the determination, whether the reactive oxygen and nitrogen species generated by inflammatory cells, such as leucocytes recruited to the inflammatory foci to kill infectious agents, may cause mutagenic assaults and result in tumour initiation.[46] Nowadays, it has been realized that the development of cancers from inflammation might be a process driven by inflammatory cells and by a variety of mediators, including cytokines, chemokines, and enzymes, which altogether establish an inflammatory microenvironment.[47]
Inflammation is a process in response to tissue damage caused by microbial pathogen infection, chemical or mechanical induced wounding.[48] At the very early stage of inflammation, neutrophils are the first cells to migrate to the inflammatory sites under the regulation of molecules produced by rapidly responding macrophages and mast cells present in tissues.[47, 49] As the inflammation progresses, various types of leucocytes, lymphocytes, and other inflammatory cells are activated and attracted to the inflamed site by a signalling network involving a great number of growth factors, cytokines and chemokines.
Surgical trauma induces an acute phase response, during which tissue damage will be controlled, infective organisms will be killed and the repair process will be induced. The acute phase response is initiated by macrophages and monocytes entering the surgical traumatized site, which release pro-inflammatory cytokines, tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β).[50] TNF-α and IL-1β stimulate the production and release of other cytokines, like interleukin 6 (IL-6).[51, 52] These cytokines are potential modulators of the expression of cell adhesion molecules (CAMs) and regulate in that respect the adhesiveness between leucocytes and the endothelium.[53] Parallel to the endothelium, the inflammatory cascade following surgical trauma in the peritoneal cavity, might create an outstanding environment for residual or spilled tumour cells to adhere to mesothelial cells.

Adhesion Molecules

Adhesion molecules are complex membrane proteins that play important roles in the interaction between leukocytes and mesothelial cells in the inflammatory process.
Adhesion molecules are divided into three major classes. Each class regulates a particular step in the adhesion cascade. Selectins constitute one class. Selectins are transmembrane proteins involved in the early phase of adhesion cascade. They facilitate the establishment of relatively weak bonds between leukocytes and endothelial cells.[54]
The second class of adhesion molecules are integrins. Integrins are complex transmembrane proteins made of 2 subunits, α and β. They are arranged in subgroups according to the type of subunit.[55, 56] The integrins are in a nonactive form and can be stimulated by cytokines, where after strong bonds between cells can be assembled.
The third class of adhesion molecules is the immunoglobulin-like superfamily, including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-

1). They have been detected in a variety of cell types including leukocytes, endothelial cells, and epithelial cells.[57-59] These molecules can be co-expressed on the same cell and cell adhesion can be considered as their core business. ICAM-1 binds to integrin LFA-1 (aL/β2) and VCAM-1 to VLA-4 (α4/β1, α4/β7).[57, 60, 61]
A further elucidation of the mechanism by which mesothelial cells regulate their expression of adhesion molecules and receptors will help in our understanding of the invasion and metastasis of tumours.

Previous Studies

The development of peritoneal tumour recurrence after surgical trauma has been studied extensively in our department. Eggermont et al. demonstrated the stimulating effect of enhanced surgical trauma on peritoneal tumour development in a murine model.[62] A randomized clinical study of Busch et al. investigated the effect of blood transfusions on survival after colorectal surgery. In this study the effects of autogenic and allogeneic blood transfusion in patients with colorectal cancer were investigated. This trial concluded that the negative effect of blood transfusion and local cancer recurrence is indirect and probably caused by the circumstances during the surgery that necessitate the transfusion. [63, 64]
Van den Tol investigated initially the effect of surgical trauma on adhesion formation in an *in vivo* rat model and concluded that the extent of adhesion formation correlates significantly with the degree of peritoneal trauma.[65, 66] These investigations showed a correlation between the amount of peritoneal trauma with the degree of tumour take.[67]
Minimal invasive techniques and local recurrence were studied by Bouvy et al, and this experimental *in vivo* rat model ended up with the conclusion that laparoscopic surgery is associated with less tumour growth compared with conventional surgical techniques.[68]
In the established *in vivo* rat model red blood cell-derived factors did appear to prevent tumour cell adhesion to the peritoneum, but did not inhibit tumour growth.[69] Ensuing experiments done by van Rossen exhibit that the antioxidant enzymes superoxide dismutase (SOD) and catalase, both components of the red blood cell, are responsible for the inhibition of tumour adherence.[70]
Following *in vivo* experiments, in which lavage fluid from traumatized peritoneal cavities was injected in the abdomen of naïve recipients, revealed an enhancement of the peritoneal recurrence in the naïve recipients. In the same *in vivo* rat model the lavage fluid was separated in a cellular and supernatant component and injected intra-peritoneal without inflicting additional surgical trauma. Intra-abdominal injection of the cellular component, composed of 75% PMNs, 10% lymphocytes and 5% macrophages, resulted in the highest tumour load compared to the effect of the supernatant component in this passive transfer model. Although the effect of the supernatant component was less impressive, the outcome was still significant. The supernatant component contained cytokines

and growth factors, mainly produced by macrophages and monocytes. These factors were studied in successive *in vitro* studies with rat mesothelial and tumour cells. These investigations demonstrated that IL-1β and EGF are significant promoting factors in cell adhesion *in vitro*.[71]

In summary, these previous studies showed that surgical abdominal trauma stimulates tumour adhesion and growth in a rat model, probably caused by factors produced during the inflammatory response after surgical trauma.

AIM OF THE THESIS

The complexity of the metastatic process necessitates the use and development of model systems that simulate human *in vivo* conditions as closely as possible. In this study we have reconstructed human mesothelium monolayers by culturing human mesothelial cells, derived from omental tissue of patients who underwent abdominal surgery. The mesothelial cells were cultured on collagen coated flasks, which is a substitute of the extracellular matrix, since it is known that mesothelial cells rest upon a subcellular matrix. This system was employed to investigate the early interactions between human colon and pancreatic cancer cells and human mesothelial cells under well defined conditions. Besides the development of the *in vitro* model, the aim of our study was to investigate the influence of inflammatory factors (i.c. cytokines, X/XO, PMN's, latex and serum) on the adhesion capacity of the mesothelium. In addition to the effect of these factors on the adhesion, we studied the expression and upregulation of adhesion molecules on the mesothelial cells and tumour cells.

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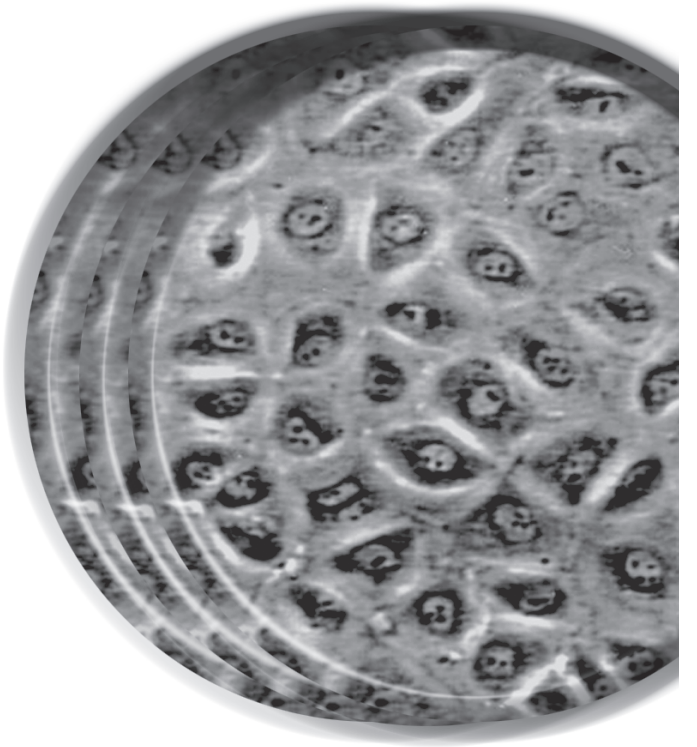
CHAPTER 2

Inflammatory cytokines stimulate the adhesion of colon carcinoma cells to mesothelial monolayers

Digestive Diseases and Science 2007



ABSTRACT

Surgical handling of the peritoneum has a dual character regarding cancer. In case of massive tissue damage, the ensuing inflammatory reaction produces a potentially lethal cocktail of active mediators, including cytokines and growth factors. The aim of this study was to investigate the effects of inflammatory cytokines, IL-1β, IL-6 and TNF-α, on the interaction between tumour cells and mesothelial cells. An experimental *in vitro* model was designed using Caco2 and HT29 colon carcinoma cells. For all studies, primary cultures of omentum derived mesothelial cells were used. Tumour cell adhesion to a mesothelial monolayer was assessed after overnight pre-incubation of the mesothelium with IL-1β, IL-6 and TNF-α.

Pre-incubation of the mesothelial monolayer with IL-1β or TNF-α resulted in enhanced tumour cell adhesion of the Caco2 and HT29 cells. This effect was dose dependent. Maximum stimulation was achieved at 5 ng/ml ($p < 0.01$). The amount of stimulation for the Caco2 cells was between 20-40% versus the control situation and for HT29 cells between 30-70%. IL-6 did not affect the tumour cell adhesion. Additional blocking experiments with anti-IL-1β and anti-TNF-α resulted in significant inhibition of the cytokine stimulated tumour cell adhesion, demonstrating the specificity of this effect.

The cocktail of intra-abdominal produced mediators have conflicting demeanor regarding wound healing and local tumour recurrence. The presented results prove that IL-1β and TNF-α are significant stimulating factors in tumour cell adhesion *in vitro* and may therefore account for tumour recurrence to the peritoneum *in vivo*.

INTRODUCTION

Colorectal cancer accounts for over half of the cases of gastro-intestinal malignancy. Several new adjuvant treatment modalities improved the overall 5-year survival rate for colorectal carcinomas in the last decade. The most recent 5 year survival rates are, according to a study from France, 36% for local recurrence and 24% for distant metastases.[1] In the United States 80% of the patients with colorectal cancer present with local or locally advanced disease and for this group curative surgery can be attempted. However, up to 40% of these patients develop recurrent disease.[2] Despite of the adjuvant chemotherapeutical therapies locoregional and distant recurrence is still a major problem with colorectal cancer. The most common site for colorectal adenocarcinoma to recur is the site of the primary tumour, the second is the peritoneal surface.[3] Therefore, elimination of locoregional recurrence in the peritoneal cavity may be benefit a significant proportion of patients in terms of survival or quality of life. A profound understanding of the pathophysiology of tumour recurrence may lead to more specific tools to confront the initial process of implantation.

Despite all peroperative precautions tumour spill and subsequent implantation, colonization and growth of these cells in the peritoneal cavity on either intact or traumatized mesothelium and extracellular matrix is an important mechanism accounting for tumour recurrence. Wound healing after surgical trauma induces an inflammatory response during which high amounts of cytokines and growth factors are produced.[4-6] These inflammatory mediators are known to upregulate adhesion molecules and in that manner offering an exceptional environment for the tumour cells to adhere.[7-9]

Aim of this study was to elucidate the role of cytokines with regard to influencing seeding and colonization of tumour cells to the surgically traumatized peritoneal surface. Therefore we developed an *in vitro* model in which we investigated the role of several major inflammatory cytokines, i.c. IL-1β, IL-6 and TNF-α, on the interaction between human mesothelial cells and colon carcinoma cells. We studied the adhesion of the Caco2 and HT29 cells on omental derived mesothelial cells. Furthermore, we studied the expression of adhesion molecules and counterparts on the cells used in this study by means of immunohistochemical staining in order to determine the potential interaction sites between the cells.

MATERIALS AND METHODS

Culture and identification of omental mesothelial cells

Mesothelial cells (MC) were obtained from the omental tissue of patients undergoing elective abdominal surgery. All patients have given informed consent. The MC were isolated according to techniques

modified from Nicholson et al. and Wu et al.[10,11] The omentum was transferred to fluid containing 0.05% trypsin-0.02% EDTA (Invitrogen, Karlsruhe, Germany). After 15 minutes the detached MC were pelleted by centrifugation at 450 g for 10 minutes and were resuspended in RPMI 1640 supplemented with 10% foetal calf serum, L-glutamin (200 mmol/L) and penicillin (5000 U/ml)(Invitrogen, Karlsruhe, Germany). MC were grown to confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (75 cm^2, Corning BV, Schiphol-Rijk, The Netherlands), pre-coated with collagen type I (17.5 µg/ cm^2). The identity of MC was demonstrated by the absence of Von Willebrand factor staining[12] and the presence of intracellular cytokeratins[13] via immunohistological staining with monoclonal antibodies (Dako, Amsterdam, The Netherlands).

Tumour cell line

The human colon adenocarcinoma Caco-2 and HT29 were kind gifts of Dr. W. Dinjens and are both mucinous adenocarcinoma. Both cell lines were cultured in RPMI medium supplemented with 10% foetal calf serum, L-glutamin (200 mmol/L) and penicillin (5000 U/ml). All supplements were obtained from Invitrogen, Karlsruhe, Germany. Before use, cells were trypsinized (5 minutes at 37°C), centrifuged (5 minutes at 400 g), resuspended in RPMI and counted. Viability was measured by trypan blue exclusion and always exceeded 95 percent.

Cytokine pre-incubation

Interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) were obtained from R&D Systems, Uithoorn, The Netherlands. Pre-incubation of the mesothelial monolayer and tumour cells was performed using increasing concentrations of these mediators.
Anti-human IL-1β and anti-human TNF-α were obtained from R&D systems. To inactivate IL-β, pre-incubation was performed with a hundred-fold excess of anti- IL-1β during 1 hour at 37 °C according to the instructions of the manufacturer. After this, the formed IL-1β/anti-IL-1β complex was added to the mesothelial monolayer for pre-incubation. Inactivation of TNF-α was performed in the same way as the inactivation of IL-1β.

Calcein-AM solution and incubation

The dye solution, calcein-AM, used to quantify tumour cell adhesion was prepared by dissolving 50 µg calcein (Molecular Probes, Leiden, The Netherlands) in 5 µl anhydrous dimethyl sulphoxide (Sigma-Aldrich, Zwijndrecht, The Netherlands) and adding this solution to 5 ml of RPMI 1640 medium supplemented with 0.5% bovine serum albumin (RPMI 1640/0.5%BSA, BSA obtained from Sigma-Aldrich). Trypsinized Caco2 and HT29 cells (1x10^6 cells/ml) were incubated in RPMI1640/0.5%BSA at 37 °C for 30 minutes with occasional mixing.

Adhesion assay

To quantify tumour cell adhesion to a monolayer mesothelium, a standardised cell adhesion assay was developed according to methods from Catterall et al.[14] Mesothelial monolayers were established in 96

well plates (Canberra Packerd, Groningen, The Netherlands), pre-coated with collagen type I (15 µg/cm^2, coating according to instructions of the manufacturer). To do this, confluent cells were washed with phosphate buffered saline (PBS) and harvested with trypsin (0.05%) and EDTA (0.02%) and 2.5 x 10^4 mesothelial cells were added in 200 µl of medium to each well. The plates were incubated at 37 °C, in a humidified atmosphere of 5% CO_2 in air; medium was replaced daily with fresh medium. Monolayers reached confluence in 5 to 7 days as determined by light microscopy. In order to determine the effect of the inflammatory cytokines on tumour cell adhesion, mesothelial monolayers were pre-incubated with increasing doses. Non pre-incubated mesothelial monolayers served as controls.
Tumour cells were labelled with calcein as described above. Before adding to the mesothelial monolayers, the labelled cells were washed twice with RPMI1640/0.5%BSA to remove free dye.
Medium from the experimental wells was removed and 200 µl RPMI1640/0.5%BSA containing 30,000 calcein labelled tumour cells was added. Plates were centrifuged for 1 minute at 80 g on a Perkin Elmer centrifuge and incubated at 37 °C for 60 minutes. After this, the medium of each well was removed and washed twice with 200 µl RPMI1640/0.5% BSA medium. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 excitation and 530 emission filters. On each plate a standard was prepared by adding different numbers of labelled tumour cells to the wells. The amount of tumour cells adhered was determined by calibrating the measured fluorescence of the experimental wells on the standard.

DNA-assay

To investigate whether overnight incubation of the mesothelial monolayer with the different cytokines was influencing the cell growth, a DNA measurement was performed. In this assay, the DNA content of the mesothelial cells was measured using the bisbenzimide fluorescent dye (Roch Diagnostics) as previously described by Hofland et al.[15] In short, at the end of the incubation period the plates were washed twice with saline and stored at -20°C until further analysis. The cells were extracted with ammonia solution (1 mmol/L) – Triton x 100 (0.2% v/v) by sonification during 5 seconds at amplitude 15 (Soniprep 150; MSE). Thereafter assay buffer (100 mmol/L NaCl, 10 mmol/L EDTA, 10 mmol/L Tris; pH 7.0) was added. The remaining solution was centrifuged at 2000 g during 5 min and 100 µl aliquots of the supernatant was mixed with 2 ml Hoechst dye H33258 (100 µg/L). Fluorescence was measured after 10 min with the excitation and emission wavelengths set at 350 and 455 nm respectively. The fluorescence of experimental samples were referenced to a standard curve of calf thymus DNA (type II; Sigma, Zwijndrecht, The Netherlands).

Growth curves

The effect of cytokines on tumour cell growth was investigated by incubating the tumour cell lines with 10 ng/ml IL-1β, IL-6 or TNF-α overnight. Pre-incubation was initiated immediately after seeding of the tumour cells in 24 well plates. Growth response to IL-1β, IL-6 and TNF-α (R&D systems,Uithoorn, The Netherlands) was studied at the indicated time-points.

Expression of cytokine receptors and adhesion molecules

Mesothelial cells and the colon carcinoma cell lines were prepared for staining by cytospin preparation, fixed in acetone for 1 minute and stored at –20 °C until use. The cytospins were pre-incubated for 15 minutes with 10% normal goat serum, diluted in PBS, and incubated overnight at 4 °C with goat anti human monoclonal antibodies to IL-1 Ra, IL-6 R, TNF-RI, TNF-RII, ICAM-1, VCAM-1, CD44, LFA-1 and VLA-4 (all obtained from R&D systems).

Statistical analysis

All data were analysed using analysis of variance (ANOVA) to determine overall differences between group means. If the ANOVA was significant on a 5% level, the post-hoc Newman-Keuls test was carried out to make a comparison between groups. $P \leq 0.05$ was considered to be statistically significant. Experiments were carried out at least twice with comparable results.

RESULTS

Validation of assay

Loading of the tumour cells with calcein did not affect their viability (>95% using trypan blue). To determine the stability of calcein uptake, the release of fluorescence in the supernatant of labelled cells after an incubation time of up to 120 minutes was measured. The fluorescence of the washed cells stayed constant for at least 90 minutes indicating retention of the dye in the cells (data not shown). A dilution series was made by using labelled Caco2 and HT29 cells on a mesothelial monolayer. There was a direct relationship between the cell number added and measured fluorescence resulting in a linear correlation which was used as a standard to calibrate the measured fluorescence. In this way the amount of adhered tumour cells in the experimental wells could be determined.

Tumour cell adhesion to a mesothelial monolayer

Tumour cell adhesion to a non pre-incubated mesothelial monolayer was relatively slow and temperature dependent. Maximum adhesion was seen after 60 minutes. At this time point a steady state situation was achieved and cell adhesion did not increase hereafter. The tumour cells remained rounded up during these adhesion assays. After more than 60 minutes, the first signs of spreading out could be detected under the light microscope. Therefore, for all subsequent experiments, 60 minutes was taken as a cut-off point. Non-stimulated cell adhesion was always between 25-50% of the total amount of tumour cells added.

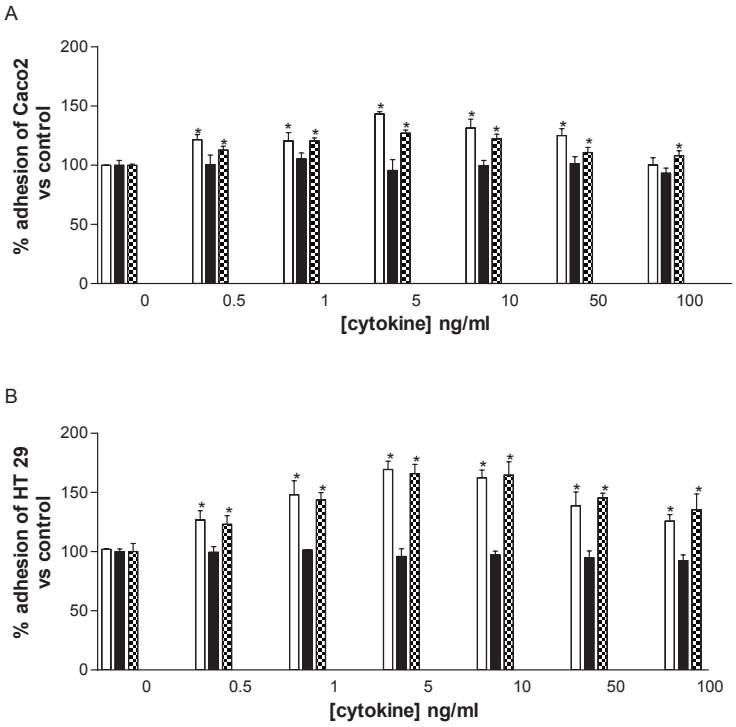


Figure 1

Tumour cell adhesion of Caco2 (A) and HT29 (B) to a mesothelial monolayer after pre-incubation of the monolayer with 0.5, 1, 5, 10, 50 and 100 ng/ml cytokine. Open bars represent IL-1β pre-treated monolayers, closed bars the effect of IL-6 pre-treated monolayers and the blocked bars the effect of TNF-α. Values are the mean (n=6) +SEM. *p<0.01

Effect of cytokine pre-incubation

Pre-incubation of the mesothelial monolayer with IL-1β or TNF-α resulted in enhanced tumour cell adhesion of the Caco2 and HT29 cells. This effect was dose dependent. Maximum stimulation was achieved at 5 ng/ml ($p < 0.01$). The amount of stimulation for the Caco2 cells was between 20-40% versus the control situation and for HT29 cells between 30-70%. Contrary to IL-1β and TNF-α, IL-6 (1-100 ng/ml) did not altered the tumour cell adhesion to a mesothelial monolayer. Results are shown in figure 1. After blocking with anti-IL-1β, stimulation with 1, 5 and 10 ng/ml IL-1β (0.1, 0,5 and 1 μg/ml anti-IL-1β) was completely inhibited (figure 2).

Blocking with a hundred-fold excess of anti-TNF-α inhibited the stimulatory effect of TNF-α as well (figure 2).

Pre-incubation of the tumour cells with IL-1β, TNF-α or IL-6 did not result in a significant increase of adhered tumour cells to the mesothelial monolayers (table 1).

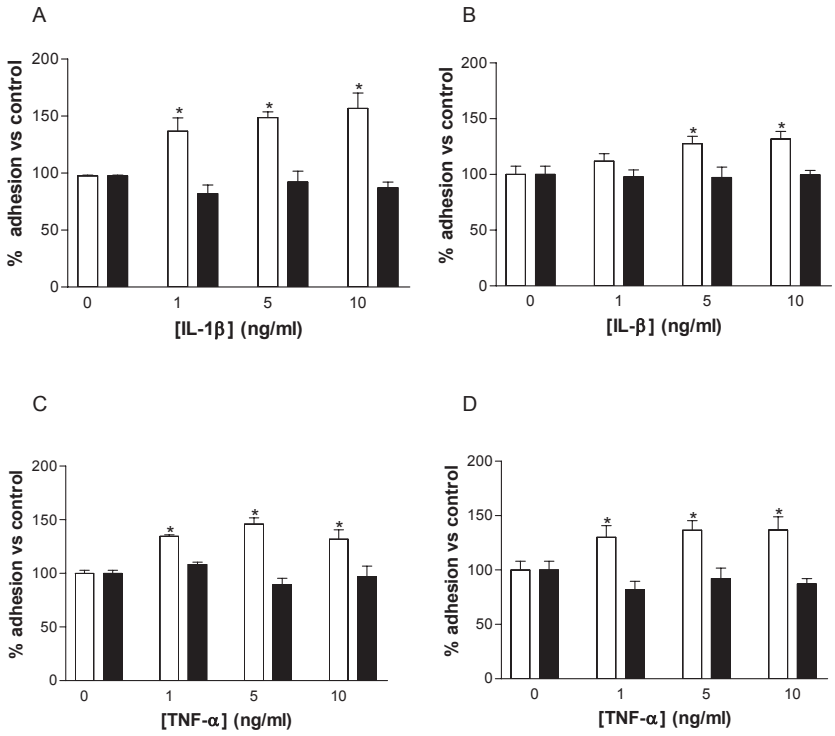


Figure 2
Tumour cell adhesion of Caco2 (A/C) and HT29 (B/D) to a mesothelial monolayer after pre-incubation of the monolayer with 1, 5 and 10 ng/ml IL-1β (open bars figure A and B) or TNF-α (open bars figure C and D). The closed bars represent the additional blocking with anti IL-1β (A/B) or anti TNF-α (C/D). Values are the mean (n=6) +SEM. ∗p<0.01

Table 1

	Control	IL-1β	TNF-α	IL-6
% adhesion Caco2	45 ± 2	47 ± 3	35 ± 3	41 ± 4
% adhesion HT29	29 ± 2	31 ± 3	26 ± 4	33 ± 2

Tumour cell adhesion to a mesothelial monolayer after pre-incubation of the tumour cells with cytokines [5 ng/ml]. Value are the mean of n=6 ± SD.

DNA-assay

Overnight pre-incubation of a mesothelial monolayer with IL-1β, IL-6 or TNF-α did not modify total DNA content of the mesothelial monolayer and therefore did not affect cell growth (figure 3). To investigate the influence of these cytokines on growing tumour cells, we measured the total DNA after 2 and 4 days of incubation of the tumour cells with IL-1β, IL-6 or TNF-α (10 ng/ml). The growth of Caco2 and HT29 cells was inhibited after

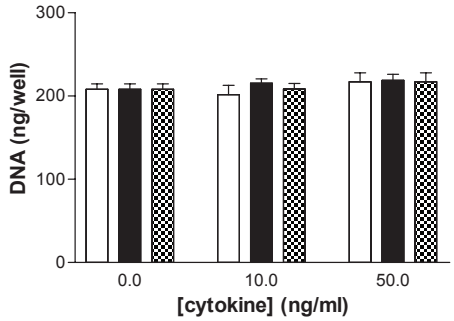


Figure 3
The effect of IL-1β (open bars), IL-6 (closed bars) and TNF-α (blocked bars) on the proliferation of a mesothelial monolayer. Data are expressed as the mean (n=6) +SEM. Pre-incubation did not significantly modify total DNA of the monolayer.

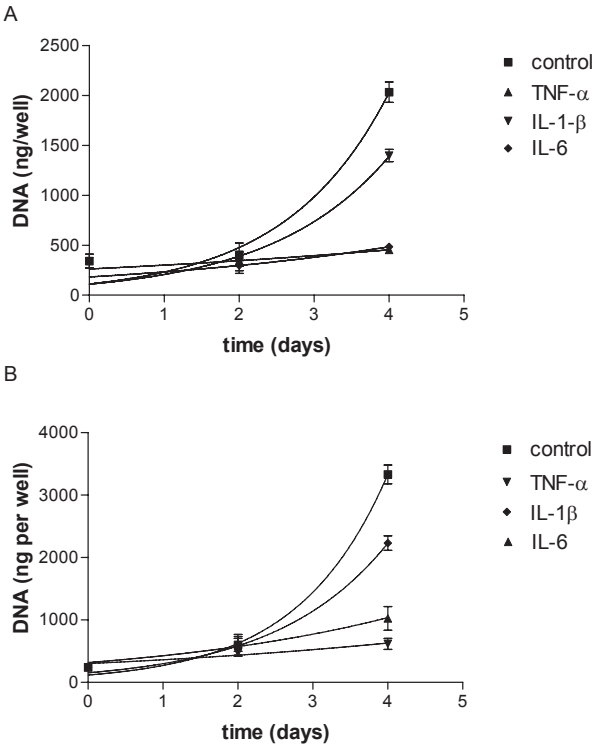


Figure 4
Growth rate of 20,000 Caco2 (A) and HT29 (B) cells incubated with cytokines IL-1β, IL-6 and TNF-α [10 ng/ml] during 4 days.

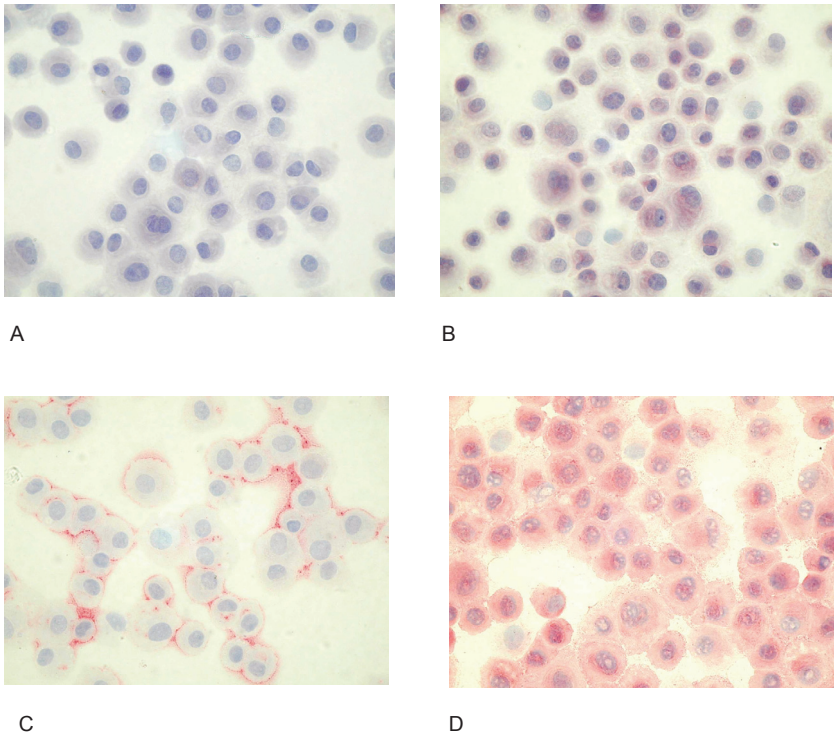


Figure 5
Expression of cytokine receptors by mesothelial cells. Figure A: negative control; B: IL-1β receptor; C: IL-6 receptor; D: TNF-α receptor.

Table 2

	ICAM-1	VCAM-1	CD 44	LFA-1	VLA-4
mesotheel	+	+	+	-	-
Caco2	+	+	+	+	+
HT29	+	+	+	+	+

Cell adhesion molecules and counterparts expressed by mesothelial, Caco2 and HT 29 cells according to the immunohistochemical staining.

4 days when the cells were grown in RPMI 1640 10% FCS enriched with TNF-α or IL-6 (10ng/ml). IL-1β (10ng/ml) incubation of Caco2 and HT29 cells resulted in a minor growth inhibition. (figure 4) These results prove the functionality of the cytokine receptors on the tumour cells.

Expression of cytokine receptors and adhesion molecules

The results of the staining of mesothelial cells are shown in figure 5 and clearly shows that these cells express receptors for IL-1β, IL-6 and TNF-α.

Table 2 exhibit the results of the immunohistochemical staining of the mesothelial cells and colon carcinoma cancer cell lines with the adhesionmolecules and counterparts. The mesothelial cells stain positive for ICAM-1, VCAM-1 and CD44. HT29 and Caco2 cell lines are both positive for ICAM-1, VCAM-1, CD44, LFA-1 and VLA-4. The negative controls, the positive stained mesothelial cells and the counterparts on the colon carcinoma cell lines are shown in figure 6.

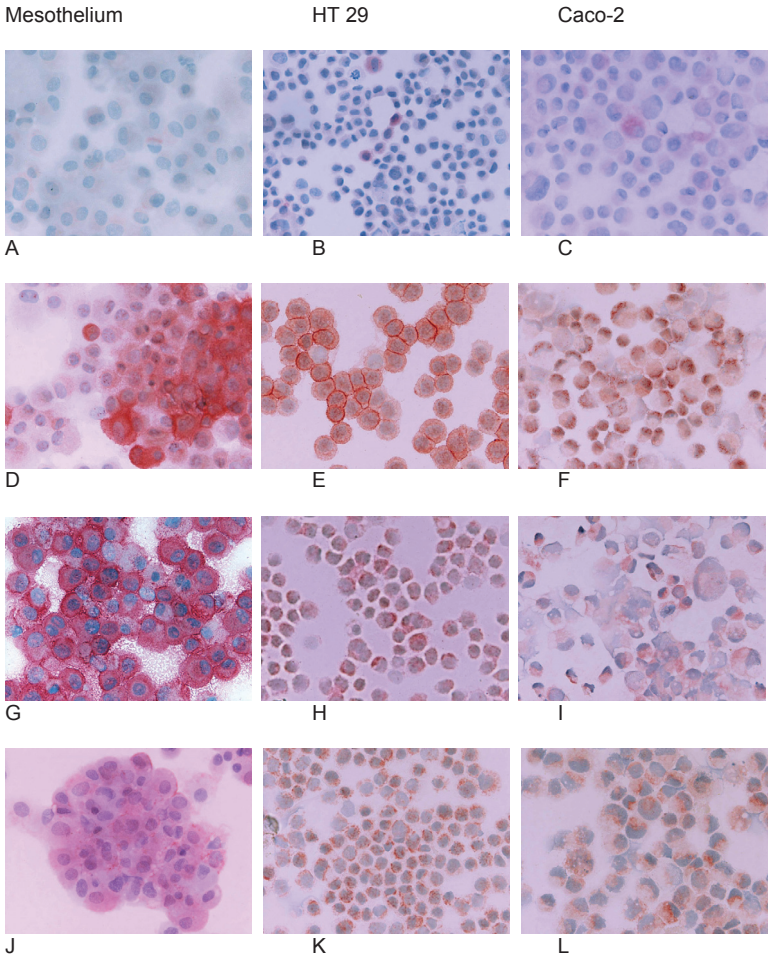


Figure 6
Expression of cell adhesion molecules and counterparts after immunohistochemical staining of mesothelial, HT29 and Caco-2 cells. Figures A,B,C: negative control; D,E,F: CD44; G: ICAM-1; H, I: LFA-1; J: VCAM-1; K,L; VLA-4.

DISCUSSION

Local recurrence of colorectal cancer is major clinical problem. Spilled tumour cells are preferential distributed to the resection site, and combined recurrences to the resection site and peritoneal surfaces are common.[3] The mechanism behind this process has not been unravelled yet.

Following abdominal surgery there will be a reactive inflammatory response, the acute phase response, during which cytokines and growth factors are produced.[16] These cytokines are known to be conducive to wound healing by initiating the cellular cascade in the course of which macrophages and leukocytes migrate to the injured site.[17] In addition to the predominantly production of cytokines by inflammatory cells, the mesothelial cells can also produce several chemokines during peritoneal injury.[4,6] In our experiments we evaluated the effects of the cytokines IL-1β, Il-6 and TNF-α on the adhesion of colon carcinoma cells to mesothelial monolayers. IL-1β is one of the most investigated pro-inflammatory cytokine and the concentration in postoperative peritoneal fluid increased significantly and therefore might be of great importance in the peritoneal cavity.[18] IL-6 is an early marker of tissue damage and the production of IL-6 by mesothelial cells is influenced by IL-1 and TNF-α in a time and dose-dependent manner.[19-21] TNF-α has a significant function in the abdominal cavity and might modulate the inflammatory response by inducing the production of ILs from mesothelial cells.[22,23] Postoperative concentrations of TNF-α in the peritoneal cavity are compared to IL-1β increased as well.[18]

Our data advocate the enhancement of adhesion of Caco2 and HT29 cells to a mesothelial monolayer after incubation with IL-1β or TNF-α in a dose-dependent way. This effect was completely inhibited by blocking with anti-IL-1β and anti-TNF-α, which underline the specificity of IL-1β and TNF-α stimulation and the involvement of a specific related pathway at which enhanced tumour cells adhesion to the mesothelial monolayer might be induced. Pre-incubation of the mesothelial monolayers with the different cytokines used in this study did not change the total DNA content and therefore demonstrate that the increased adhesion of colon carcinoma cells to the mesothelium is not caused by an enhanced number of mesothelial cells. Other investigators demonstrate the same effect of cytokines on the enhancement of adhesion with different tumour cell lines, like gastric tumour cells, melanoma cells and ovarian tumour cells.[24-27] IL-1β and TNF-α are also potent stimulators of adhesion of colon carcinoma cells to endothelial cells.[28]

The bell shape curves of cytokine stimulated adhesion are most likely caused by the fact that a high concentration of cytokine might stimulate mesothelial cells to produce mediators which can inhibit the stimulation of adhesion.[29] Another explanation might be the down-regulation of cytokine receptors at high concentrations of the ligand.

The cytokines are not influencing the growth of mesothelial cells in contrast to their inhibitory effect on the growth of the colon carcinoma cell lines. This inhibitory effect

demonstrate the functionality of the cytokine receptors on the colon carcinoma cell lines. Other studies have shown this suppressive effect of IL-1β and TNF-α on proliferating colon carcinoma cell lines as well.[30-32] The inhibition of IL-6 on colon carcinoma cell growth was not studied before.

The enhancement of adhesion in this study is most likely originated by an upregulation of adhesion molecules. According to our immunohistochemical results mesothelial cells express ICAM-1, VCAM-1 and CD44. Caco2 and HT29 cells express, adjacent to the molecules expressed by the mesothelial cells, also the counterparts LFA-1 and VLA-4. These results suggest abundant possibilities for cell-cell and cell-matrix interactions under non-stimulated conditions. The expression patterns are similar to those observed in the literature. Several studies investigated the influence of TNF-α and IL-1β on the expression of adhesion molecules on mesothelial cells. ICAM-1 and VCAM-1 expression is upregulated by these cytokines, which manifest the importance of these molecules in the interaction with colon carcinoma cells after stimulation of the mesothelium with IL-1β and TNF-α.[7,8]

After stimulation of the colon carcinoma cell lines with the inflammatory cytokines there is no remarkable enhancement of the adhesion to the mesothelial monolayer, suggesting that the mesothelial monolayer is the primary cell involved in increased adhesion induced by inflammatory cytokines. Expression of ICAM-1 on HT-29 and Caco2 cells is upregulated by TNFa and IL-β, however mesothelial cells do not express its counterpart LFA-1, which might be the explanation for the fact that there was not an enhancement of adhesion after stimulation of the colon carcinoma cell lines with the cytokines used in this study.[33,34]

In conclusion, inflammatory cytokines released following surgical wounding can cause an environment which is beneficial to tumour cell adhesion and possibly increases the metastatic potential of local tumour cells. This study demonstrate that the enhancement of colon carcinoma cells to a monolayer mesothelium is induced by IL-β and TNFa. This enhancement might be derived from an upregulation of ICAM-1, VCAM-1 and CD44 on mesothelial cells. Future experiments has to source out which of the cell-cell and cell-matrix interactions are the most important in tumour cell adhesion in order to develop site-specific drug delivery systems and in that way block the tumour cells to adhere.

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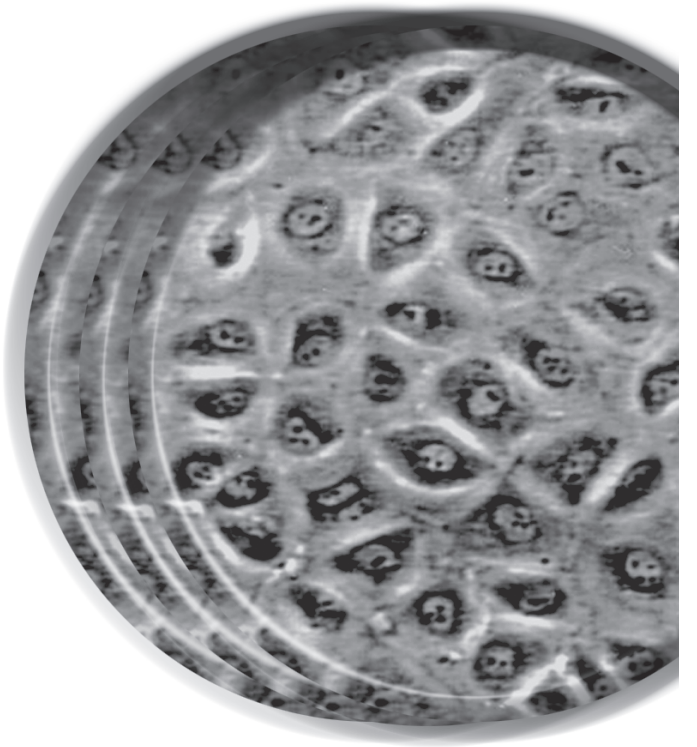
CHAPTER 3

The expression of adhesion molecules and the influence of inflammatory cytokines on the adhesion of human pancreatic carcinoma cells to mesothelial monolayers

Pancreas 2006



ABSTRACT

Pancreatic cancer has a tremendously deplorable prognosis. Peritoneal dissemination frequently occur after surgical resection of the tumour. The aim of this study was to investigate the effects of inflammatory cytokines, IL-1β, IL-6 and TNF-α, on the interaction between pancreatic tumour cells and mesothelial cells. Primary cultures of mesothelial cells were incubated with the inflammatory cytokines and after the incubation the adherence of the different pancreatic cell lines was measured. By means of immunocytochemical staining and enzyme immunoassay, the expression of adhesion molecules was investigated.

Pre-incubation of the mesothelial monolayer with IL-1β or TNF-α resulted in enhanced tumour cell adhesion of the Mia-PaCa-2 and BxPC-3 cells. The amount of stimulation for the Mia-PaCa-2 cells was more than 100% versus the control situation and for BxPC-3 cells between 20-35%. IL-6 did not affect the tumour cell adhesion of the Mia-PaCa-2 and BxPC-3 cells. The adherence of Panc-1 was not enhanced after pre-incubation of the mesothelial monolayers with the inflammatory cytokines. Mesothelial cells show an significant enhancement of expression of ICAM-1, VCAM-1 and CD44 after stimulation with IL-1β and TNF-α.

The presented results prove that IL-1β and TNF-α are significant stimulating factors in pancreatic tumour cell adhesion *in vitro* and may therefore account for tumour recurrence to the peritoneum *in vivo*. The immunocytochemical staining results demonstrate that ICAM-1 and CD44 important adhesion molecules and interference with their function may decrease the incidence of peritoneal tumour recurrence after curative resection of pancreatic cancer.

INTRODUCTION

Pancreatic cancer has a very poor prognosis and is often in an advanced state of disease at the time of diagnosis. In the first year more than 90% patients die and the 5-year survival is less than 5%. The intra-abdominal recurrence rate after curative resection ranged between 38% and 86%.[1-3] The most common site of local recurrence is the resection site, but all the traumatized peritoneal sites are at risk for tumour cell entrapment. The mechanism of local recurrence is still poorly understood.

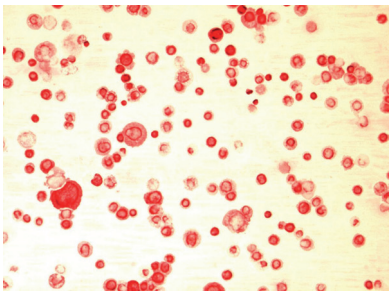
In blood-borne metastasis the attachment of carcinoma cells to endothelial cells is a crucial step. The endothelium is activated by inflammatory cytokines and polymorphonuclear leukocytes or lymfocytes adhere to the activated endothelium. In the same manner tumour cells attach to the endothelium. This process is mediated by E-selectin on the endothelial cells and Sle^a on the pancreatic tumour cells.[4] Contrary to blood-borne metastasis studies on pancreatic cancer, very few studies report on the mechanism of local tumour recurrence. The first study in which local recurrence of pancreatic tumour cells to components of the extracellular matrix has been investigated was done in 1981 by McIntyre *et al*. This study shows that the adhesion of MiaPaCA-2 cells to collagen is stimulated by fibronectin, present in fetal bovine serum.[5] In other studies the pancreatic tumour cells are stimulated and exposed to the extracellular matrix.[6,7] Beside the extracellular matrix the traumatized peritoneum may induce the release cytokines and growth factors. Our study is based on the theory that these inflammatory mediators upregulate the adhesion molecules on the mesothelial cells and in that way causes an enhancement of adhesion of tumour cells to the peritoneum.[8-12]

In the present study, we developed an *in vitro* adhesion model to quantify the adhesion of three human pancreatic cell lines to cytokine activated mesothelial monolayers. The pancreatic tumour cell lines we used were Panc-1 and Mia-PaCa-2, which are non-metastatic cell lines, and BxPC-3, which is a metastatic cell line.[13] The aim of this reproducible model was to demonstrate the effect of inflammatory cytokines, produced after surgical trauma, on the adhesion of tumour to the mesothelium *in vitro*. Little is known about the expression of adhesion molecules (ICAM-1 and VCAM-1) and counterparts (LFA-1 and VLA-4) on the pancreatic tumour cell lines. Therefore the pancreatic carcinoma cell lines and the mesothelium were analyzed by means of immunocytochemical staining to investigate the expression of adhesion molecules and counterparts in order to verify important interactions between the investigated cell types. Following the results of the immunocytochemistry, analysis of the upregulation of adhesion molecules was done by means of Enzyme Immuno Elisa (EIA).

MATERIALS AND METHODS

Culture and identification of omental mesothelial cells

Mesothelial cells (MC) were obtained from the omental tissue of patients undergoing elective abdominal surgery who had given informed consent. The experimental protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam, The Netherlands. The MC were isolated according to techniques modified from Nicholson et al. and Wu et al.[14,15] The omentum was transferred to fluid containing 0.05% trypsin-0.02% EDTA (Invitrogen, Karlsruhe, Germany). After 15 minutes the detached MC were pelleted by centrifugation at 1800 rpm for 10 minutes and were resuspended in RPMI 1640 supplemented with 10% foetal calf serum, L-glutamin (200 mmol/L) and penicillin (5000 U/ml)(Invitrogen, Karlsruhe, Germany). MC were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (75 cm^2, Corning BV, Schiphol-Rijk, The Netherlands) pre-coated with collagen type I (17.5 µg/ cm^2). The identity of MC was demonstrated by the absence of Von Willebrand factor (F8) and the presence of intracellular keratin and vimentin via immunocytochemical staining with monoclonal antibodies (Dako, Amsterdam, The Netherlands).[16,17] We only used pure cultures of MC for our experiments. The immunocytochemical stainings of the MC are shown in figure 1.



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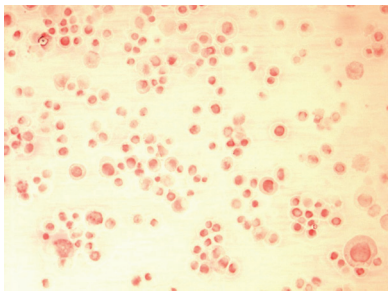

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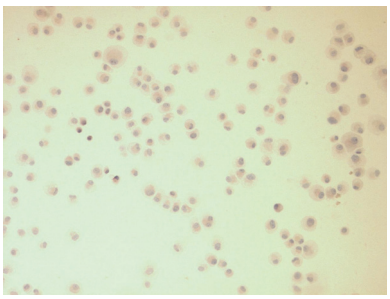

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Figure 1
Immunocytochemical staining of mesothelial cells for keratin (A), vimentin (B) and Von Willebrand factor (C).

Tumour cell line

The pancreatic carcinoma cell lines, Panc-1, Mia-PaCa-2 and BxPC-3 were kind gifts of Dr. W. Dinjens. All cell lines were cultured in RPMI1640 medium supplemented with 10% foetal calf serum, L-glutamin (200 mmol/L) and penicillin (5000 U/ml). All supplements were obtained from Invitrogen, Karlsruhe, Germany. Before use, cells were trypsinized (5 minutes at 37°C), centrifuged (5 minutes at 1500 rpm), resuspended in RPMI 1640 and counted. Viability was measured by trypan blue exclusion and always exceeded 95 percent.

Cytokine pre-incubation

Interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) were obtained from R&D Systems, Uithoorn, The Netherlands. Pre-incubation of the mesothelial monolayer and tumour cells was performed using increasing concentrations of these mediators.

Calcein-AM solution and incubation

The dye solution, calcein-AM, used to quantify tumour cell adhesion was prepared by dissolving 50 µg calcein (Molecular Probes, Leiden, The Netherlands) in 5 µl anhydrous dimethyl sulphoxide (Sigma-Aldrich, Zwijndrecht, The Netherlands) and adding this solution to 5 ml of RPMI 1640 medium supplemented with 0.5% bovine serum albumin (RPMI 1640/0.5%BSA, BSA obtained from Sigma-Aldrich). Trypsinized Panc-1, MiaPaCa-2 and BxPC-3 cells ($1x10^6$ cells/ml) were incubated in RPMI1640/0.5%BSA at 37 °C for 30 minutes with occasional mixing.

Adhesion assay

To quantify tumour cell adhesion to a monolayer mesothelium, a standardized cell adhesion assay was developed according to methods from Catterall et al..[18] Mesothelial monolayers were established in 96 well plates (Canberra Packerd, Groningen, The Netherlands), pre-coated with collagen type I (15 µg/cm^2, coating according to instructions of the manufacturer). To do this, confluent cells were washed with phosphate buffered saline (PBS) and harvested with trypsin (0.05%) and EDTA (0.02%) and 2.5×10^4 mesothelial cells were added in 200 µl of medium to each well. The plates were incubated at 37 °C, in a humidified atmosphere of 5% CO_2 in air; medium was replaced daily with fresh medium. Monolayers reached confluence in 5 to 7 days as determined by light microscopy. In order to determine the effect of the inflammatory cytokines on tumour cell adhesion, mesothelial monolayers were pre-incubated with increasing doses. Non pre-incubated mesothelial monolayers served as controls.
Tumour cells were labeled with calcein as described above. Before adding to the mesothelial monolayers, the labeled cells were washed twice with RPMI1640/0.5%BSA to remove free dye.
Medium from the experimental wells was removed and 200 µl RPMI1640/0.5%BSA containing 30,000 calcein labeled tumour cells was added. Plates were centrifuged for 1 minute at 80 g on a Perkin Elmer centrifuge and incubated at 37 °C for 60 minutes. After this, the medium of each well was removed and washed twice with 200 µl RPMI1640/0.5% BSA medium. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 excitation and 530 emission filters. On each plate a

standard was prepared by adding different numbers of labeled tumour cells to the wells. The amount of tumour cells adhered was determined by calibrating the measured fluorescence of the experimental wells on the standard.

Expression of adhesion molecules and their counterparts

Mesothelial cells and the pancreatic tumour cell lines were prepared for staining by cytospin preparation, fixed in acetone for 1 minute and stored at –20 °C until use. The cytospins were pre-incubated for 15 minutes with 10% normal goat serum, diluted in PBS, and incubated overnight at 4 °C with goat anti human monoclonal antibodies to ICAM-1, VCAM-1, CD44, LFA-1 and VLA-4 (all obtained from R&D systems).

Enzyme Immuno Assay (EIA)

An enzyme immunoassay was used to determine the effect of the cytokines on the expression of adhesion molecules. Mesothelial cells were grown to confluence in 96-well flat-bottomed multititer plates and pre-incubated with either cell culture medium alone or combined with IL-1β, IL-6 or TNF-α for 12 hours. Medium from the experimental wells was removed and the cells were fixed in 95% ethanol/ 5% methanol solution for 45 minutes. Subsequently, the cells were pre-incubated for 10 minutes with 1% normal goat serum, diluted in PBS, to inactivate unspecific binding. Mouse anti-human monoclonal antibodies to ICAM-1 (CD54, R&D Systems), VCAM-1 (CD106, R&D Systems), CD44 (CD106, R&D Systems) was added for 60 minutes. The cells were subsequently incubated with biotinylated goat antimouse antibody (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) in a dilution of 1 : 500 for another 60 minutes. Finally the cells were incubated with a Streptavidin-peroxidase complex for 30 minutes, washed, and substrate (2,2'-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) in 0,05M phosphate-citrate buffer containing 0,03% sodium perborate was added. Incubation of tumour cells or mesothelial cells without the primary antibody served as a negative control. After 40 minutes, the reaction was stopped using 0,4% sodium fluoride. Photometrical evaluation was performed with a computer-controlled ELISA-plate reader at λ = 405 nm.

Statistical analysis

All data were analyzed using analysis of variance (ANOVA) to determine overall differences between group means. If the ANOVA was significant on a 5% level, the post-hoc Newman-Keuls test was carried out to make a comparison between groups. $P \leq 0.05$ was considered to be statistically significant. Experiments were carried out at least twice with comparable results.

RESULTS

Validation of assay

Loading of the tumour cells with calcein did not affect their viability (>95% using trypan blue). To determine the stability of calcein uptake, the release of fluorescence in the

supernatant of labeled cells after an incubation time of up to 120 minutes was measured. The fluorescence of the washed cells stayed constant for at least 90 minutes indicating retention of the dye in the cells (data not shown). A dilution series was made by using labeled Panc-1, MiaPaCa-2 and BxPC-3 cells on a mesothelial monolayer. There was a direct relationship between the cell number added and measured fluorescence resulting in a linear correlation which was used as a standard to calibrate the measured fluorescence. In this way the amount of adhered tumour cells in the experimental wells could be determined.

Tumour cell adhesion to a mesothelial monolayer

Tumour cell adhesion to a non pre-incubated mesothelial monolayer was relatively slow and temperature dependent. Maximum adhesion was seen after 60 minutes. At this time point a steady state situation was achieved and cell adhesion did not increase hereafter. The tumour cells remained rounded up during these adhesion assays. After more than 60 minutes, the first signs of spreading out could be detected under the light microscope. Therefore, for all subsequent experiments, 60 minutes was taken as a cut-off point.

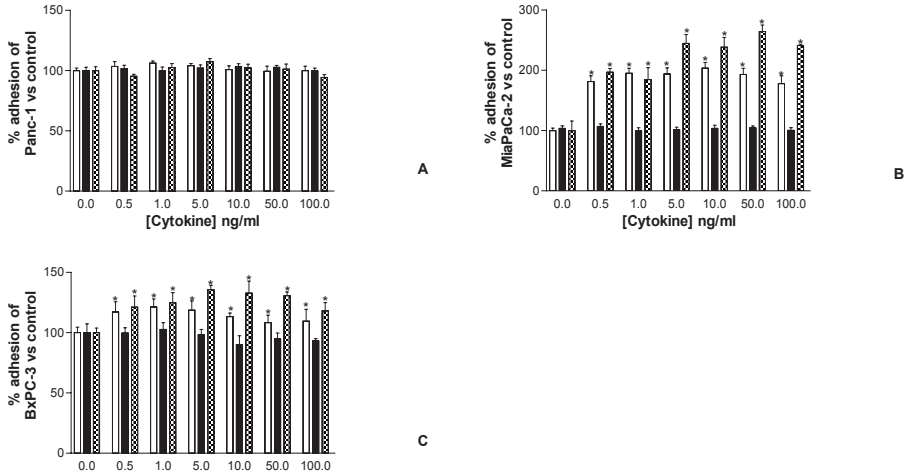

Figure 2

Tumour cell adhesion of Panc-1 (A), MiaPaCa-2 (B) and BxPC-3 (C) to a mesothelial monolayer after pre-incubation of the monolayer with 0.5, 1, 5, 10, 50 and 100 ng/ml cytokine. Open bars represent IL-1-β pre-treated monolayers, closed bars the effect of IL-6 pre-treated monolayers and the blocked bars the effect of TNF-α. Values are the mean (n=6) +SEM. *p<0.01 vs. control.

Effect of cytokine pre-incubation

Overnight incubation of a mesothelial monolayer with IL-1β, IL-6 or TNF-α did not modify total DNA content of the mesothelial monolayer and therefore did not affect cell growth (data not shown).

The adhesion of the Panc-1 cells in the control situation was 49%. Pre-incubation of the mesothelial monolayer with IL-1β, IL-6 or TNF-α did not resulted in enhanced tumour cell adhesion of the Panc-1 cells (figure 2A).

In the control situation the adhesion of Mia PaCa-2 cells was 13%. Pre-incubation of the mesothelial cells with IL-1β or TNF-α resulted in an significant enhancement of adhesion of the MiaPaCa-2 cells of more than 100%. Pre-incubation with IL-6 had no effect on the adhesion (figure 2B).

In the control group 28% of the BxPC-3 cells adhere to the mesothelium. Pre-incubation of the mesothelial cells with IL-1β or TNF-α caused an enhancement of the adhesion of the BxPC-3 cells. This enhancement was for IL-1β and TNF-α dose-dependent, with a maximum stimulation at respectively 1 and 5 ng/ml ($p<0.01$). The amount of stimulation was for the BxPC-3 cells between 20-35% versus the control situation. Contrary to IL-1β and TNF-α, IL-6 (1-100 ng/ml) did not altered the tumour cell adhesion to a mesothelial monolayer (figure 2C).

Table 1

	ICAM-1	VCAM-1	CD 44	LFA-1	VLA-4
mesothelium	+	+	+	-	-
Panc1	+	+	+	-	-
Miapaca2	-	-	+	-	-
Bxpc3	+	-	+	+	-

Cell adhesion molecules (ICAM-1, VCAM-1 and CD 44) and counterparts (LFA-1, VLA-4) expressed by mesothelial, Panc-1, MiaPaCa-2 and BxPC-3 cells.

Expression of adhesion molecules and counterparts

The results of the immunocytochemical staining of the mesothelial cells and the three pancreatic cancer cell lines are shown table 1. The mesothelial cells stain positive for ICAM-1, VCAM-1 and CD44. Panc-1 is positive for ICAM-1, VCAM-1 and CD44. MiaPaCa-2 is positive only for CD44 and the BxPC-3 cells stain positive for ICAM-1, CD44 and LFA-1.

The EIA is a semi quantitative assay giving the opportunity to investigate the dynamics of expression of adhesion molecules on mesothelial cells with or without stimulating the cells with IL-1β, IL-6 or TNF-α (in optical density (OD) units measured at 405 nm). The staining intensity for ICAM-1 of unstimulated mesothelial cells was 0.623, whereas the negative control gave an intensity of 0.118, indicating a marked basal expression of ICAM-1 on mesothelial cells (figure 3A). Optical density (OD) for VCAM-1 on mesothelial cells

and the negative control were 0.505 and 0.137 respectively. And the optical density for CD44 on mesothelial cells was 0.342 with a negative control of 0.184, indicating that mesothelial cells have a basal expression of VCAM-1 and CD44 as well (figure 3B,C). Overnight incubation of the mesothelial cells with TNF-α resulted in an enhanced expression of ICAM-1 (mean OD 0.73) and VCAM-1 (mean OD 0.65). Stimulating the mesothelial cells with IL-1β for 12 hours a significant overexpression of ICAM-1

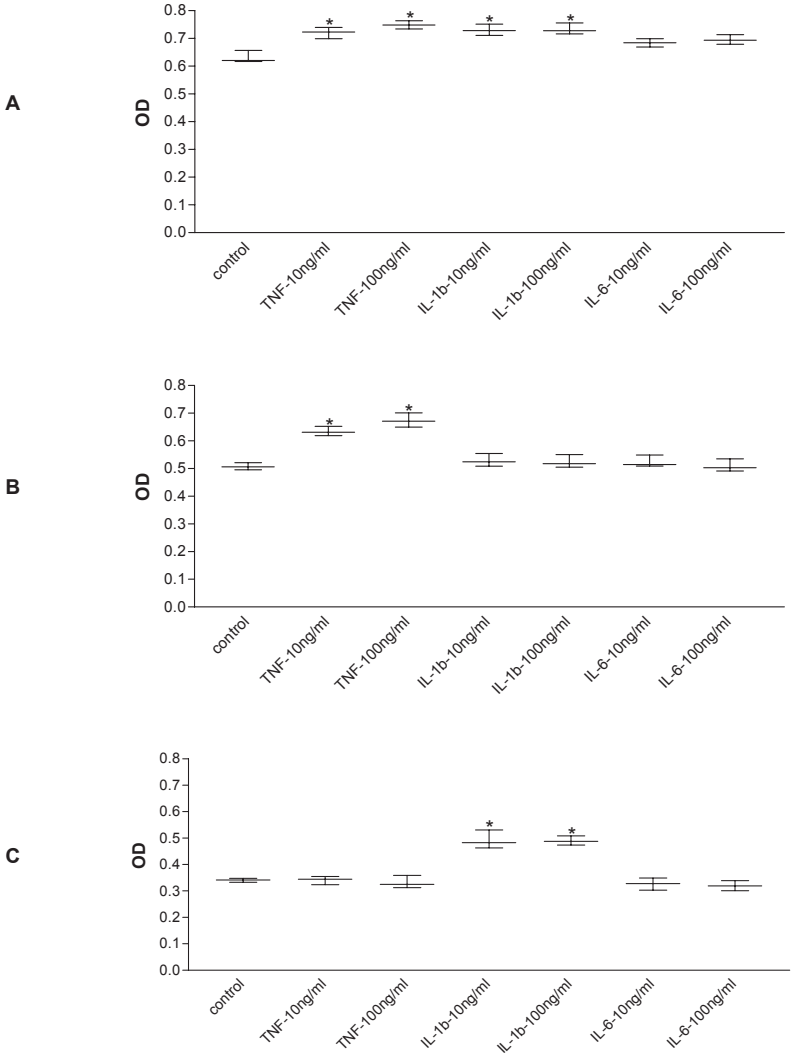

Figure 3
Expression of ICAM-1 (A), VCAM-1 (B) and CD44 (C) on mesothelial cells stimulated with IL-1β, TNF-α or IL-6. *p<0.01 vs. control. (OD = optical density units measured at 405 nm)

(mean OD 0.74) and CD44 (mean OD 0.49) could be observed. Incubation of the mesothelial cells with IL-6 has no influence on the expression of the adhesion molecules (figure 3A-C).

DISCUSSION

Pancreatic cancer has a poor prognosis. Only 15-20% of the patients with pancreatic cancer have a resectable tumour at the time of diagnosis. Complete macroscopically resection of the tumour does not prevent the development of early recurrences at the resection site or the peritoneum. This study provides an insight in the mechanism of invasion and implantation of cancer cells to the resection site or peritoneum after curative surgery.

Cytokines produced during surgery are known to influence tumour recurrence *in vivo*.[19-21] Surgical trauma causes an inflammatory response during which leucocytes and macrophages migrate to the injured site in order to promote wound healing.[22] Mesothelial cells, lining the peritoneum have an active part in the inflammatory process by producing a variety of cytokines such as IL-1β and IL-8.[8,23] This will cause a rich micro-environment at traumatized peritoneal sites with high concentrations cytokines and growth factors. This study investigated the adherence pattern of three pancreatic tumour cell lines to mesothelial monolayers. Pre-treatment of the monolayer mesothelium with IL-1β or TNF-α causes an upregulation of adhesion of the MiaPaCa-2 and BxPC-3 cells. The adhesion of Panc-1 cells is not enhanced after stimulation of the mesothelium with the cytokines used in this study, probably caused by the high amount of adhered tumour cells in the control situation. Overnight pre-incubation of the mesothelial monolayer with IL-1β, IL-6 or TNF-α did not modify total DNA content of the mesothelial monolayer and therefore did not affect cell growth. This suggests that the increased adhesion induced by IL-1β and TNF-α is not caused by an increased number of mesothelial cells and that these cytokines are not influencing confluent monolayers.

Studies on cell adhesion have been done to investigate the adhesion of leucocytes on endothelial and mesothelial cells during inflammation. These studies conclude that leucocyte enrolling is caused by an upregulation of ICAM-1, VCAM-1, E-selectin and P-selectin on endothelial or mesothelial cells and in that way induces leucocyte attachment and extravasation.[8, 24-26] Tumour cells may utilize this upregulated expression of adhesion molecules and in that way adhere to the mesothelium.

The mechanism of adhesion of pancreatic cells to mesothelial cells has not been investigated extensively. Hosono et al. studied the adhesion of the SW1990 cell line to mesothelial cells and investigated that antibodies against integrin β1 and CD44 can block the adhesion to mesothelium. CD44 and integrin play important roles in the

initial attachment of SW1990 cells to mesothelial cells.[27] In haemotogenic metastasis of pancreatic tumours E-selectin and the counterpart sialylated Lewis^a on tumour cells plays an important role. This process is enhanced by cytokines IL-1β and TNF-α.[4]

Investigations on expression of integrins and attachment to the extracellular matrix proteins are ample. β1 integrins on different pancreatic carcinoma cell types have various levels of constitutive activity and pancreatic carcinoma cells with higher constitutive activity of β1 integrin are more invasive. Blocking this integrin inhibited the invasion of pancreatic carcinoma cells. Cytokines modulate the integrin expression and in that way enhance the attachment of pancreatic tumour cells to the extracellular matrix.[6, 28-30]

Very few studies report on the expression of adhesion molecules and counterparts on pancreatic tumour cells. The expression of adhesion molecules on the cells used in this study are conclusive for different possibilities for cell-cell and cell-matrix interaction under non stimulated condition. In the stimulated situation IL-1β causes an significant enhancement of expression of VCAM-1 and CD44 on mesothelial cells. Incubation of the mesothelial cells with TNF-α increased the expression of ICAM-1 and VCAM-1. IL-6 is not influencing the expression of the studied adhesion molecules on mesothelial cells, and therefore not enhancing the adhesion of pancreatic tumour cells to mesothelial monolayers in the adhesion assay. From the known interaction of adhesion molecules, these results suggest that CD44 and ICAM-1 are important molecules in the adhesion of pancreatic tumour cells to mesothelial cells. Although TNF-α upregulates VCAM-1 as well, VCAM-1 is not a key adhesion molecule in this model, because the counterpart VLA-4 is not expressed by the pancreatic tumour cell lines. Only BxPC-3 can benefit from overexpression of ICAM-1 on the mesothelial cells because this pancreatic cell line expresses the counterpart LFA-1.

Tumour cell invasion and metastasis is a multistep process that involves also a variety of cell surface receptors that mediate interactions with the mesothelial cells, extracellular matrix and endothelial cells. This study comes up with an comprehension of the adhesion pattern of three different pancreatic tumour cell lines to mesothelial cells and an overview of the expression of adhesion molecules and their counterparts on the tumour and mesothelial cells. Our data suggest that the adhesion is enhanced by stimulation of the mesothelial cells with cytokines IL-1β and TNF-α. These cytokines are produced during surgery and upregulates adhesion molecules and can cause in that manner local tumour recurrence of spilled tumour cells. Interference with the function of cell adhesion molecules may decrease the incidence of local and distant metastasis and enhance the prognosis of patients with pancreatic cancer after surgery. In future studies the mechanism of adhesion of tumour cells has to be unraveled further and in that way prevent tumour recurrence after curative resection of pancreatic cancer.

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CHAPTER 4

Surgery derived reactive oxygen species produced by pmn promote tumour recurrence, studies in an *in vitro* model

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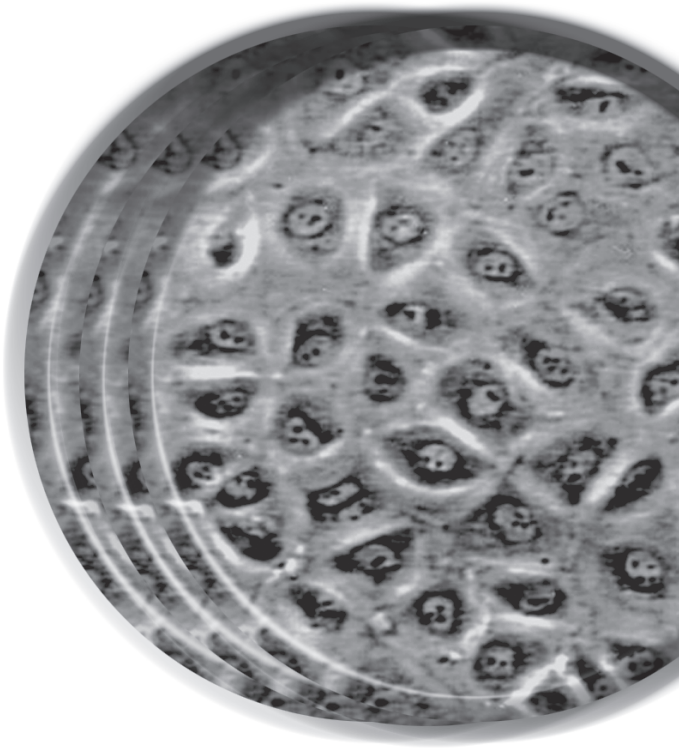

ABSTRACT

Tissue injury induces the acute phase response, aimed at minimizing damage and starting the healing process. Polymorphonuclear leukocytes (PMN) respond to the presence of specific chemoattractants and begin to appear in large numbers. The aim of this study is to investigate the influence of reactive oxygen species (ROS) produced by PMN on the interaction between colon carcinoma cells and mesothelial cells. Tumour cell adhesion to a mesothelial monolayer was assessed after pre-incubation of the mesothelium with stimulated PMN and unstimulated PMN. Mesothelial cells were also incubated with Xanthine/Xanthine Oxidase (X/XO) complex producing ROS after which adhesion of Caco-2 cells was investigated and the expression of adhesion molecules (ICAM-1, VCAM-1 and CD44) by means of enzyme immunoassay (EIA).

In the control situation the average adhesion of Caco2 cells to the mesothelial monolayers was 23%. Mesothelial monolayers incubated with unstimulated PMN showed a 25% increase of tumour cell adhesion ($p<0.05$). The adhesion of tumour to the monolayers incubated with the FMLP stimulated PMN increased with 40% ($p<0.01$). Incubation of the mesothelium with X/XO resulted in an enhancement of adhesion of Caco-2 cells of 70% and an upregulation of expression of ICAM-1, VCAM-1 and CD44.

This study reveals an increase of tumour cell adhesion to the mesothelium induced by incubating the mesothelial monolayers with PMN. PMN are producing a number of products, like proteolytic enzymes, cytokines and reactive oxygen species (ROS). These factors upregulate the expression of adhesion molecules and in that way stimulate the adhesion of tumour to the mesothelium.

INTRODUCTION

Surgical treatment of gastro intestinal malignancies is often complicated by locoregional tumour recurrence [1]. Regardless this detrimental adversity, surgery remains the best treatment option. Therefore, many attempts have been undertaken, in clinical and research settings, to elucidate the effect of surgical trauma on tumour recurrence. In a clinical trial, Busch *et al.* put forward an association between tumour recurrence and the extent of surgical injury [2]. In experimental models, post surgically produced factors have been shown to augment local and remote tumour growth. Experimental and clinical studies have demonstrated that enhanced tumour cell adherence and tumour growth are inevitable repercussions of surgical trauma [1,3-7].

Nowadays, there is a growing interest in the pathophysiological mechanism on the basis of which surgical trauma leads to enhanced tumour recurrence.

Surgery is an inflammatory stimulus that activates the body's immune response. The ensuing influx of polymorph nuclear leukocytes (PMN) and mononuclear cells to the surgically traumatized site is mediated through chemotactic factors and pro-inflammatory cytokines such as IL-1β or TNF-α and is the first line of defense.[8,9]

Insights in the host defense mechanisms of the peritoneum have demonstrated that peritoneal lymphocytes, submesothelial monocytes, PMN and mesothelial cells act in an orchestrated response under the control of locally expressed cytokines, chemokines and adhesion molecules. The peritoneal membrane and the mesothelium in particular, as a site for the production of mediators, play a pivotal role in the activation and control of inflammation.[10,11]

In previous studies we demonstrated that the inflammatory sequelae after surgery promote tumour recurrence and that this effect is mainly based on the cellular component of the inflammatory process. Analysis of the cellular fraction reveals a trauma related influx of PMN into the abdominal cavity (van Rossen et al. unpublished). Comparable shifts in cell differentiation have also been demonstrated in other animal models following peritoneal trauma as well.[9,12] PMN are known to produce reactive oxygen species (ROS) to destroy invading organisms, beside this beneficial effect, the overwhelming oxidative potential can result in additional tissue destruction.[13,14,15]

The aim of this study was to investigate the effect of unstimulated and FMLP (N-formyl-methionyl-leucyl-phenylalanine) stimulated PMN on the adhesion of Caco-2 cells, a human colon carcinoma cell line, on mesothelial monolayers. To evaluate our hypothesis that ROS, produced by PMN, are the source of the enhancement of adhesion we also investigated the direct effect of reactive oxygen species produced by the interaction of xanthine (X) and xanthineoxidase (XO) on tumour cell adhesion. Finally, we studied the expression of adhesion molecules on mesothelial cells after incubation with X/XO products by means of enzyme immunoassay (EIA).

MATERIALS AND METHODS

Culture and identification of omental mesothelial cells

Mesothelial cells (MC) were obtained from the human omental tissue. The experimental protocol was approved by the Medical Ethical Committee of the Erasmus Medical Centre Rotterdam, The Netherlands. The MC were isolated according to techniques modified from Nicholson *et al.*[16] and Wu *et al.*[17]. The omentum was transferred to fluid containing 0.05% trypsin-0.02% EDTA (Invitrogen, Karlsruhe, Germany). After 15 minutes the detached MC were pelleted by centrifugation at 1800 rpm for 10 minutes and were resuspended in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamin (2 mmol/L) and penicillin (10^5 U/ml) (Invitrogen, Karlsruhe, Germany). MC were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (75 cm^2, Corning BV, Schiphol-Rijk, The Netherlands) pre-coated with collagen type I (17.5 µg/ cm^2). The identity of MC was demonstrated by the absence of Von Willebrand factor staining [18] and the presence of intracellular cytokeratins [19] via immunohistological staining with monoclonal antibodies (Dako, Amsterdam, The Netherlands).

Caco-2 cell line

The human colon adenocarcinoma Caco-2 was a kind gift of Dr. W. Dinjens. The cell line was cultured in RPMI1640 medium supplemented with 10% fetal calf serum, L-glutamin (2 mmol/L) and penicillin (10^5 U/ml). All supplements were obtained from Invitrogen, Karlsruhe, Germany. Before use, cells were trypsinized (5 minutes at 37°C), centrifuged (5 minutes at 1500 rpm), resuspended in RPMI 1640 and counted. Viability was measured by trypan blue exclusion and always exceeded 95 percent.

Isolation of Polymorphonuclear leukocytes (PMN)

Human blood was drawn by venipuncture from healthy volunteers into a sterile syringe containing heparin. PMN were prepared using the standard techniques of dextran sedimentation followed by Histopaque 1077 gradient centrifugation. Residual erythrocytes were removed by hypotonic lysis and PMN were resuspended in buffered salt solution (BSS) (138 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, pH 7.4) supplemented with 1 mg/ml glucose and 1 mg/ml human serum albumine (HAS). Purity of the final cell suspension and cell viability, assessed by trypan blue exclusion test, were > 95% in all cases.

Superoxide (O_2^-) production by stimulated PMN with FMLP

PMN were suspended in buffer saline solution (BSS); they were pretreated with cytochalasin B (5 g/ml) for 5 min at 37°C to maximize the measured response, then challenged with the test compound for 15-25 min at 37°C before exposure to 10^{-7}M FMLP (N-formyl-methionyl-leucyl-phenylalanine) for a further 5 min. O_2^- production was determined spectrophotometrically by measuring the superoxide dismutase inhibitable reduction of cytochrome c reduced/10^6 PMN/min as described below.

Xanthine / Xanthine Oxidase superoxide generating system

The reactive oxygen species (ROS) superoxide and hydrogen peroxide were generated by Xanthine Oxidase (XO) (0.05 U/ml, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) upon addition of Xanthine (X) (0.1 mmol/l, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). The oxygen-free radicals are produced by the following enzymatic reaction: Xanthine + O_2 + H_2O à Uric Acid + H_2O_2 + O_2, catalyzed by Xanthine Oxidase (XO).

Superoxide generation by X/XO was determined using the ferricytochrome C reduction techniques modified from Leslie et al.[20] In brief, cytochrome c (cytc) was prepared in HBSS without phenol red and used at a final concentration of 150µM. All solutions were pre-warmed to 37°C. The assay was performed as six-fold replicates by filling the wells in vertical rows. Initially cytc or cytc + xanthine (100 µl/well) was distributed in the rows. The rows were then filled with xanthine oxidase solution (100 µl/well). An extra row was filled with cytc and HBSS only to provide a control. Immediately after adding the xanthine oxidase the plate was read (t=0) and every 10 minutes afterwards, with 120 minutes as endpoint. The plate was kept at a constant temperature of 37°C. The amount of cytc reduction at 37°C was determined by taking kinetic measurements of absorbance at 540 and 550 nm using a Thermomax double-beam microplate reader. The cumulative superoxide production is shown as optical density (OD) by deducting the OD at 550 nm from the OD measured at 540 nm. The super oxide production is expressed in nanomoles per minutes per 10^7 cells.[21]

Tumour cell labeling and incubation

The dye solution, calcein-AM, used to quantify tumour cell adhesion was prepared by dissolving 50 µg calcein (Molecular Probes, Leiden, The Netherlands) in 5 µl anhydrous dimethyl sulphoxide (Sigma-Aldrich, Zwijndrecht, The Netherlands) and adding this solution to 5 ml of RPMI 1640 medium supplemented with 0.5% bovine serum albumin (RPMI 1640/0.5%BSA, BSA obtained from Sigma-Aldrich). Trypsinized Caco2 cells ($1x10^6$ cells/ml) were incubated in RPMI1640/0.5%BSA at 37 °C for 30 minutes with occasional mixing. Before adding to the mesothelial monolayers, the labelled cells were washed twice with RPMI1640/0.5%BSA to remove free dye.

Tumour cell adhesion assay

To quantify tumour cell adhesion to a monolayer mesothelium, a standardized cell adhesion assay was developed according to methods from Catterall *et al.*[22] Mesothelial monolayers were established in 96 well plates (Canberra Packerd, Groningen, The Netherlands), pre-coated with collagen type I (15 µg/cm^2, coating according to instructions of the manufacturer). To do this, confluent cells were washed with phosphate buffered saline (PBS) and harvested with trypsin (0.05%) and EDTA (0.02%) and 2.5×10^4 mesothelial cells were added in 200 µl of medium to each well. The plates were incubated at 37 °C, in a humidified atmosphere of 5% CO_2 in air; medium was replaced daily with fresh medium. Monolayers reached confluence in 5 to 7 days as determined by light microscopy.

Mesothelial monolayers were incubated with unstimulated PMN and FMLP stimulated PMN in a transwell system (Corning Incorporated Life Sciences, U.S.A.)overnight. The next day the transwells were removed en

the mesothelial cells were incubated with the Caco-2 cells. Non pre-incubated mesothelial monolayers served as controls.

Tumour cells were labeled with calcein as described above. Before adding to the mesothelial monolayers, the labeled cells were washed twice with RPMI1640/0.5%BSA to remove free dye. Medium from the experimental wells was removed and 200 µl RPMI1640/0.5%BSA containing 30,000 calcein labeled tumour cells was added. Plates were centrifuged for 1 minute at 80 g on a Perkin Elmer centrifuge and incubated at 37 °C for 60 minutes. After this, the medium of each well was removed and washed twice with 200 µl RPMI1640/0.5% BSA medium. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 nm excitation and 530 nm emission filters. On each plate a standard was prepared by adding different numbers of labeled tumour cells to the wells. The amount of tumour cells adhered was determined by calibrating the measured fluorescence of the experimental wells on the standard.

In order to determine the effect of ROS (*in casu* superoxide) on tumour cell adhesion, mesothelial monolayers were pre-incubated with Xanthine, Xanthine Oxidase and the combination of Xanthine and Xanthine Oxidase overnight. Medium from the experimental wells was removed and 200 µl RPMI1640/0.5%BSA containing 30,000 calcein labeled tumour cells was added. Non pre-incubated mesothelial monolayers served as controls. The mesothelial cells were incubated with the Caco-2 cell according to the adhesion protocol described above.

Enzyme immuno-assay (EIA)

Enzyme immunoassay was used to determine the effect of X/XO on the expression of adhesion molecules. Mesothelial cells were grown to confluence in 96-well flat-bottomed multititer plates and pre-incubated with either cell culture medium alone or combined with X/XO overnight. Medium from the experimental wells was removed and the cells were fixed in 95% ethanol/ 5% methanol solution for 45 minutes. Subsequently, the cells were pre-incubated for 10 minutes with 1% normal goat serum, diluted in PBS, to inactivate unspecific binding. Mouse anti-human monoclonal antibodies to ICAM-1 (CD54, R&D Systems), VCAM-1 (CD106, R&D Systems) and CD44H (CD106, R&D Systems) was added for 60 minutes. The cells were subsequently incubated with biotinylated goat antimouse antibody (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) in a dilution of 1 : 500 for another 60 minutes. Finally the cells were incubated with a Streptavidin-peroxidase complex for 30 minutes, washed, and substrate (2,2'-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) in 0,05M phosphate-citrate buffer containing 0,03% sodium perborate was added. Incubation of the mesothelial cells without the primary antibody served as a negative control. After 40 minutes, the reaction was stopped using 0,4% sodium fluoride. Photometrical evaluation was performed with a computer-controlled ELISA-plate reader at λ = 405 nm.

Statistical analysis

All data were analyzed using analysis of variance (ANOVA) to determine overall differences between group means. If the ANOVA was significant on a 5% level, the post-hoc Newman-Keuls test was carried out to make a comparison between groups. $P \leq 0.05$ was considered to be statistically significant. Experiments were carried out at least twice with comparable results.

RESULTS

Validation of assay

Loading of the tumour cells with calcein did not affect their viability (>95% using trypan blue). To determine the stability of calcein uptake, the release of fluorescence in the supernatant of labeled cells after an incubation time of up to 120 minutes was measured. The fluorescence of the washed cells stayed constant for at least 90 minutes indicating retention of the dye in the cells (data not shown). A dilution series was made by using labeled Caco-2 cells on a mesothelial monolayer. There was a direct relationship between the cell number added and measured fluorescence resulting in a linear correlation which was used as a standard to calibrate the measured fluorescence. In this way the amount of adhered tumour cells in the experimental wells could be determined.

Tumour cell adhesion to a mesothelial monolayer

Tumour cell adhesion to a non pre-incubated mesothelial monolayer was relatively slow and temperature dependent. Maximum adhesion was seen after 60 minutes. At this time point a steady state situation was achieved and cell adhesion did not increase hereafter. The tumour cells remained rounded up during these adhesion assays. After more than 60 minutes, the first signs of spreading out could be detected under the light microscope. Therefore, for all subsequent experiments, 60 minutes was taken as a cut-off point.

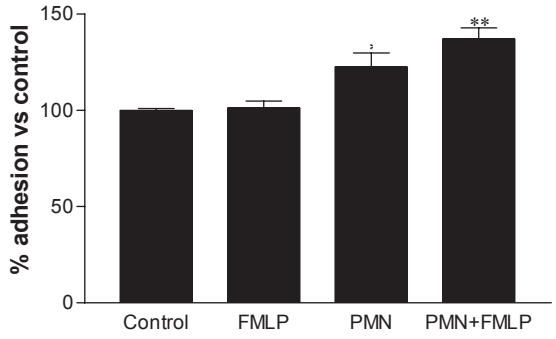

Figure 1
Adhesion of Caco-2 to a mesothelial monolayer after pre-incubation with PMN and FMLP stimulated PMN overnight. The adhesion of Caco-2 cells to mesothelial monolayers in the control situation is set to a 100% adhesion. Data are expressed as the mean (n=6) + SEM. *=p<0.05, **=p<0.01 vs control.

Effect of PMN pre-incubation

Overnight incubation of a mesothelial monolayer with PMN in a transwell system did not modify total DNA content of the mesothelial monolayer and therefore did not affect cell growth (data not shown).

The adhesion of the Caco-2 cells in the control situation was 23%. Pre-incubation of the mesothelial cells with unstimulated PMN resulted in an significant enhancement of adhesion of the Caco-2 cells of 25% ($p<0.05$). FMLP stimulated PMN increased the adhesion of Caco-2 cells to the mesothelium with 40% ($p<0.01$) (figure 1).

Ferricytochrome-C measurement

The assay is based on the turn over of ferric(Fe^{3+}) iron to ferrous (Fe^{2+}) iron, that can be measured at 550 nm. The turn over is a measure of the cumulative production of superoxide and is expressed as the optical density. The optical density was measured at intervals of 10 minutes over a period of 120 minutes. Xantine and xantine oxidase alone did not produce significant amounts of reactive oxygen species. However when combining xantine and xantine oxydase a marked increase in the optical density (figure 2) is seen. After an initial burst the superoxide production slowed down and almost no further superoxide production is seen after 120 minutes.

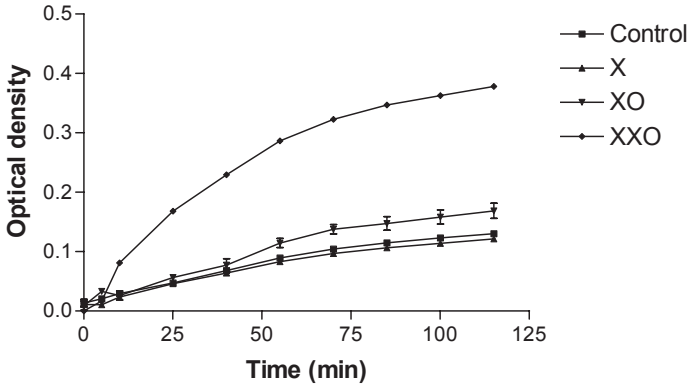

Figure 2

This figure shows the results of the Ferricytochrome C reduction assay. It is a measure of the cumulative production of superoxide and is expressed as the optical density. Xanthine (X) and Xanthine Oxidase (XO) alone does not produce significant amounts of reactive oxygen species (ROS). Combined X/XO caused a significant enhancement of the optical density in the first 50 minutes, denoting an significant burst of production of ROS.

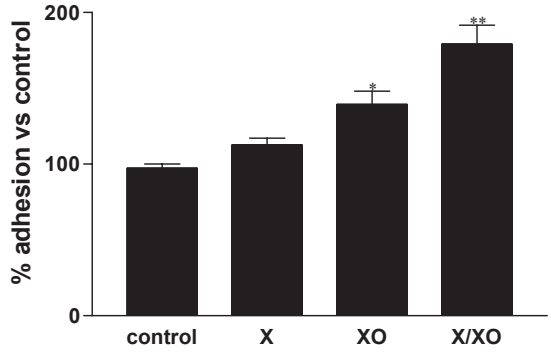

Figure 3
Adhesion of Caco-2 to a mesothelial monolayer after pre-incubation with X, XO and X/XO overnight. The adhesion of Caco-2 cells to mesothelial monolayers in the control situation is set to a 100% adhesion. Data are expressed as the mean (n=6) + SEM. *=p<0.05, **=p<0.01 vs control.

Effect of X/XO pre-incubation

Adhesion of Caco-2 to non-stimulated mesothelial cells was 23%. Preliminary studies showed that at least 6 hours of pre-incubation were needed to induce enhanced cell adhesion (data not shown). Maximum enhancement of tumour cell adhesion was achieved after 12 hours of pre-incubation with X/XO (70% enhancement compared to the control situation, p<0.01) (figure 3). With light microscopy, marked morphologic changes were seen in cell appearance of the monolayer after 24 hours. After 12 hours pre-incubation, stretching of cells could be observed, but the monolayer was still intact.

Expression of cell adhesion molecules

EIA is a semiquantitative assay investigating the dynamics of expression of adhesion molecules on mesothelial cells or tumour cells with or without stimulating the cells with X/XO (in optical density units measured at 405 nm). The staining intensity for ICAM-1 of unstimulated mesothelial cells was 0.285, whereas the negative control gave an intensity of 0.118, indicating a marked basal expression of ICAM-1 on mesothelial cells. Optical density for VCAM-1 and CD44H was 0.137 and 0.184 respectively, indicating that mesothelial cells have a basal expression of VCAM-1 and CD44H as well. When stimulating the mesothelial cells with X/XO for 12 hours, which is the optimal time point for adhesion of Caco-2 to mesothelial cells, a significant (p<0.01 vs control) increase in expression of ICAM-1, VCAM-1 and CD44H could be observed (figure 4).

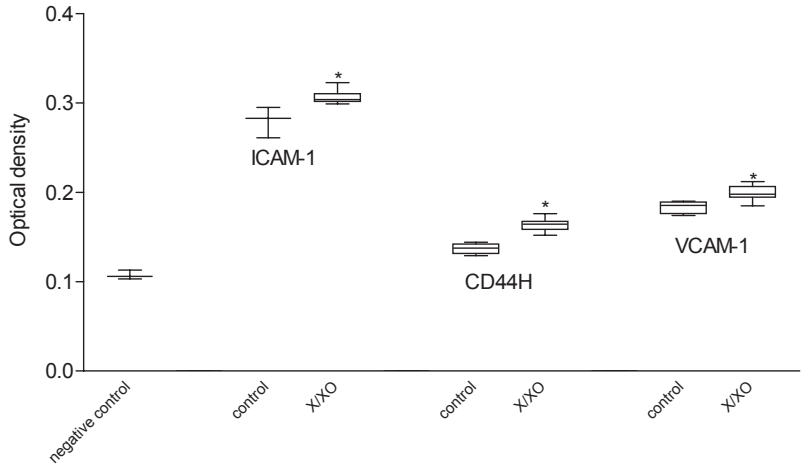

Figure 4
Expression of adhesion molecules, ICAM-1, CD44 and VCAM-1 on mesothelial cells and stimulated mesothelial cells with X/XO overnight. *=p< 0.01 vs. control.

DISCUSSION

Surgical treatment of patients with gastro intestinal cancer is complicated by high recurrence rates. In case of colorectal carcinoma more than 40% of patients, who undergo potentially curative resection of advanced colorectal cancer, do develop recurrence of the disease. Peroperatively spilled tumour cells or circulating tumour cells apparently profit from the inflammatory sequelae provoked by surgery, ultimately leading to manifest tumour recurrence [1].

The direct effects of surgery lead to damage to the mesothelial lining of the abdominal cavity. This causes a cascade of events, commonly termed the sterile inflammatory response after surgery.[23,24] During this response, inflammatory cells, like macrophages, monocytes and polymorph nuclear leukocytes (PMN), are attracted to the site of injury and inflammation.[25] This attraction takes place through the release of chemo attractants (*e.g.* IL-8) and transsection of blood vessels.

Macrophages, monocytes and also mesothelial cells are known to produce cytokines, providing mutual interaction. IL-1β and TNF-α have been shown to induce activation of transcription factors like NF-κB and AP-1, leading to an augmentation of both the inflammatory response and tissue damage.[26] Cytokine-activated mesothelial cells produce chemo-attractants, like IL-8, leading to recruitment of PMN. In this way, the mesothelium and inflammatory cells in the abdominal cavity may perpetuate a cytokine loop, resulting in extreme activation of the inflammatory process. In addition, cytokines (like IL-1β and

TNF-a) have been shown to upregulate adhesion molecules on mesothelial cells and in that way enhanced tumour cell adhesion.[27]

In previous studies we provide evidence that passive transfer of lavage fluid, collected after inflicting surgical trauma, can lead to enhanced tumour recurrence in naïve recipients. The cellular component of the lavage fluid gave rise to the greatest enhancement of tumour recurrence (van Rossen et al. unpublished). Detailed analysis of this cellular fraction revealed a trauma related influx of PMN into the abdominal cavity. There is a correlation between the amount of surgical trauma and an enlarging shift of primarily monocytes to PMN in the lavage fluid. This phenomenon will evidently affect the homeostatic milieu of the peritoneum. These PMN seem to play a pivotal role in the enhanced tumour recurrence seen after surgical trauma.

The oxygen-dependent and oxygen-independent processes of the PMN participate in the killing of bacteria, but those same processes can also result in damage to host tissue. Especially in ischemia and reperfusion injury research, evidence became available about the damage ROS can inflict on transplanted organs, leading to possible decreased graft function. ROS, like superoxide can inflict damage to mesothelial surfaces by inducing adenine nucleotide depletion[28], membrane damage[29] and DNA damage.[30] ROS can, besides their well known direct toxic effects, also function as signal transduction molecules to activate the chemotactic cytokine interleukin-8 (IL-8) and the transcription factors NF-κB and AP-1, resulting in upregulation of the cell surface adhesion protein, intercellular adhesion molecule-1 (ICAM-1).[31] Together, IL-8 and ICAM-1 orchestrate the transendothelial migration of PMN to sites of inflammation and injury. So, there seems to be a positive feedback in which PMN, through the production of ROS, stimulate the influx of even more PMN.

Yokoyama reported that massive quantities of Xanthine Oxidase (XO) are released into the circulation after hepatic ischemia-reperfusion injury.[32] These products can serve as a potential source of circulating oxygen radical production, resulting in tissue injury at sites distal to its origin. Therefore the hypothesis of this study was that ROS produced by PMN after surgical trauma influence the adhesion of tumour cells to mesothelial monolayers.

This study demonstrate the enhancement of adhesion of colon carcinoma cells on mesothelium after pre-incubation of the mesothelium with PMN. By means of the superoxide production measurement assay, we demonstrated that PMN do produce ROS. Because isolation of PMN from whole-blood might possibly stimulate the PMN in a non-physiological way, we used the ROS producing combination of X and XO in our *in vitro* experiments to mimic the influence of ROS produced by PMN. Mesothelial cells have the disposal of endogenous amounts of X and XO. When incubated with exogenous XO, they were able to generate superoxide in small amounts. This amount of superoxide proved to be sufficient to induce a moderate increase in tumour cell adhesion to mesothelial monolayers. The amount of endogenous XO was too low to generate superoxide in the presence

of exogenous X. In the mesothelial experiment the combination of X and XO, releasing superoxide in larger quantities, resulted in the highest increase of tumour cell adhesion. This outcome might be explained by an upregulation of the adhesion molecules, ICAM-1, VCAM-1 and CD44, on the mesothelial cells according to the results of the enzyme immunoassay.

In conclusion, this study demonstrated that PMN produce ROS and that incubation of mesothelial cells with activated PMN increased colon carcinoma adhesion. This effect is likely mediated via the ROS produced by PMN, since incubation of the mesothelial monolayers with ROS increased tumour cell adhesion. The results give evidence that the inflammatory response, i.c. PMN influx after surgery promote tumour recurrence. Preventing tissue damage by inflammatory cells therefore may provide a novel strategy to defeat progression and metastasis of cancer. Manipulation of the intricate cytokine network may cause unwanted side effects in the wound healing process. Attacking of the reactive oxygen products or blocking the adhesion molecules however seems a feasible way of preventing cellular tissue damage ultimately resulting in diminished tumour recurrence.

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CHAPTER 5

The influence of reactive oxygen species on the adhesion of pancreatic carcinoma cells to the peritoneum

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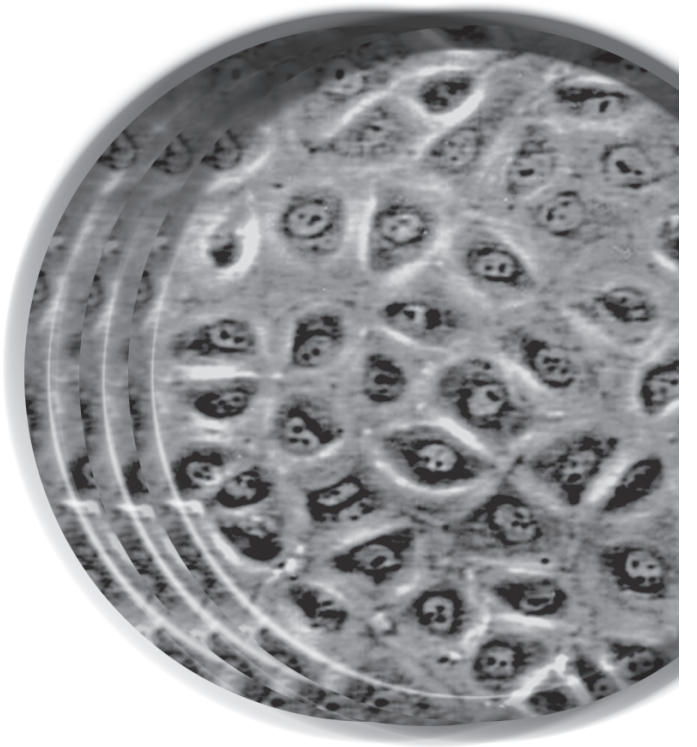

ABSTRACT

Postoperative peritoneal carcinomatosis is a significant clinical problem after curative resection of pancreatic carcinoma. Peroperative surgical trauma activates a cascade of peritoneal defence mechanisms responsible for postoperative intra-abdominal tumour recurrence. Reactive oxygen species (ROS) play a pivotal role in this postoperative inflammatory reaction. This study explores the influence of ROS on adhesion of human pancreatic carcinoma cells to human mesothelial cells. Furthermore this study explores the influence of ROS on the presentation of adhesion molecules on Panc-1 and mesothelial cells. ROS were produced using the enzymatic reaction of xanthine with xanthine oxidase (X/XO).

Mesothelial monolayers were incubated with ROS produced prior to adhesion of the tumour cells. Incubation of the mesothelial cells with X/XO resulted in a significant increase (69.5%) in adhesion of Panc-1 in all patients. SOD/catalase, anti-oxidants, could reduce this increase by 56.7%. ROS significantly influenced the expression of the adhesion molecules ICAM-1, VCAM-1 and CD44 on mesothelial cells, but did not influence adhesion molecule expression on Panc-1.

The ROS released during the post-operative inflammatory reaction may play an important role in the adhesion of pancreatic tumour cells to the mesothelium. Possibly by influencing adhesion molecule expression on mesothelial cells. Therefore ROS can partly be responsible for the enhanced post-operative intra-abdominal tumour recurrence.

INTRODUCTION

Pancreatic carcinoma has the lowest 5-year survival rate of any cancer. Surgical resection is the only chance for cure, but the overall 5-year survival is only 6.8 – 25%, and up to 50% of those who survive 5 years may die of recurrent cancer.[1] In 70-80% local recurrence and/or peritoneal dissemination is found after potentially curative resection of pancreatic carcinoma.

Early preoperative tumour cell seeding and peroperative intra-abdominal shedding of tumour cells, due to handling the tumour and leakage from dissected lymphatic channels, are the most likely causes of peritoneal carcinosis.[2, 3] Previous experimental data have demonstrated that the proliferative and metastatic potentials of these spilled tumour cells are very well preserved.[4] Peroperative occurrence of tumour cells in the peritoneal cavity has been shown to negatively correlate with the postoperative survival rate.[4, 5] Previously described clinical and experimental studies showed that surgical trauma promotes intra-abdominal tumour recurrence.[6-8] In an animal experimental study Raa et al.[9] showed that this effect is mainly due to enhanced adhesion of tumour cells rather than enhanced growth of already adhered tumour cell clusters.

The dynamic cascade of peritoneal healing, induced by peritoneal damage, seems to be important in the process of intra-peritoneal adhesion of tumour cells.[8, 10, 11] We demonstrated earlier that within a few hours after infliction of peritoneal trauma, factors in the abdominal cavity could be captivated in a lavage fluid enhancing tumour recurrence in naive, non-operated recipients.[8] Separated components of these lavage fluids i.e. inflammatory cells and soluble factors, could each enhance tumour recurrence, however the cellular fraction led to the greatest tumour load. More detailed analysis of the cellular fraction revealed a peritoneal trauma related influx of polymorph nuclear leucocytes (PMN) in the abdominal cavity. Similar shifts in cell differentiation following peritoneal trauma have been demonstrated in other animal models.[12-14] The exact role of PMN in post-surgical enhanced tumour development is not yet clear. In the early post-operative inflammatory reaction PMN are responsible for clearing dead tissue and invading organisms by producing and releasing reactive oxygen species (ROS). Especially oxygen-free radicals and hydrogen peroxide are formed. Despite this beneficial effect, the overwhelming oxidative potential can result in additional tissue destruction.[15, 16] Van Rossen et al.[17] showed in an animal model that inhibition of ROS with the use of the anti-oxidant enzymes superoxide dismutase (SOD) and catalase lead to diminished tumour recurrence. The exact effect of ROS on the peritoneum is unknown. Whether the effects shown by van Rossen et al. is fully explained by reducing additional tissue damage by ROS, or whether ROS also have a direct effect on the mesothelial cells remains unclear. The aim of the current in-vitro study was to evaluate the effect of ROS on pancreatic tumour cell adhesion to a monolayer of human mesothelial cells. And whether anti-oxidants could be used to reduce post-operative enhanced tumour adhesion. Moreover we

analyse the effects of ROS by assessing the expression of adhesion molecules on both mesothelial cells and tumour cells. To produce ROS, the reaction of xanthine with xanthine oxidase was used that is known to produce superoxide and hydrogen peroxide.[18]

MATERIALS AND METHODS

Culture and identification of omental mesothelial cells

Mesothelial cells (MC) were obtained from the human omental tissue. The experimental protocol was approved by the Medical Ethical Committee of the Erasmus Medical Centre Rotterdam, The Netherlands. The MC were isolated according to techniques modified from Nicholson *et al.*[19] and Wu *et al.*[20]. The omentum was transferred to fluid containing 0.05% trypsin-0.02% EDTA (Invitrogen, Karlsruhe, Germany). After 15 minutes the detached MC were pelleted by centrifugation at 1800 rpm for 10 minutes and were resuspended in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamin (2 mmol/L) and penicillin (10^5 U/ml) (Invitrogen, Karlsruhe, Germany). MC were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (75 cm^2, Corning BV, Schiphol-Rijk, The Netherlands) precoated with collagen type I (17.5 µg/ cm^2). The identity of MC was demonstrated by the absence of Von Willebrand factor staining [21] and the presence of intracellular cytokeratins [22] via immunohistological staining with monoclonal antibodies (Dako, Amsterdam, The Netherlands).

Panc-1 cell line

The human pancreas carcinoma cell line Panc-1 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamin (2mM) and penicillin (10^5 U/L) and maintained by serial passage after trypsinization using 0.05% trypsin/ 0.02% EDTA. Before the adhesion assay, tumour cells were trypsinized and maintained in suspension culture for 2 hrs to regenerate cell-surface proteins.

Drugs

The cell cultures were exposed to the reactive oxygen species (ROS) superoxide and hydrogen peroxide generated by xanthine oxidase (0.05 U/ml, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) upon the addition of xanthine (0.1 mmol/l, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) (X/XO). The oxygen-free radicals are produced by the following enzymatic reaction: xanthine + O_2 + H_2O Uric acid + H_2O_2 + O_2^-, catalyzed by xanthine oxidase.

The anti-oxidant enzymes SOD (5000 U/mg) from Roche Diagnostics BV, Almere, the Netherlands and catalase (2350 U/mg) from Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands were added to the cell cultures in a 400U/ml and 200U/ml final concentration, respectively, to counteract the ROS production specifically.

Ferricytochrome c reduction

Superoxide generation by xanthine/xanthine oxidase (X/XO) was determined using the ferricytochrome c (cytc) reduction assay modified from Leslie et al.[23] In brief, cytc was prepared in HBSS without phenol red and used at a final concentration of 150µM. After filling each well with 100 µl of cytc, cytc + xanthine or cytc + xanthine + SOD/catalase , the reaction was started by adding 100 µl of xanthine oxidase solution (100 µl/well). Wells filled with cytc in HBSS served as a blank. The plate was kept at a constant temperature of 37°C using a thermostatted microplate reader (Versamax, Molecular Devices), and read every 10 minutes up to 120 minutes. The amount of reduced cytc reduction was calculated from the absorbance at 550 nm with 540 nm as the reference using molar absorbance coefficient of 12.2×10^3 M^{-1}.[23]

Apoptosis assay

To examine if overnight incubation of the mesothelial monolayer with X/XO could induce apoptosis, a cell death detection ELISA-kit (Roche Diagnostics BV, Almere, the Netherlands), was used based on a quantitative sandwich-enzyme-immunoassay with mouse monoclonal antibodies directed against human DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates.

Adhesion assay

To quantify tumour cell adhesion to mesothelium, a previously standardized and validated cell adhesion assay was used.[24] In short, mesothelial cells were transferred to 96-well plates (Perkin Elmer, Groningen, the Netherlands) pre-coated with collagen type-1 and grown to confluency as confirmed by light microscopy. In order to determine the effect of ROS on tumour cell adhesion, mesothelial monolayers or Panc-1 were pre-incubated with X/XO for 12 hours. Untreated cells served as a control.
Tumour cells were labelled with calcein-AM (Molecular Probes, Leiden, NL) by incubating 1×10^6 Panc-1 cells/ml RPMI medium with 1% FCS containing 10 µM calcein-AM for 45 minutes at 37 °C with occasional mixing. After washing the labeled Panc-1 twice with RPMI containing 1% FCS to remove free dye, supernatant from the experimental wells was removed and 30.000 labelled Panc-1 cells in 200 µl RPMI/1% FCS were added. Plates were centrifuged for 1 minute at 80 g and incubated at 37 °C for one hour. After washing away unbound cells, the numbers of adherent tumour cells were assessed by measuring fluorescence at 485 nm excitation and 535 nm emission using a standard curve prepared from various numbers of labelled tumour cells.

Enzyme immuno-assay (EIA) of cellular adhesion molecules

A previously described enzyme immunoassay was used to determine the effect of X/XO on the expression of adhesion molecules. Mesothelial cells and Panc-1 were grown to confluence in 96-well flat-bottomed multititer plates and pre-incubated with either cell culture medium alone or in combination with X/XO or X/XO and SOD/catalase for 12 hours. Medium from the experimental wells was removed and the cells were fixed in 95% ethanol/ 5% methanol solution for 45 minutes. Subsequently, the cells were

pre-incubated for 10 minutes with 1% normal goat serum, diluted in PBS, to block non-specific binding. Mouse anti-human monoclonal antibodies to ICAM-1 (CD54, R&D Systems; diluted 1 : 500), VCAM-1 (CD106, R&D Systems; diluted 1 : 500), LFA-1 (CD11a, Leinco Technologies; diluted 1 : 10), VLA-4 (CD49d, R&D Systems; diluted 1 : 50), CD44 (CD106, R&D Systems; diluted 1 : 500) were added for 60 minutes. Tumour cells or mesothelial cells incubated without one of these primary antibody served as a negative control. After a washing step with phosphate buffered saline (room temperature, pH 7.4) the cells were subsequently incubated with biotinylated goat antimouse antibody (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) in a dilution of 1 : 500 for another 60 minutes. Finally the cells were washed with phosphate buffered saline and incubated with a streptavidin-peroxidase complex for 30 minutes, washed, and substrate (2,2'-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) in 0.05M phosphate-citrate buffer containing 0.03% sodium perborate was added. After 40 minutes, the reaction was stopped using 0.4% sodium fluoride and the absorbance was read at 405 nm. Membrane antigen expression is expressed as OD units.

Statistical analysis

All data were analyzed using analysis of variance (ANOVA) to determine overall differences between group means. If the ANOVA was significant on a 5% level, the post-hoc Newman-Keuls test was carried out to make a comparison between groups. $P \leq 0.05$ was considered to be statistically significant. Experiments were carried out at least twice with comparable results.

RESULTS

Quantification of ROS generation by xanthine/xanthine oxidase

To appreciate the effect of the xanthine/xanthine oxidase (X/XO) system on the adhesion of Panc-1 cells to mesothelial monolayers, the amount of ROS produced by X/XO was determined over time. The results showed that X and XO alone did not produce significant amounts of ROS. However when combining X and XO, superoxide was produced at a rate of 0.4097 nmol/ml/min during the observation period of 60 min (figure 1). After that the amount of superoxide did not increase much showing that the rate of superoxide production approached zero. In the presence of SOD, the superoxide generated by X/XO was almost completely inactivated (figure 1).

Effect of ROS on mesothelial cell viability

Inspection by light microscopy showed that up to 12 hours of incubation with X/XO, the monolayer was still intact, but marked morphologic changes in cell appearance of the monolayer occurred after 24 hours. Simultaneous adding of SOD and catalase with X/XO prevented this effect of X/XO.

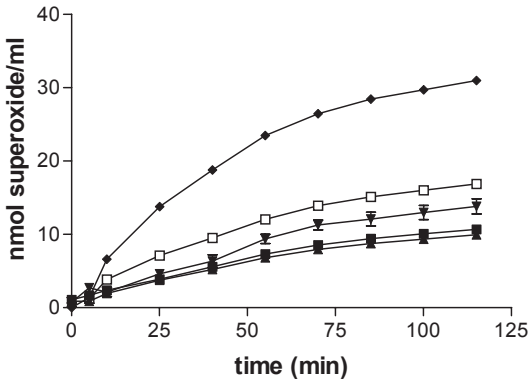

Figure 1
Ferricytochrome C reduction assay. Superoxide production by control (-■-), X (-▲-), XO (-▼-), X/XO (-◆-) or X/XO and SOD/catalase (-□-). Cumulative superoxide production is displayed nmol/ml ± SEM (n=6/condition).

Further study showed that a significant number of the mesothelial cells already became apoptotic when incubated with X/XO for 12 hours (figure 2). XO only induced some apoptosis, while X alone had no effect (figure 2). Addition of SOD/catalase inhibited the apoptosis-inducing effect of X/XO almost completely.

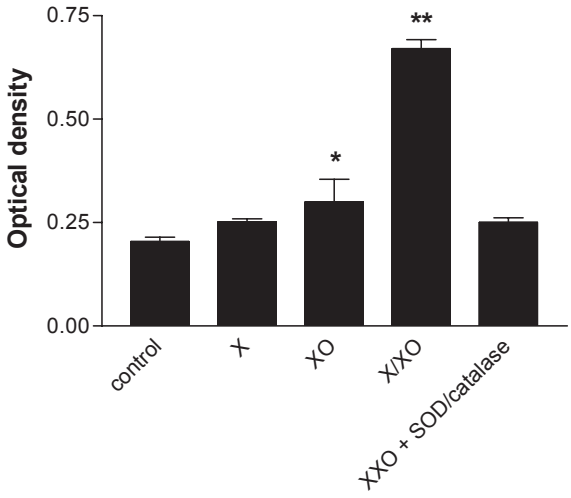

Figure 2
Apoptosis assay. * = $p < 0.05$ vs. control, ** = $p < 0.001$ vs. control.

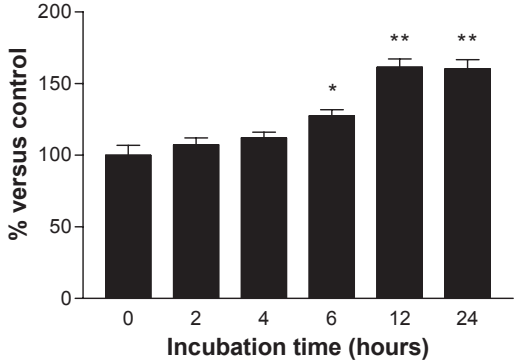

Figure 3
Adhesion of Panc-1 to a mesothelial monolayer after pre-incubation with X/XO at varying time intervals. Data are expressed as the mean (n=6) and SEM.
* = $p < 0.05$; ** = $p < 0.001$.

Effect of ROS on Panc-1 adherence

Tumour cell adhesion to non-stimulated mesothelial monolayers reached a maximum after 60 minutes. The mean adhesion of Panc-1 cells in the control situation was 21%. After incubation of the mesothelial monolayer with X/XO, the number of adhering Panc-1 cells did not differ from the control up to 6 hr, but increased to 61% stimulation (SD = 14%); $p < 0.001$) after 12 hours of pre-incubation with X/XO (figure 3). If SOD and catalase

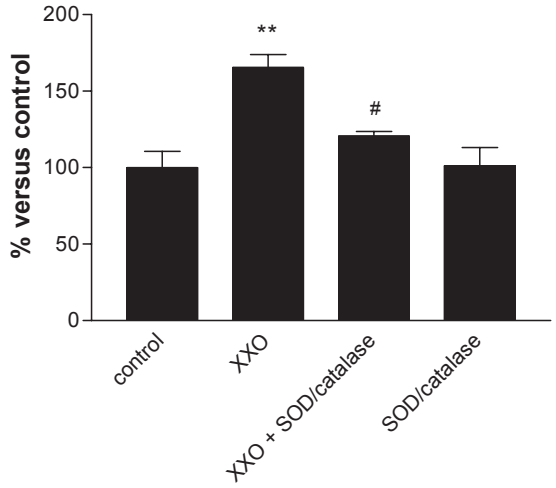

Figure 4
Adhesion of Panc-1 to a mesothelial monolayer after pre-incubation with X/XO with or without SOD/catalase. Data are expressed as the mean (n=6) and SEM. ** = $p < 0.001$; # = $p < 0.05$.

were added during pre-incubation of the mesothelial monolayer with X/XO, the stimulatory effect of ROS on the adherence of Panc-1 cells to the mesothelial cells could be inhibited almost completely (figure 4), while pre-incubation of the mesothelium with SOD and catalase alone did not affect tumour cell adhesion. Incubation of Panc-1 with X/XO did not affect adhesion of the tumour cells to the mesothelial monolayer.

Expression of cell adhesion molecules

To investigate if an increased expression of cellular adhesion molecules by the mesothelium underlies the increase in adherence of Panc-1 cells to ROS-treated mesothelial cells, the expression of various adhesion molecules was studied. The results showed that normal mesothelial cells express ICAM-1 (141% above the negative control), while the expression of CD44 and VCAM-1 was low (16%) and moderate (56%) (figure 5). When stimulating the mesothelial cells with X/XO for 12 hours, after which the adhesion of Panc-1 to mesothelial cells was maximal, a significant increase in expression of ICAM-1, VCAM-1 and CD44 could be observed (figure 5). Adding SOD and catalase to X/XO prevented the increase in expression of these adhesion molecules. Mesothelial cells did

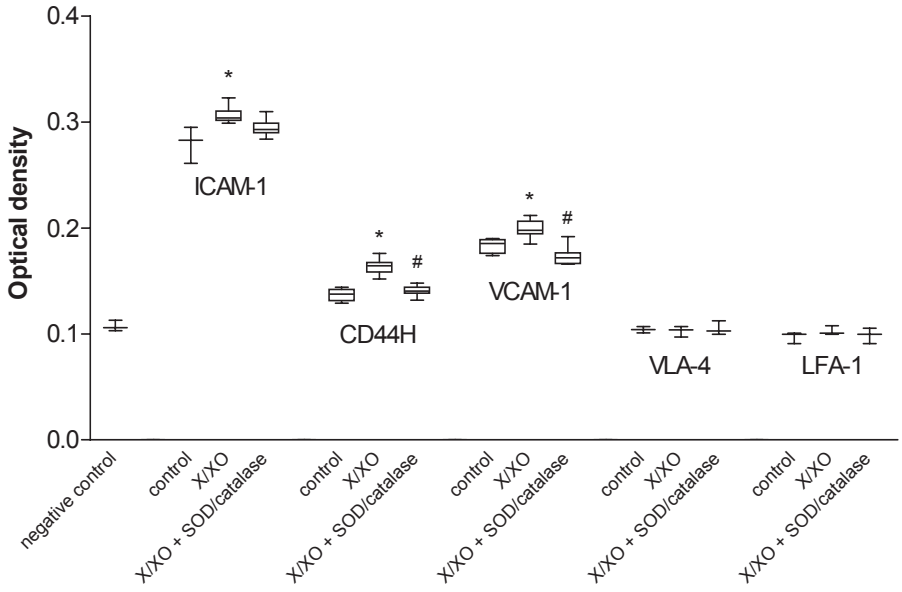

Figure 5
Expression of adhesion molecules on mesothelial cells stimulated with X/XO with or without SOD and catalase. Optical density represents the median absorbance values and range at 405 nm. * = p < 0.001 vs. control.

not express the adhesion molecules VLA-4 and LFA-1 and this pattern did not change upon stimulation with X/XO (figure 5).

Panc-1 cells express the adhesion molecules CD44 (89% above neg. control), ICAM-1 (77%) and only low levels of VLA-4 (20%) (figure 6). Panc-1 does not express LFA-1. The expression levels of these adhesion molecules did not change upon stimulation by X/XO (results not shown).

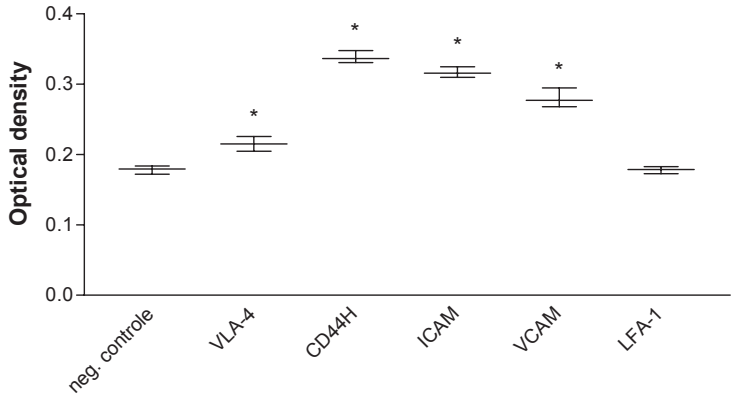

Figure 6
Expression of adhesion molecules on Panc-1. * = $p < 0.01$ vs. negative control. No changes were seen upon stimulation with X/XO. Optical density represents the median absorbance values and range at 405 nm.

DISCUSSION

For local or peritoneal tumour recurrence to develop, adhesion and growth of per-operatively spilled tumour cells is necessary. Cytokines and growth factors produced in great amount after surgical trauma, are known to promote tumour recurrence in *in vivo* and *in vitro* animal models.[8, 11, 25]

The present study demonstrates that ROS stimulate tumour cell adhesion to the peritoneum in a human *in vitro* model. The mechanism by which ROS are able to enhance tumour cell adhesion is dual. Firstly, we found that ROS are able to directly damage the peritoneal lining (through induction of apoptosis of mesothelial cells). The subsequent exposing of the ECM will create a preferential adhesion site for the tumour cells.[26] Under the present conditions, however, the mesothelial monolayer was still intact as examined by phase-contrast microscopy after 12 hr of stimulation by ROS.

Another mechanism by which ROS could stimulate tumour cell adhesion is by enhancing the expression of adhesion molecules, either on mesothelial cells or pancreatic carcinoma cells. The expression of adhesion molecules on human mesothelial cells under the influence of

inflammatory cytokines (TNFa, IL-1β, IL-8) has been analysed. Generally these studies show an increase in ICAM-1, VCAM-1 and CD44 upon stimulation with different pro-inflammatory cytokines.[27-29]

In the present study we found that ROS induced the expression of adhesion molecules VCAM-1, ICAM-1 and CD44 on mesothelial cells as well. ROS did not alter the expression pattern of adhesion molecules on the Panc-1 cell membrane and therefore the observed increase in Panc-1 tumour cell adhesion to the mesothelial cells is dependent on changes of the mesothelium. Hosono et al.[30] showed that CD44 plays an essential role in the initial attachment of pancreas carcinoma cell-line (SW1990) to mesothelial cells and Ziprin et al.[29] demonstrated that ICAM-1 blockade reduced the ability of pancreas carcinoma cell line PSN-1 to adhere to the mesothelium. These results are supported by findings of van Grevenstein et al.[28] who demonstrated the effect of inflammatory cytokines (TNFa, IL-1β and IL-8) on adhesion of pancreatic carcinoma cell lines (MiaPaCa-2 and BxPc3) to mesothelial cells. Surprisingly, no effects of these cytokines were seen on adhesion of Panc-1 to a monolayer of mesothelial cells in their study. They already discussed the problem of high basal adhesion of Panc-1, which was not seen in the present study. Moreover, no expression of VLA-4 was found on Panc-1 by classical immunohistochemical staining. However, the more sensitive enzyme immunoassay did show expression of VLA-4 on mesothelial cells. Possibly the tripsinisation step used in their experiments influenced the expression of VLA-4. Important is that VLA-4 is the counterpart of VCAM-1 which has shown to be upregulated in mesothelial cells upon stimulation with ROS and inflammatory cytokines. Therefore VCAM-1 might play an important role in the adhesion of Panc-1 to mesothelial monolayers. In the present study ICAM-1 might be of less importance, as the mesothelial cells as well as Panc-1 did not express its natural ligand LFA-1. Although Ziprin et al.[27] showed CD43 as an important ligand for ICAM-1 and mediator in adhesion of pancreas carcinoma as well as colon carcinoma cell-lines to mesothelial cells.

Taking the expression patterns of most pancreas carcinoma cell-lines into account, adhesion to mesothelial cells have shown to be influenced by the adhesion molecules ICAM-1, VCAM-1 or CD44. ROS upregulate the expression of these adhesion molecules on mesothelial cells and have shown to enhance adhesion of Panc-1 to mesothelial cells. It is very well conceivable that ROS will enhance adhesion of other pancreas carcinoma cell-lines as well, although this was not investigated in the present study.

In conclusion, despite this emerging complex picture, the present study indicated that ROS released during the post-operative inflammatory reaction could play an important part in the adhesion of pancreatic carcinoma cells to the mesothelium. Although in this study only Panc-1 carcinoma cells were used and general statements are therefore premature, these findings may indicate a rationale for using anti-oxidants as a treatment modality to prevent/reduce activation of the mesothelium and subsequent tumour recurrence after surgical trauma.

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CHAPTER 6

The influence of serum, taken during surgery, on tumour cell adhesion

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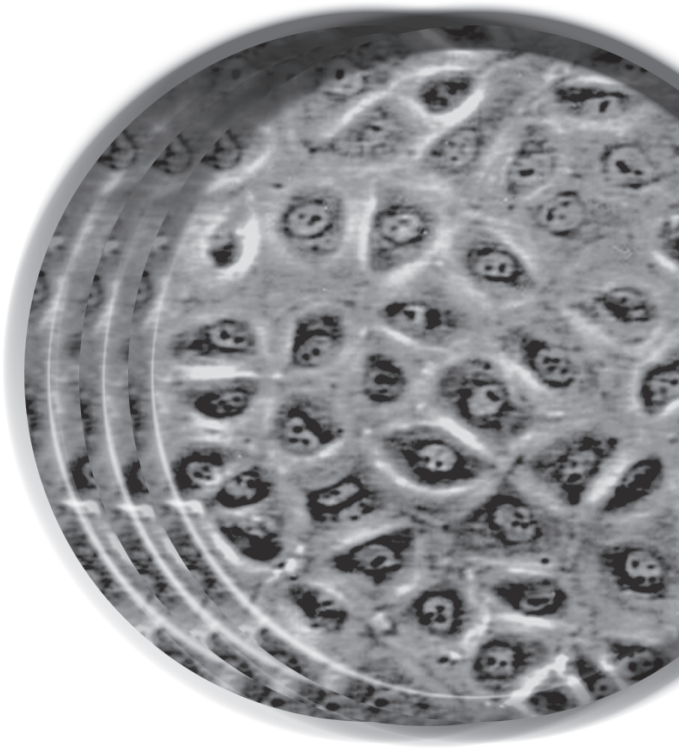


ABSTRACT

The inflammatory response followed by abdominal surgery induces the production of active mediators in the abdominal cavity. Beside the influence of these local produced factors on local tumour recurrence, we hypothesize that during surgery systemically produced mediators are potential stimulators of tumour cell adhesion as well. The aim of this study was to investigate the influence of serum samples, obtained at 8 different time periods before, during and after surgery on the interaction between human colon carcinoma cells and mesothelial cells.

In the control situation the average adhesion of the Caco2 cells to the mesothelial monolayers was 29%. No enhancement in adhesion was observed after incubation of the mesothelial cells with the pre- or post-operative serum of the patients.

This study suggests that systemic factors produced directly after surgery are not influencing tumour adhesion to mesothelial monolayers. Therefore, local tumour recurrence is probably only influenced by locally produced factors.

INTRODUCTION

Local tumour recurrence after curative surgery of colorectal tumours is a persistent problem. The local recurrence rate is almost 50 per cent after curative resection. The most common sites of local recurrence are the site of the primary tumour and the peritoneal surface.[1] There are several theories on the mechanism of local recurrence of spilled tumour cells. According to the theory of metastatic efficiency, implantation of tumour cells onto raw tissue surfaces is an efficient process compared to inefficient implantation.[1] The fibrin entrapment hypothesis proposes that peroperatively spilled tumour cells are trapped in fibrin at the resection site and on damaged peritoneal surfaces.[2] Another hypothesis on which our study is based, suggests that after surgical trauma the acute inflammatory response will produce a great amount of growth factors and cytokines which are very beneficial for the healing process, but also for adhesion and growth of tumour cells.[3-7] Surgical handling of the peritoneum is ambiguous regarding cancer. In case of massive tissue damage, the ensuing inflammatory reaction produces a potentially lethal cocktail of active mediators. These mediators are known to influence tumour recurrence to the peritoneum, probably caused by an upregulation of adhesion molecules, like ICAM-1 and VCAM-1. Besides these locally produced cytokines and adhesion molecules, systemically produced mediators might be potential stimulators of local tumour cell adhesion as well. Recently, a soluble form of ICAM-1 has been described and elevated levels have been associated with advance disease in gastric, colon, gallbladder, pancreatic and renal carcinomas.[8] Pre-operative serum levels of ICAM-1, E-selectin and VCAM-1 are also correlated in gastric and colorectal cancer with invasion, lymph node involvement and distant mestastasis.[9,10] Serum levels of these adhesion molecules, measured at day 7, decreased significantly after radical resection of the tumour compared with pre-operative levels. The serum levels of these adhesion molecules remain unchanged in patients with unresectable tumours and in patients who underwent surgery for benign gastric disease.[10]

We hypothesized that during surgery, systemically produced mediators might be potential stimulators of local tumour recurrence. Therefore, in the present study we investigated the influence of serum, obtained at 8 different time periods before, during, and after surgery from patients who underwent elective abdominal surgery for colorectal carcinoma, on the interaction between colon carcinoma cells and mesothelial cells. We developed an *in vitro* model, in which we studied the adhesion of colon carcinoma cells on mesothelial cells pre-incubated with serum from the same patient.

MATERIALS AND METHODS

Serum samples of patients, culture of omental mesothelial cells and cell line

In total, we studied 9 patients undergoing elective abdominal surgery who had given informed consent to donate 8 samples of blood at 8 different time points and to donate a piece of omental tissue during surgery. The experimental protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam, The Netherlands.

Blood was drawn one day before surgery, time point -24, at the beginning of the operation, time point 0, and 2, 4, 8, 12, 24 and 48 hours after surgery. One sample of blood was taken at every time point. From each sample we obtained approximately 4 ml of serum.

Mesothelial cells (MC) were obtained from the omental tissue of the same patients. The MC were isolated according to techniques modified from Nicholson et al.[11] and Wu et al.[12] and cultured in RPMI1640 medium supplemented with 10% human pooled normal serum, L-glutamin (200 mmol/L) and penicillin (5000 U/ml).

The human colon adenocarcinoma cell line Caco-2 was a kind gift of Dr. W. Dinjens (Department of Pathology, Erasmus Medical Center Rotterdam, The Netherlands). The cell line was cultured in RPMI1640 medium supplemented with 10% human pooled serum, L-glutamin (200 mmol/L) and penicillin (5000 U/ml). All supplements were obtained from Invitrogen (Karlsruhe, Germany). For experiments, the cells were trypsinized (5 minutes at 37°C), centrifuged (5 minutes at 450 g), resuspended in RPMI 1640 and counted. Viability was measured by trypan blue exclusion and always exceeded 95 percent.

Adhesion assay

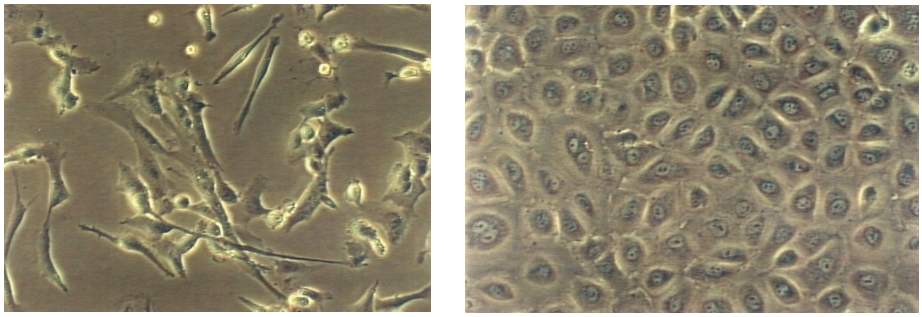
To quantify tumour cell adhesion to a monolayer mesothelium, a standardised cell adhesion assay was developed according to methods from Catterall et al.[13] Mesothelial monolayers were established in 96 well plates (Canberra Packerd, Groningen, The Netherlands), pre-coated with collagen type I (15 $\mu g/cm^2$, coating according to instructions of the manufacturer). To do this, confluent cells were washed with phosphate buffered saline (PBS) and harvested with trypsin (0.05%) and EDTA (0.02%) and 2.5 x 10^4 mesothelial cells were added in 200 µl of medium to each well. The plates were incubated at 37 °C, in a humidified atmosphere of 5% CO_2 in air; medium was replaced daily with fresh medium. Monolayers reached confluence in 5 to 7 days as determined by light microscopy. In order to determine the effect of the serum samples on tumour cell adhesion, mesothelial monolayers were pre-incubated overnight with the 8 serum samples of the same patient. We used a 10% serum-RPMI medium. The control mesothelial monolayers were incubated overnight in medium with 10% human pooled normal serum.

Tumour cells were labelled with calcein. Before adding to the mesothelial monolayers, the labelled cells were washed twice with RPMI1640/0.5%BSA to remove free dye. Medium from the experimental wells was removed and 200 µl RPMI1640/0.5%BSA containing 30,000 calcein labelled tumour cells was added. Plates were centrifuged for 1 minute at 80 g on a Perkin Elmer centrifuge and incubated

at 37 °C for 60 minutes. Thereafter, the medium of each well was removed and washed twice with 200 µl RPMI1640/0.5% BSA medium. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 excitation and 530 emission filters. On each plate, a standard was prepared by adding different numbers of labelled tumour cells to the wells. The amount of tumour cells adhered was determined by calibrating the measured fluorescence of the experimental wells on the standard.

Statistical analysis

All data were analysed using analysis of variance (ANOVA) to determine overall differences between group means. If the ANOVA was significant on a 5% level, the post-hoc Newman-Keuls test was carried out to make a comparison between groups. Values represent ± SEM of n=6 wells per treatment. $P \leq 0.05$ was considered to be statistically significant.



A B

Figure 1
Growth features of mesothelial cells. During the first days of culture, the mesothelial cells spread out to make contact to each other in order to form a monolayer (1A). After 3-5 days there is a monolayer, notice the typical cobblestone appearance (1B).

RESULTS

During the first day of culture, the mesothelial cells spread out to make contact to each other in order to create a monolayer (figure 1A). After 3-5 days, a monolayer is formed with a typical cobblestone appearance (figure 1B). The morphology of the monolayers did not change after pre-incubation with the serum samples.
Loading of the tumour cells with calcein did not affect their viability (>95% using trypan blue). To determine the stability of calcein uptake, the release of fluorescence in the supernatant of labelled cells after an incubation time of up to 120 minutes was measured. The fluorescence of the washed cells remained constant for at least 90 minutes, indicating

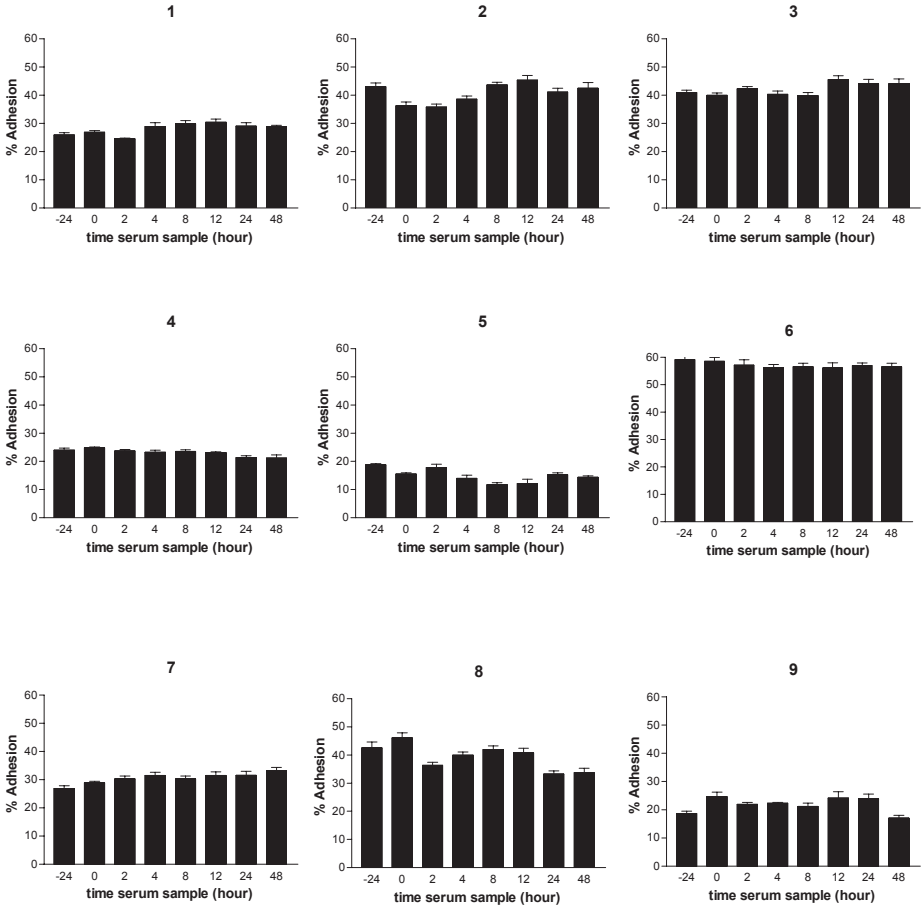


Figure 2
Bargrafts showing the adhesion of Caco-2 cells to monolayers mesothelium of 9 different patients pre-incubated with serum samples of the same patient at 8 different periods. Data are expressed as the mean (n=8) +SEM.

retention of the dye in the cells (data not shown). A dilution series was made by using labelled Caco2 cells on a mesothelial monolayer. There was a direct relationship between the cell number added and measured fluorescence, resulting in a linear correlation which was used as a standard to calibrate the measured fluorescence. In this way the number of adhered tumour cells in the experimental wells could be determined.

Tumour cell adhesion to a mesothelial monolayer in medium with 10% human pooled serum was relatively slow and temperature dependent. Maximum adhesion was seen after 60 minutes. At this time point a steady state situation was achieved and cell adhesion did not increase hereafter. The tumour cells remained rounded up during these adhesion

assays. After more than 60 minutes, the first signs of spreading out could be detected under the light microscope. Therefore, for all subsequent experiments, 60 minutes was taken as a cut-off point.
The bar graphs in figure 2 show the results of adhesion after preincubation of the meso-

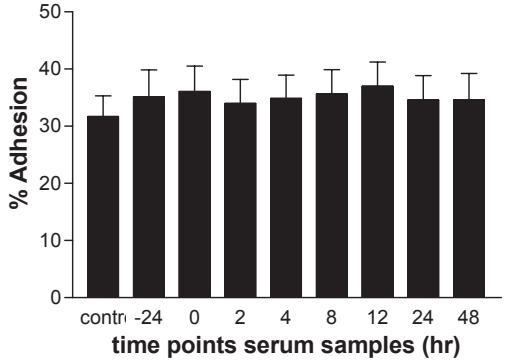


Figure 3
Mean adhesion of Caco-2 cells to monolayer mesothelium pre-incubated with serum samples of the 9 patient at 8 different periods. Data are expressed as the mean (n=8) +SEM. The first bar shows the control adhesion of Caco-2 cells on mesothelial cells incubated in human pooled serum overnight.

thelial cells with the different serum samples of the 9 individual patients. Compared with the percentage adhesion observed with serum obtained one day prior to surgery (-24 hr), no statistically significant changes in the percentage adhesion were observed after incubation with serum at the start of the surgical procedure (0 hr) and 2, 4, 8, 12, 24 and 48 hr during and after surgery. As shown in figure 2 the adhesion of Caco-2 cells to the mesothelial cells from the different patients varies between 20-55%. The variability of the adhesion of Caco-2 cells to the different donors was also observed within incubation with pooled human serum, suggesting intrinsic differences between the mesothelial cells obtained from different patients in their capacity to cause Caco-2 adhesion. Figure 3 shows the mean adhesion of the 9 patients, including the control with human pooled serum. No significant difference in adhesion was observed between the control group and the serum samples taken from the patients.

DISCUSSION

Tumour metastasis following curative surgery has been a relentless hurdle for patients with colorectal cancer. Tumour recurrence after surgery has been studied in various animal models. These studies concluded that tumour recurrence is enhanced by surgical trauma

and that the expanse of tumour growth is dependent on the severity of surgical trauma.[14-19] Despite all these animal models, the mechanism of tumour recurrence is still unclear. Which factors are involved and whether it is a systemic or local stimulated process is still open to question.

Local tumour recurrence is a multistep process in which several factors are involved. Serum is a the total complex of cytokines, soluble adhesion molecules and growth factors. In this model we have chosen to use whole serum, because it is not known if tumour recurrence is influenced by systemic factors. It is important to investigate first if the serum is influencing tumour adhesion, before you extract different factors from the serum and study them individually.

In this study we have evaluated whether systemic influences on local tumour recurrence can be involved. In our cell adhesion model post-operative serum did not enhance adhesion of Caco-2 cells to incubated mesothelium. This suggests that systemic factors are not a main cause influencing the adhesion of spilled tumour cells.

However, several other explanations for the absence of an influence of post-surgical serum on tumour cell adhesion need to be discussed as well.

1) The concentration of factors that could potentially influence tumour cell adhesion may be too low. In previous studies we showed that 1 ng/ml IL-1β or TNF-α can enhance tumour cell adhesion to the mesothelium.[20] The concentration of IL-6 in serum in normal healthy patients is < 100 pg/ml and levels of IL-1β and TNF-α are not detectable in serum. [21]

2) The absence of enhancement of tumour adhesion may be explained by blockade of ICAM-1 and VCAM-1 receptors by soluble ICAM-1 and VCAM-1. *In vivo*, the function of soluble ICAM-1 and VCAM-1 is that they have been shed of the tumour cells, in order to block the counterligands on immunocompetent recognition lymphocytes and in that way promote metastasis development.[8,9]

In addition, another interesting observation is that inflammatory cytokines such as IL-1β or TNF-α are detectable in higher concentrations (>1000 pg/ml) in the drainage fluid of the thorax and abdominal cavity.[21] Taken this consideration, local tumour recurrence may be more potently influenced by locally produced factors.

The basal adhesion of colon carcinoma cells to the mesothelium, after incubation with serum a day before surgery and control human pooled serum, varies between 20-60%. Variations in basal adhesion are caused by inter-individual differences of mesothelial cells, which is the only variable in this model, but of the utmost importance *in vivo*. These differences of the mesothelium may lead to the inter-individual variations in tumour recurrence after curative colon surgery.

In conclusion tumour recurrence might be influenced by substances of the cocktail of intra-abdominal produced mediators, which have conflicting demeanor regarding wound healing and local tumour recurrence. In addition to the influence of these locally

produced factors, this study shows that systemic factors produced directly after surgery are not a main factor influencing tumour cell adhesion. More research is required to investigate if systemic factors can stimulate distant metastasis and in which manner local tumour recurrence is influenced. Such knowledge may lead to novel therapeutic options using compounds interfering with this process.

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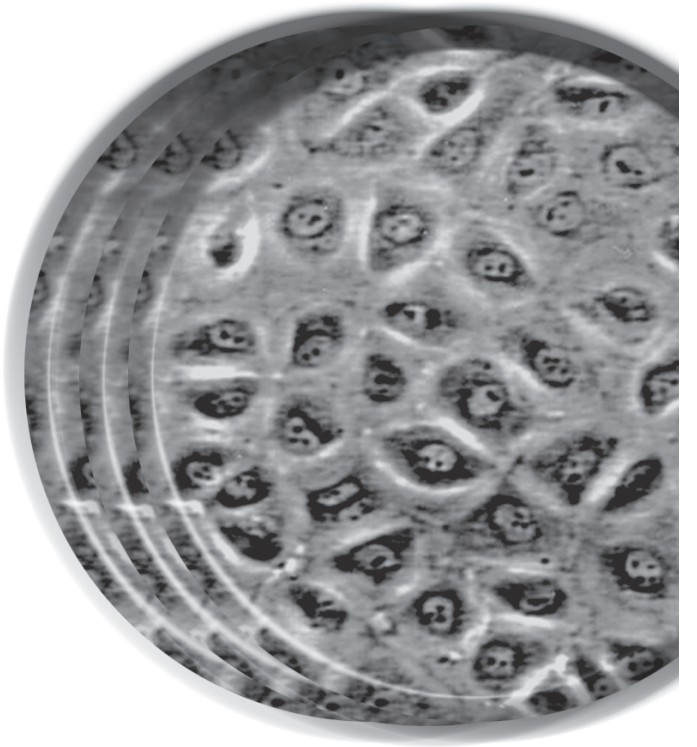
CHAPTER 7

The influence of latex on tumour cell adhesion

Adhesion News & Views 2004



ABSTRACT

The presence of foreign material in the abdominal cavity during surgery irritates the peritoneal surface leading to an inflammatory reaction of the peritoneum. This defensive mechanism can provoke adhesion formation. The same peritoneal defence cascade is thought to play a role in the process of intra-abdominal tumour recurrence. The aim of this study was to compare 4 different types of gloves and their influence on tumour cell adhesion to human mesothelial cells in a *in vitro* model.

An experimental human *in vitro* model was designed using Caco2 colon carcinoma cells. For all studies, primary cultures of omentum derived mesothelial cells were used. Tumour cell adhesion to a mesothelial monolayer was assessed after pre-incubation of the mesothelium with powdered and non- powdered gloves solutions. In this model we have used Baxter (powdered), Romed (powdered), Biogel (non-powdered) and non-latex Biogel(non-powdered) gloves.

These results showed that only the non-latex powder free glove solution does not increase the tumour cell adhesion to a mesothelial monolayer. From previous *in vivo* rat studies we know that powder leads to increased adhesion and growth of free intra-abdominal tumour cells. This study shows that beside powder the pure latex particles can enhance the tumour recurrence as well. The presence of latex proteins may aggravate the normal inflammatory reaction to tissue injury and therefore enhance tumour adhesion to the peritoneum.

INTRODUCTION

The presence of foreign material in the abdominal cavity during surgery may irritate the peritoneal surface leading to an inflammatory reaction of the peritoneum. This defensive mechanism can provoke adhesion formation. The same peritoneal defence cascade is thought to play a role in the process of intra-abdominal tumour recurrence.

Following potentially curative resection of gastro-intestinal carcinoma, local recurrence and peritoneal dissemination are common in tumour recurrence.[1] Distribution patterns of first peritoneal recurrence show that the resection site is preferential, and combined recurrence on peritoneal surfaces and resection site is common.[2] Early preoperative tumour cell seeding and preoperative shedding of tumour cells, due to handling the tumour and leakage from dissected lymphatic channels, are the most likely causes of tumour recurrence. The tumour cell entrapment theory proposes that the fibrinous exudates, formed as an initial response to surgical trauma of the peritoneum, facilitates implantation of cancer cells onto raw tissue.[2] The dynamic cascade of peritoneal healing, induced by peritoneal damage, leading to adhesion formation also seems to be important in the process of intra-peritoneal adhesion and growth of tumour cells. Indeed, previously described clinical and experimental studies showed that surgical trauma may promote intra-abdominal recurrence.[3-5] The degree of inflicted trauma correlated with the extent of intra-abdominal tumour load.[5] In previous studies we investigated the contribution of glove powder on local tumour take *in vivo*.[6] It appears that glove powder as well as pure starch leads to enhanced tumour take. Since good powder free alternatives are available, most hospitals are not using powdered gloves anymore during intra-abdominal surgery. The aim of this study was to compare four different types of powdered and non-powdered gloves and their influence on tumour cell adhesion to human mesothelial cells in a *in vitro* model.

MATERIALS AND METHODS

Culture of omental mesothelial cells

Mesothelial cells (MC) were obtained from the omental tissue of patients undergoing elective abdominal surgery who had given informed consent. The MC were isolated according to techniques modified from Wu et al.[7] The omentum was transferred to fluid containing 0.05% trypsin-0.02% EDTA (Invitrogen, Karlsruhe, Germany). After 15 minutes the detached MC were pelleted by centrifugation at 1800 rpm for 10 minutes and were resuspended in RPMI 1640 supplemented with 10% foetal calf serum, L-glutamin (200 mmol/L) and penicillin (5000 U/ml)(Invitrogen, Karlsruhe, Germany). MC were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (75 cm^2, Corning BV, Schiphol-Rijk, The Netherlands) pre-coated with collagen type I (17.5 µg/ cm^2).

Tumour cell line

The human colon adenocarcinoma Caco-2 was a kind gift of Dr. W. Dinjens. The cell line was cultured in RPMI1640 medium supplemented with 10% foetal calf serum, L-glutamin (200 mmol/L) and penicillin (5000 U/ml). All supplements were obtained from Invitrogen, Karlsruhe, Germany. Before use, cells were trypsinized (5 minutes at 37°C), centrifuged (5 minutes at 1500 rpm), resuspended in RPMI 1640 and counted. Viability was measured by trypan blue exclusion and always exceeded 95 percent.

Surgical gloves; isolation of latex

The gloves we have used were manufactured by three different companies. In this study we have used Baxter (powdered), Romed (powdered), Biogel (non-powdered) and non-latex Biogel(non-powdered) gloves. The gloves were cut in pieces of 1x1 cm. The latex allergens were prepared by extracting the pieces in phosphate buffered saline (PBS), pH 7.4, containing 0.03% human serum albumin and 0.5% phenol during 72 hours at 4°C. The supernatants were collected and dialysed with a 3500 d molecular weight cutoff membrane (Molecularpouros Dialyses Membrane, Spectrum, Canada) against deionised water for 72 hours. The dialyzate was centrifuged at 13,000 g for 15 minutes, and the supernatant were filter sterilised with a 0.22 µm Millex GS filter (Millipore, The Netherlands) and freeze-dried. A 1% dilution in PBS was made from the freeze-dried material.[8]

Calcein-AM solution and incubation

The dye solution, calcein-AM, used to quantify tumour cell adhesion was prepared by dissolving 50 µg calcein (Molecular Probes, Leiden, The Netherlands) in 5 µl anhydrous dimethyl sulphoxide (Sigma-Aldrich, Zwijndrecht, The Netherlands) and adding this solution to 5 ml of RPMI 1640 medium supplemented with 0.5% bovine serum albumin (RPMI 1640/0.5%BSA, BSA obtained from Sigma-Aldrich). Trypsinized Caco2 and HT29 cells ($1x10^6$ cells/ml) were incubated in RPMI1640/0.5%BSA at 37 °C for 30 minutes with occasional mixing.

Adhesion assay

To quantify tumour cell adhesion to a monolayer mesothelium, a standardised cell adhesion assay was developed according to methods from Catterall et al.[9] Mesothelial monolayers were established in 96 well plates (Canberra Packerd, Groningen, The Netherlands), pre-coated with collagen type I (15 µg/cm^2, coating according to instructions of the manufacturer). To do this, confluent cells were washed with phosphate buffered saline (PBS) and harvested with trypsin (0.05%) and EDTA (0.02%) and 2.5×10^4 mesothelial cells were added in 200 µl of medium to each well. The plates were incubated at 37 °C, in a humidified atmosphere of 5% CO_2 in air; medium was replaced daily with fresh medium. Monolayers reached confluence in 5 to 7 days as determined by light microscopy. In order to determine the effect of the latex on tumour cell adhesion, mesothelial monolayers were pre-incubated with increasing doses. Non pre-incubated mesothelial monolayers served as controls.

Tumour cells were labelled with calcein as described above. Before adding to the mesothelial monolayers, the labelled cells were washed twice with RPMI1640/0.5%BSA to remove free dye.

Medium from the experimental wells was removed and 200 µl RPMI1640/0.5%BSA containing 30,000 calcein labelled tumour cells was added. Plates were centrifuged for 1 minute at 80 g on a Perkin Elmer centrifuge and incubated at 37 °C for 60 minutes. After this, the medium of each well was removed and washed twice with 200 µl RPMI1640/0.5% BSA medium. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 excitation and 530 emission filters. On each plate a standard was prepared by adding different numbers of labelled tumour cells to the wells. The amount of tumour cells adhered was determined by calibrating the measured fluorescence of the experimental wells on the standard.

DNA-assay

To investigate whether overnight incubation of the mesothelial monolayer with the different latex solutions was influencing the cell growth, a DNA measurement was performed. In this assay, the DNA content of the mesothelial cells was measured using the bisbenzimide fluorescent dye (Roch Diagnostics) as previously described by Hofland et al.[10] In short, at the end of the incubation period the plates were washed twice with saline and stored at -20°C until further analysis. The cells were extracted with ammonia solution (1 mmol/L) – Triton x 100 (0.2% v/v) by sonification during 5 seconds at amplitude 15 (Soniprep 150; MSE). Thereafter assay buffer (100 mmol/L NaCl, 10 mmol/L EDTA, 10 mmol/L Tris; pH 7.0) was added. The remaining solution was centrifuged at 2000 g during 5 min and 100 µl aliquots of the supernatant was mixed with 2 ml Hoechst dye H33258 (100 µg/L). Fluorescence was measured after 10 min with the excitation and emission wavelengths set at 350 and 455 nm respectively. The fluorescence of experimental samples were referenced to a standard curve of calf thymus DNA (type II, no D-3636; Sigma, Zwijndrecht, The Netherlands).

Statistical analysis

All data were analysed using analysis of variance (ANOVA) to determine overall differences between group means. If the ANOVA was significant on a 5% level, the post-hoc Newman-Keuls test was carried out to make a comparison between groups. $P \leq 0.05$ was considered to be statistically significant. Experiments were carried out at least twice with comparable results.

RESULTS

Morphology and growth of mesothelial cells

Mesothelial cells were isolated from omental tissue and brought into culture. The first days of culture the mesothelial cells spread out to make contact to each other and form a monolayer. After 5 to 7 days there is a monolayer and also the typical cobblestone appearance (figure 1, chapter 6).

Before we started the adhesion experiments the vitality of the mesothelial cells was measured after incubation with different concentrations of the latex solutions by means

of trypan blue coloring. The ideal concentration for all the different latex solutions was 0.125%. Incubating the mesothelial cells with this concentration did not influence the morphology of the cells microscopically. This concentration of latex was not influencing the growth of the mesothelial monolayers as well (data not shown).

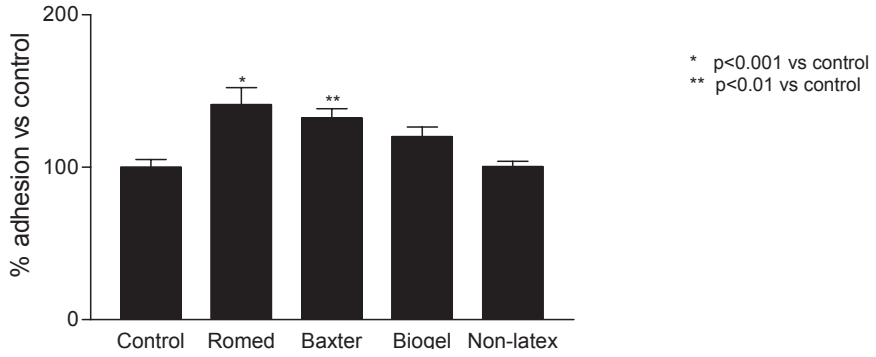


Figure 1
The effect of the number of wash steps after 1 hour incubation of HT29 and Caco2 on collagen I. Means (n=6) ± SEM are shown.

Adhesion assay

In the control situation the average adhesion of Caco2 cells to the mesothelial monolayers was 20%. Pre-incubation of the monolayer mesothelium with a 0.125% Romed solution resulted in an average of 40% enhanced tumour cell adhesion compared to the control situation ($p<0.001$). Pre-incubation with 0.125% Baxter solution increased the tumour cell adhesion with 30% ($p<0.01$). Biogel solution resulted in a 20% enhancement of tumour cell adhesion ($p>0.05$). The non-latex Biogel solution did not affect the amount of tumour cell adhesion (figure 1).

Table 1 shows the significance levels between all groups. There was a significant difference in tumour cell adhesion between the Romed gloves and the non-powdered Biogel gloves.

Table 1

	Control	Romed	Baxter	Biogel	Non-latex
Control		<0.001	<0.01	ns	ns
Romed	<0.001		ns	<0.05	<0.001
Baxter	<0.01	ns		ns	<0.01
Biogel	ns	<0.05	ns		ns
Non-latex	ns	<0.001	<0.01	ns	

Levels of significance between all groups. $P \leq 0.05$ was considered to be statistically significant.

DISCUSSION

Since the introduction of gloves by William S. Halsted in 1896, several materials and lubricants have been used in the glove industry to facilitate manufacturing and to lessen the hazards associated with glove use.[11] Modern gloves are made of natural rubber (latex) or synthetic rubber (vinyl or polyvinyl). The glove lubricants are powder (starch) or hydrogel. Glove lubricants have been associated with a number of iatrogenic problems in surgical patients. Especially starch powder in surgical gloves can lead to serious complications such as granulomatous peritonitis, adhesion formation and infection potentiation.[12] Previous experiments done in our laboratory showed that powder increases adhesion formation as well as tumour cell adhesion and growth in an *in vivo* rat model.[6] In the current *in vitro* experiments the latex solutions increase the tumour adhesion as well. The non-powdered Biogel and the non-latex Biogel solution did not increase adhesion of tumour cells. The enhancement of tumour adhesion by the presence of latex proteins may be caused by an aggravation of the normal inflammatory reaction to tissue injury which enhances tumour adhesion to the peritoneum. In peritoneal wound healing inflammatory and mesothelial cells produce an abundance of cytokines and growth factors which might be beneficial for tumour cell adhesion and tumour growth.[13-15] What we know from other studies is that there is a significant correlation between the intensity of trauma to the peritoneum and the degree of tumour recurrence.[16] The most impressive enhancement of tumour was seen after incubation with the Romed (40%) and Baxter (30%) latex solutions. These are powdered gloves. This enhancement is probably caused by an increased release of cytokines by the mesothelial cells after pre-incubation induced by starch powder in combination with the latex particles in these solutions. Despite evidence in support of starch induced complications, a considerable number of surgeons continues to wear starch powdered gloves.[17] The non-powdered Biogel glove solution did not increase the tumour cell adhesion significantly. The non-latex gloves are not influencing the tumour cell adhesion at all. A logical conclusion might be that we should operate with non-latex gloves. However the disadvantage of non-latex gloves is inferior flexibility and tactility. Therefore the second best option is to use non-powdered gloves during operation. Moreover, the surgeon should abandon all substances which will cause enhanced trauma to the peritoneum from the operating theatre and should try to handle the operating field very carefully in order to limit surgical trauma of the peritoneum as much as possible.

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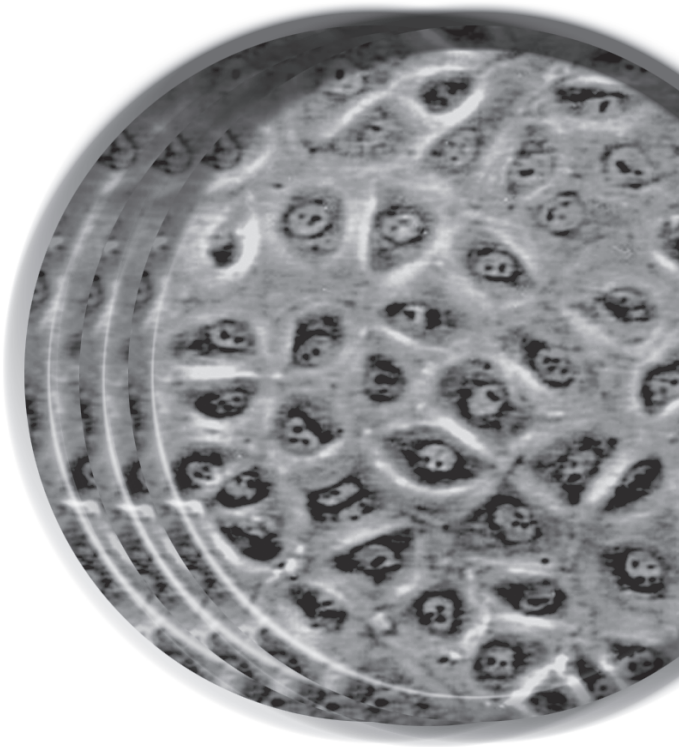
CHAPTER 8

Mechanisms of colon carcinoma cell adhesion to components of the extracellular matrix

submitted



ABSTRACT

In addition to the studies in which the adhesion to mesothelial monolayers was investigated, this chapter describes the results of the studies on the interactions between colon cancer cells and the extracellular matrix (ECM). These interactions play a major role in intravasation and subsequent extravasation during distant metastasis in colon cancer, but are also important in the process of intra-abdominal recurrences in areas of the peritoneum where mesothelial cells have been exfoliated.

In an *in vitro* model the adhesion of the HT29 and Caco2 colon carcinoma cell lines to the main components of the ECM, collagen type I and IV, fibronectin and laminin, was investigated. This adhesion was compared to the adhesion to the overlying endothelium and mesothelium. Inhibition assays with the use of monoclonal antibodies were performed to study the role of adhesion molecules in tumour cell - ECM interactions.

We showed that different colon cancer cell lines strongly adhere to the ECM, although they express different adhesion patterns to these components. Lower adhesion was seen to the endothelium and mesothelium. Knowledge of the integrins involved in tumour cell - ECM interactions may lead to new therapeutic strategies to prevent metastasis.

INTRODUCTION

Despite the development of modern treatment strategies for colon cancer, recurrences after intentionally curative surgery remain a major problem. Recurrence rates up to 40% have been reported and preferential sites are locally, as well as liver and lungs.[1-4] The majority of death of colorectal cancer patients is caused by metastatic disease rather than the primary tumour.[5]

Key events in the metastasic cascade comprise of invasion in tumour stroma, intravasation of the circulatory system at the primary site, survival from defence mechanisms, extravasation at the secondary site and outgrowth of new tumours.

At the secondary site, binding to mesothelial or endothelial cells results in retraction with discontinuity of the cell layer, thereby exposing the underlying extracellular matrix (ECM).[6] Binding to components of the ECM stimulates the production of matrix metalloproteinases which degrade the ECM.[7] In this way, the tumour cells can enter the underlying tissue, proliferate and grow out to form a metastasis. Tumour cells show a higher binding affinity to the ECM compared to the overlying endothelium or mesothelium.[8,9] This difference in binding affinity causes a gradient for the tumour cells into the underlying tissue. In the case of trauma to the mesothelial or endothelial cell layer, it is likely that more circulating tumour cells are able to adhere and successfully form a metastasis. Trauma may consist of direct trauma like the disruption of the mesothelium in case of abdominal surgery or indirect trauma like inflammation.

Major components of the ECM are collagen type I (C I) and IV (C IV), fibronectin (FN) and laminin (LN). Therefore, these components are thought to play an important role in cancer metastasis.

Binding to these components is accomplished by adhesion molecules on tumour cells. Both the cellular adhesion molecules ICAM and VCAM and the integrins may play a role in this interaction. The very late activation (VLA) antigens form a major subclass of integrins which are transmembrane heterodimers consisting of an α and β_1 chain. It is known that VLA-1 (α1β1), VLA-2 (α2β1) and VLA-3 (α3β1) are able to bind to C I and IV. FN act as a ligand for VLA-3 till VLA-5 and LN for VLA-1, -2, -3, -6 and -7.[10,11]

To study the adhesion of colon cancer cells to components of the ECM and to compare this adhesion with adhesion to the covering layer consisting of endothelium or mesothelium we developed a human *in vitro* model. We used two different human colon cancer cell lines, namely the poorly differentiated HT29 cell line and the moderately differentiated Caco2 cell line. The role of the VLA-antigens as well as ICAM-1 and VCAM-1 in binding to particular components was evaluated.

MATERIALS & METHODS

Cells

The human colon carcinoma cell lines HT29 and Caco2 were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, glutamine (2 mM) and penicillin (10^5 U/L) and maintained by serial passage after trypsinization using 0.05% trypsin / 0.02% EDTA (all, except penicillin, obtained from Invitrogen, Breda, the Netherlands; penicillin from Yamanouchi, Leiderdorp, The Netherlands). Before the adhesion assay, tumour cells were trypsinized and maintained in suspension culture for 2 hours to regenerate cell-surface proteins.

Human microvascular endothelial cells of the lung (EC) were purchased from Cambrex (Verviers, Belgium) at passage 4 and maintained in EGM-2-MV Bullet kit according to the manufacturer at 37°C, 95% relative humidity and 5 % CO_2. Confluent monolayers were passaged by 0.025% trypsin / 0.01% EDTA and cells were used up to passage 8.

Human mesothelial cells (MC) are obtained from omental tissue of patients undergoing elective abdominal surgery. Isolation of the cells was accomplished using modified techniques from Wu *et al.*[12] To detach MC from the omentum, 0.05% Trypsin with 0.02% EDTA is used. Next, MC are resuspended in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine (all Invitrogen) and penicillin (10^5 U/L) and brought into culture in collagen coated culture flasks at 37°C, 95% relative humidity and 5 % CO_2. Confluent monolayers were passaged by 0.05% trypsin / 0.02% EDTA.

Adhesion assay

To quantify tumour cell adhesion to components of the ECM, to EC and to MC monolayers, a standardised cell adhesion assay was developed according to methods from Catterall *et al.*[13] Flat-bottomed 96 well microtiter plates (Perkin Elmer, Groningen, The Netherlands) were coated with 20 µg/ml C I, C IV (Sigma-Aldrich, Zwijndrecht, the Netherlands), FN or LN (Boehringen-Mannheim, Mannheim, Germany). To block unspecific binding sites, wells were pre-incubated with 1% BSA for 30 minutes.

To obtain endothelial and mesothelial monolayers, confluent cells were trypsinized and 2×10^4 EC and 1×10^4 MC were added to each well. The plates were incubated at 37°C, 95% relative humidity, 5% CO_2 and medium was daily replaced by fresh medium. EC reached confluence in 3 to 4 days as determined by light microscopy. For MC, confluence was achieved after 5 days.

To quantify tumour cell adhesion, trypsinized tumour cells (1×10^6 cells/ml) were labelled with calcein-AM (Molecular Probes, Leiden, The Netherlands) and 3×10^4 cells per well were added.

Evaluation of the contribution of integrins in the adhesion of tumour cells to components of the ECM was done by pre-incubation of the tumour cells with the function-blocking antibodies anti-α_1 till anti-α_6, anti-β_1 (BD Biosciences, Alphen a/d Rijn, the Netherlands), anti-ICAM and anti-VCAM (Dako Cytomation, Heverlee, Belgium) in a concentration of 1 µg/ml for 30 minutes at room temperature before adding the tumour cells to the wells.

Plates were centrifuged for 1 minute at 80 × *g* in a Heraeus centrifuge and incubated at 37°C for 1 hour. After this, wells were washed twice with medium to remove non-adherent tumour cells. The remaining

fluorescence per well was measured on a Perkin Elmer plate reader using 485 nm excitation and 530 nm emission filters.

Immunocytochemistry of adhesion molecules

Tumour cells were prepared for staining by cytospin preparation, fixed in acetone for 10 minutes and stored at -20°C until use.

The cytospins were incubated for 30 minutes at room temperature with the following primary antibodies: mouse anti-human monoclonal antibodies to α_1 till α_6, β_1 (BD Biosciences, Alphen a/d Rijn, the Netherlands), ICAM-1 and VCAM-1 (Dako Cytomation, Heverlee, Belgium). Negative controls were incubated with PBS. As secondary antibodies, biotinylated goat anti-mouse antibodies were used followed by incubation with Streptavidin-biotinylated alkalin-phosphatase complex. Substrate development was done with New Fuchsine 4%. Cytospins were counterstained with Haematoxiline.

The expression of cell adhesion molecules was quantified by 2 separate observers using semi-quantitative scoring system ranging from no expression (-), weakly positive (±) to positive expression (+).

Statistical analysis

All data were analysed using analysis of variance (ANOVA) to determine overall differences between groups. The post-hoc Newman-Keuls test was carried out to compare between groups. $P \leq 0.05$ was considered to be statistically significant. Experiments (n=6) were performed at least twice with comparable results.

RESULTS

Validation of assay

Labelling tumour cells with calcein-AM did not decrease their viability (>95% using trypan blue). Incubation times not exceeding 60 minutes gave negligible leakage of calcein out of the cells into the medium (data not shown). Longer incubation times gave significant calcein loss resulting in unreliable results. Therefore, we used 60 minutes incubation time in our experiments. After incubation, three times washing resulted in thorough washing away non-adhering tumour cells and leaked calcein from the experimental wells (Fig 1). Dilution series showed a linear correlation between tumour cell number and measured fluorescence that was used as a standard to calibrate measured fluorescence. In this way the amount of adhered tumour cells in the experimental wells could be determined.

Tumour cell adhesion

Table 1 shows the adhesion of HT29 and Caco2 to the different components of the ECM as well as to endothelial and to mesothelial monolayers. For HT29, highest adhesion after 1 hour incubation was achieved to C I, namely 60.9%, followed by adhesion to C IV with 46.4%, than LN with 39.6% and lowest adhesion was to FN with 20.6%. Caco2

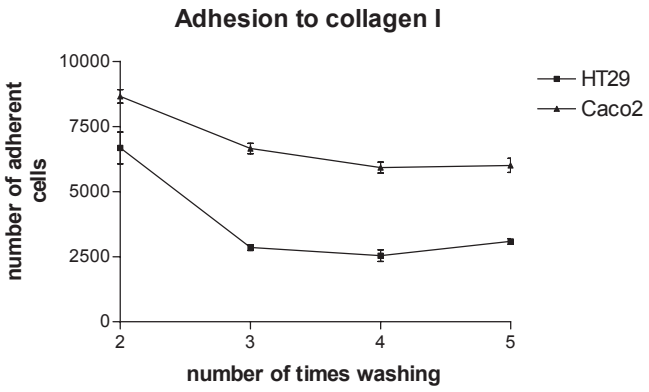


Figure 1
The effect of the number of wash steps after 1 hour incubation of HT29 and Caco2 on collagen I. Means (n=6) ± SEM are shown.

cells showed a different adhesion pattern with highest adhesion to C IV (51.4%), followed by adhesion to FN with 48.8%, than to C I with 41.8% and lowest adhesion was to LN with 21.9%. To C IV and FN, Caco2 demonstrated a significant higher adhesion compared to HT29 (resp. $p<0.05$ and $p<0.01$). HT29 showed a significantly higher adhesion to C I and LN compared to Caco2 ($p<0.01$). The adhesion to EC was for both tumour cell lines considerably lower compared to the adhesion to the ECM, namely 12.1% of the HT29 and 21.9% of the Caco2 adhered to EC ($p<0.01$). Unlike the endothelial cells, which are acquired from a single donor, are the mesothelial cells obtained from different donors. Therefore, series of adhesion experiments were performed using monolayers of 5 different patients. Highest adhesion was found for Caco2 with 34.1% of the cells adhering. The mean adhesion of HT29 to MC was 21.4% of the cells.
Both cell lines showed low affinity for uncoated wells, with only 2.4% of HT29 cells and 5.7% of Caco2 cells adhering to the plastic of the experimental wells.

Table 1.

Substrate	C I	C IV	FN	LN	EC	MC	plastic
HT29	60.9 ± 2.1	46.4 ± 1.4	20.9 ± 2.7	39.6 ± 6.8	12.1 ± 2.0	21.4 ± 5	2.4 ± 0.8
Caco2	41.8 ± 4.0	51.4 ± 3.3	48.8 ± 5.7	21.9 ± 1.6	21.9 ± 3.4	34.1 ± 5	5.7 ± 0.5

Adhesion of HT29 and Caco2 to various substrates: components of the extracellular matrix, endothelial cells, mesothelial cells and to empty experimental wells (plastic). Data are expressed as the mean (n=6, % vs total) ± SD.

Expression of integrins, ICAM and VCAM

Table 2 shows the immunocytochemistry results of the tumour cells. Except for the α4-component, all α-components are expressed on HT29. The α1- and α5-components are only weakly expressed. On Caco2, all α-components are expressed, although the α1-, α4- and α5-components show only weak expression. The β-component is expressed on both tumour cell lines, as well as ICAM-1 and VCAM-1.

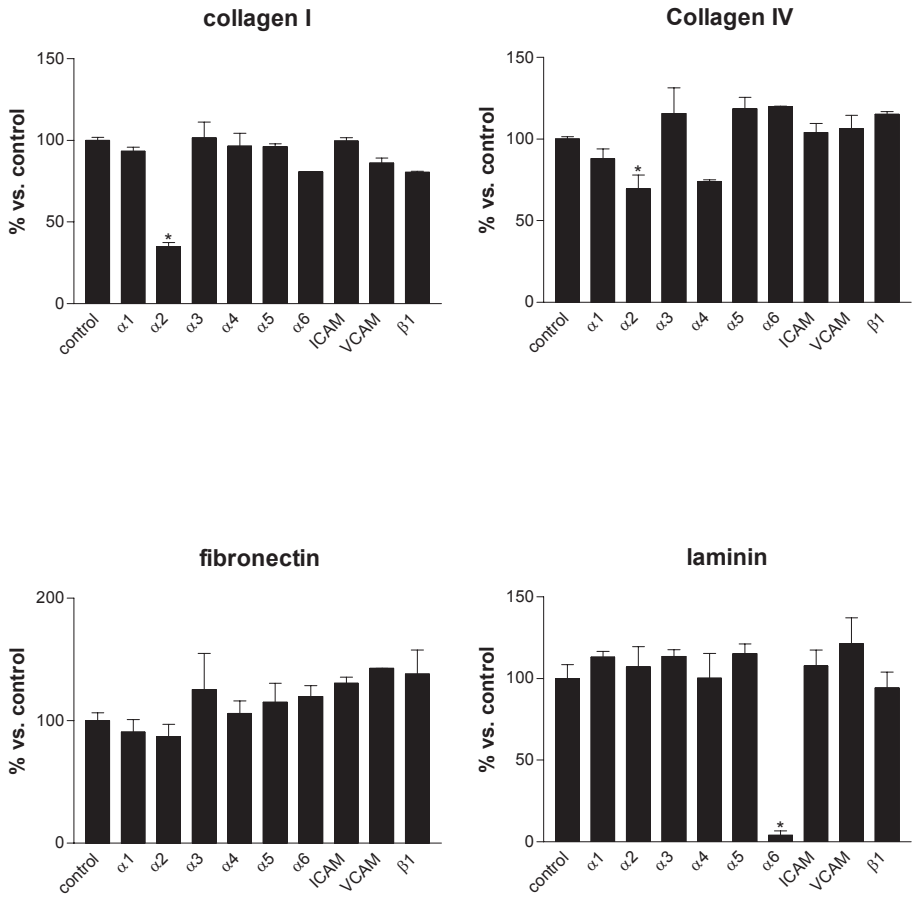


Figure 2
Adhesion of HT29 to different components of the ECM. Tumour cells were pre-incubated with antibodies against α1-6, β1, ICAM-1 and VCAM-1 (1 µg/ml). Data represent means (n=6, % *vs.* control) ± SEM. *, $p < 0.01$.

Table 2.

antibody	HT29	Caco2
α1	+/-	+/-
α2	+	+
α3	+	+
α4	-	+/-
α5	+/-	+/-
α6	+	+
β1	+	+
ICAM	+	+
VCAM	+	+

Cell adhesion molecules expressed by HT29 and Caco2 as found by immunocytochemistry.

Inhibition assays

Inhibition assays using functional blocking antibodies to components of integrins are shown in figure 2 (HT29) and 3 (Caco2). Adding antibodies against the α_2 component gave a significant reduction of 64.9% ($p<0.01$) in HT29 cell adhesion to C I and of 30% ($p<0.01$) to C IV. For Caco2 a reduction of 46.8% was found by adding the α_2-antibody in the adhesion to C I and of 39.9% to C IV. Addition of the α_4-antibody resulted only for Caco2 in a significant reduction in the adhesion to C IV, namely 26.3%. Almost complete inhibition in HT29 cell adhesion to LN was achieved by addition of the α_6-antibody, since this antibody reduces adhesion with 96.1% ($p<0.01$). For Caco2 cells, no significant reduction was observed using the α_6-antibody in the adhesion to LN. Neither antibodies to other α-components, nor antibodies to the β_1-component, ICAM-1 and VCAM-1 gave an inhibition to ECM components.

DISCUSSION

The capacity for adhesion seems a contradiction in tumour metastasis, in which the first step the detachment of the tumour cell from the primary site is. However, detachment and adhesion are necessary steps and form a delicate balance in metastasis. Interactions between colon cancer cells and components of the ECM are thought to play a major role in intravasation and subsequent extravasation during the metastatic cascade in colon cancer. The purpose of this study was to analyse these interactions.

High adhesion was found for the HT29 and Caco2 colon cancer cell lines to the various components of the ECM, although each cell line displays its own adhesion pattern. For HT29, 60.9% of the cells attached to C I and 40% or more to C IV and LN. Lowest adhesion was seen to FN with only 20.6% of HT29 adhering. More than 50% of the Caco2 cells adhered to C IV, 48.8% and 41.8% of the Caco2 cells adhered to FN and

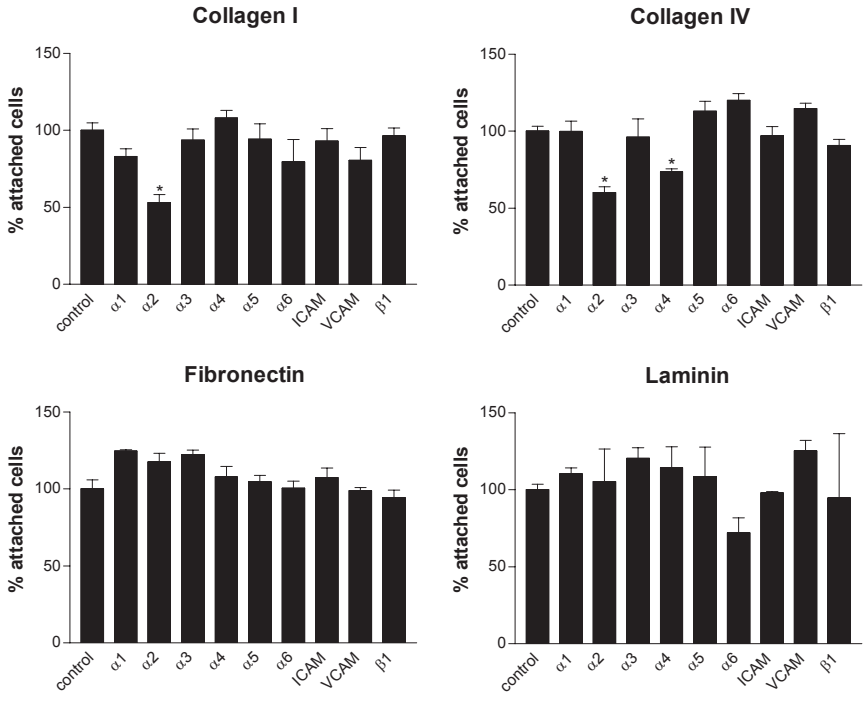


Figure 3
Adhesion of Caco2 to different components of the ECM. Tumour cells were pre-incubated with antibodies against α1-6, β1, ICAM-1 and VCAM-1 (1 μg/ml). Data represent means (n=6, % *vs.* control) ± SEM. *, $p < 0.01$.

C I respectively. Here, lowest adhesion was to LN with only 21.9% of the cells adhering. This individual adhesion pattern for various types of colon cancer cell lines was also found by Schlaeppi *et al*,[14] who compared 4 different colon carcinoma cell lines in their adhesion and invasion to various components of the ECM.

A preceding step in extravasation is adhesion to the mesothelium locoregionally or the microvascular endothelium at secondary sites. To the mesothelium the adhesion varied between different donors with each having their unique characteristics. Here, again a difference was observed between the 2 tumour cell lines, with a significant higher adhesion for Caco2 compared to HT29 to the mesothelium and to the microvascular endothelium.

In this study we found a high adhesion to individual components of the ECM. In order to be able to dissect the individual ECM component, we did not measure the adhesion to a complete ECM consisting of a mixture of the tested components and additional components like hyaluronic acid. Adhesion to a complete ECM may bring about even higher numbers of adherent cells and thereby creates a more pronounced difference in

adhesion affinity between the endothelium and ECM and perhaps also between the mesothelium and ECM leading to a stronger invasion gradient.

In our experiments, HT29 shows slightly lower adhesion to both the endothelium and mesothelium and higher adhesion to ECM components compared to Caco2 and therefore the invasion gradient seems more pronounced for HT29, a poorly differentiated colon carcinoma cell line, than for Caco2 which is a more differentiated cell line.

Recently, we investigated the influence of an inflammatory reaction on tumour cell – endothelial cell interactions.[15] In these studies we found that pre-incubation of the endothelium with pro-inflammatory cytokines or reactive oxygen species, that are produced by activated leukocytes, brings about an increase in tumour cell adhesion to the endothelium. This pre-incubation resulted in a significant increase in apoptosis of endothelial cells. In the early phase of apoptosis the cells are activated with increased expression of adhesion molecules resulting in enhancement of tumour cell adhesion. Eventually, cell death occurs with exposure of the underlying ECM. Therefore, inflammation, but also direct mechanical disruption of the covering monolayer leading to exposure of the ECM may cause enhanced tumour recurrence.

By immunocytochemistry, we showed that α2, α3, α6 and β1 are abundantly expressed on both tumour cells. ICAM, VCAM, α1, α3 and α4 have modest expression on one or both cell lines. These findings are corresponding with the literature.[14,16,17] It is known that the expression of integrins is versatile with pathways of inside-out and outside-in signalling as means of activation.[18-21] Accordingly, the expression of integrins is important, but the functionality is even more important. Consequently, inhibition assays using functional blocking antibodies were performed to get insight in integrins responsible for adhesion to ECM components. These assays clearly display that the binding of HT29 to LN seems almost completely formed by the α6-component, which forms the VLA-6 integrin with β1. The binding to LN exhibit a discrepancy between the two tumour cell lines, since the binding of Caco2 to LN is not significantly inhibitable by the α6-antibody in contrast to the binding of HT29. The α2-component was found to play a major role in the binding of both tumour cell lines to C I and to a lesser extent to C IV. These findings are partly in accordance with the results of Haier *et al*.[22] They used subclones of HT29, namely the poorly liver-metastatic colon carcinoma cell line HT29P and the highly liver-metastatic colon carcinoma cell line HT29LMM. In their study the α2-antibody comparably suppressed the adhesion of both cell lines to C I and IV, but the α6-antibody gave only a minor reduction in the adhesion to LN. Nonetheless, cell lines may not be comparable, since they used HT29 cell lines originating from liver metastases and we from primary tumours and therefore it is possible that these cell lines originate from different subgroups with different characteristics.

The next step in the metastatic cascade after binding to the ECM is invasion through this layer. It is believed that by binding to the ECM matrix metalloproteinases (MMP)

are produced or activated which degrade the ECM. As yet, the role of integrins in the degradation of the ECM has to be unravelled.

In summary, we showed that different colon cancer cell lines express a high binding affinity to components of the ECM but that their adhesion pattern varies. At present, the identity of molecules involved in these interactions remains indefinitive, because of the complexity of which the ECM consist and because of the heterogeneity between tumour cells. Knowledge on the selective or more universally expressed integrins involved in tumour cell adhesion to the ECM as well as to the EC and MC may be of help for the development of a cocktail of antibodies aimed at integrins. This cocktail may prove a powerful tool in the prevention of tumour recurrence.

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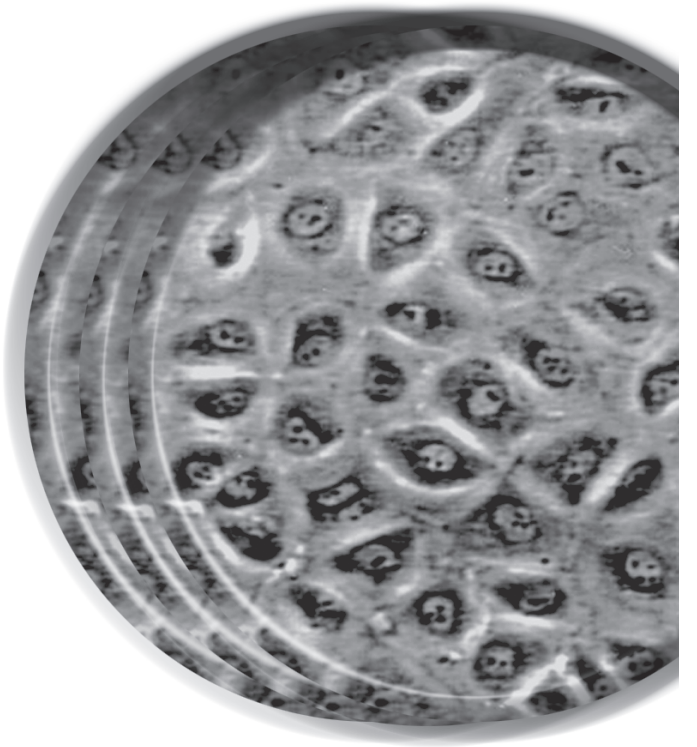
CHAPTER 9

General discussion



DISCUSSION

The process of cancer metastasis could be compared with an exhausting obstacle race, during which the tumour cell has to pass a series of sequential interrelated steps to become a clinically relevant lesion. In distant metastasis the tumour cell must succeed in invasion, embolisation, survival in the circulation, adhere to a distant capillary endothelium, followed by extravasation and multiplication in another organ.[1, 2] Locoregional tumour recurrence after intra abdominal seeding or spill of tumour cells seems to be less complicated, the tumour cell has to adhere, after which it can flourish and grow out as a lesion.

Although the process of locoregional tumour recurrence gives the impression to be highly comprehensible, peritoneal and local recurrence is a persistent hurdle after curative resection of colon or pancreatic tumours.[3-7] The resection site is preferable and recurrence to locoregional sites is common.[8] Several theories on local tumour recurrence have been advocated. The most feasible theory is the development of local recurrence after resection of locally advanced disease, which already penetrates the peritoneal surface or adjacent organs. Another source for local recurrence is the presence of cancer cells in the abdominal cavity prior or during surgery. Peritoneal washings, before manipulation of the tumour, are positive in 20-30% of the patients with colorectal cancer.[9-12] Furthermore, extensive manipulation of the tumour during surgery will cause leakage of tumour cells out of the dissected lesion or out of the transected lymphatic channels or veins. The free floating tumour cells or tumour emboli will precipitate on raw tissue, followed by an inflammatory response during which an outstanding environment for the tumour cells will be created.[8, 13]

Approximately 40% of the patients with colorectal cancer, who underwent surgery for local or locally advanced disease, develop recurrent disease.[7] The intra-abdominal recurrence rate after curative resection for pancreatic cancer is even more deplorable. Methods of prevention and treatment of locoregional metastasis, like neoadjuvant radiotherapy, brachytherapy, adjuvant chemotherapy, photodynamic therapy, (hyperthermic) intraperitoneal chemotherapy and peritonectomy, are developed and implemented in daily surgical practice.[14-20] The benefit of adjuvant chemotherapy on overall survival for patients with node-positive, locally advanced colon cancer is well established.[21] The adjuvant therapy regimen in pancreatic cancer has not been elucidated yet. The ESPAC-1 randomized controlled trial showed no survival benefit for adjuvant chemoradiotherapy after R0 resection, however it revealed a potential benefit for adjuvant chemotherapy.[22] However a study from Smeenk et al. showed that adjuvant chemoradiotherapy, after irradical resections of pancreatic cancer, gives a significant improvement of local control, nevertheless treatment with chemoradiotherapy does not improve survival.[23]

These (neo)adjuvant treatment strategies are mainly based on diminishing advanced disease. An adequate understanding of the pathophysiology of the tumour cell adherence is needed to clarify the initial step of implantation. The experiments described in this thesis were aimed at exploring and unravelling mechanisms of adherence of tumour cells to human mesothelial monolayers.

The experimental model

The process of tumour recurrence can be separated into tumour cell adhesion and tumour growth. Initially the tumour cells have to adhere before they nurture and develop into a metastatic lesion. To study the specific pathways of tumour cell adhesion we developed an *in vitro* model, in which we investigated the interactions of different human colon and pancreatic tumour cell lines on mesothelial monolayers, derived from human omental tissue. The use of primary cultures of human mesothelial cells may have potential pitfalls. For example, the culture of mesothelial cells can be contaminated by an overgrowth of fibroblasts. Therefore, it is important to identify the primary cultures of mesothelial cells by immunocytological staining with monoclonal antibodies after every isolation from omental tissue. The identity of mesothelial cells is demonstrated by the absence of von Willebrand factor and the presence of intracellular vimentin and keratin. Optimal isolations of mesothelial cells can be achieved by incubating the omental tissue for 20 minutes in 0.25% trypsin. Minimal tissue manipulation and early collection of the omental tissue during surgery may minimise fibroblast contamination.[24, 25] An additional drawback in the use of primary cultures is the early senescence of the mesothelial cells. For that reason the cells should only be used during the second and third passage. Because the amount of mesothelial cells isolated from 10 cm^2 omental tissue is limited, we used different cultures of mesothelial cells. The difficulty by using cultures from different patients is the variation in basal adhesion. These inter-individual differences are a hurdle during *in vitro* investigations, nevertheless very important *in vivo*. This objectivity might explain the inter-individual variations in tumour recurrence and responses to different cancer therapies. The experiments described in chapter 6 exhibit the inter-individual differences in basal adhesion of tumour cells to the mesothelial monolayers. The basal adhesion varied between 20-55%, although no variation in enhancement of adhesion was observed after incubation of the different mesothelial monolayers with the serum samples obtained per-operatively.

Inflammatory mediators of tumour cell adhesion

This thesis focuses on the influence of surgical derived inflammatory factors on tumour cell adhesion. Our data showed that IL-1β and TNF-α are crucial enhancing factors of tumour cell adhesion to the mesothelial monolayers. The results described in chapter 2 and 3 revealed a significant enhancement of adhesion of Caco-2, HT29, Mia-PaCa-2 and BxPC-3 to mesothelial monolayers, after incubating the mesothelium with IL-1β or

TNF-α. In addition IL-1β and TNF-α significantly upregulated the expression of adhesion molecules ICAM-1, VCAM-1 and CD44 on mesothelial cells.

These cytokines are produced during the acute inflammatory response, which is induced by peritoneal trauma and has to initiate the wound healing process.[26, 27] IL-1β, TNF-α and IL-6, produced by activated leukocytes, are the major mediators of inflammation and tumourigenesis.[28, 29] Together they generate the production of adhesion molecules, growth factors, nitric oxide, and the activation of the NF-κB pathway. In that way the pro-inflammatory cytokines will stimulate tumour adhesion, growth and invasion.[30, 31] Furthermore, mesothelial cells have additional active participation in the inflammatory response by producing pro-inflammatory cytokines and in that way stimulating the expression of adhesion molecules. TNF-α has a dominant function in the abdominal cavity and might modulate the production of ILs from mesothelial cells. Production of IL-1 and IL-8 by mesothelial cells is enhanced after stimulating the cells with TNF-α. [32, 33] In malignant disease, high dose local TNF-α selectively destroys tumour blood vessels and thereby induces apoptosis, whereas chronically produced TNF-α may act as an endogenous tumour promoter. It contributes to the modulation of the cell, i.c. cell adhesion molecules, necessary for tumour spread and growth.[30, 34] The influence of IL-1β on tumour cell metastasis is inevitable as well. In mouse metastasis models treatment with an IL-1 receptor antagonist significantly decreased tumour development. Additionally, IL-1β deficient mice are resistant for developing metastases.[35]

During the inflammatory response PMN are attracted to the site of injury, which is mediated by chemotactic factors and pro-inflammatory cytokines.[36, 37] PMN are known to aggravate an overwhelming burst of ROS (reactive oxygen species) to destroy invading organisms and inducing additional tissue destruction.[38, 39] Furthermore this oxidative burst can be induced by TNF-α, which causes an upregulating of the FMLP receptors (N-formyl-methionyl-leucyl-phenylalanine) on PMN.[40, 41] In the studies described in chapter 4 and 5 FMLP stimulated PMN induce a significant enhancement of tumour cell adhesion. Moreover the ROS producing system (X/XO) exhibits an even superior enhancement of adhesion of tumour cells to the mesothelium and this enhancement was inhibited by anti-oxidant scavengers.

A pathway of interest is the NF-κB transcription factor pathway in inflammation and tumour cell adhesion. NF-κB is activated by inflammatory stimuli and its constitutive activation is found in cancer.[42] NF-κB plays a pivotal role in cellular responses to environmental changes, such as stress, inflammation and infection. NF-κB is activated in response to pro-inflammatory cytokines and ROS, resulting in the production of growth factors, adhesion molecules, immunoreceptors and cytokines are produced.[43, 44]

Summarising, the inflammatory sequelae enhance tumour cell adhesion the mesothelium *in vitro*. The pathways involved are orchestrated in a meticulous way by pro-inflammatory factors produced per-operatively in response to surgical trauma. Interference with these

pathways may lead to specific tools to conquer the adhesion and growth of spilled tumour cells *in vivo*.

Future directions

Although we did not elucidate the exact mechanism of loco-regional tumour recurrence, we believe that we made progress in unravelling several pathways involved in the tumour cell adhesion process. However, further investigations are required to fully clarify the mechanism of tumour recurrence.

Complementary *in vitro* investigations are necessary in order to provide insights in the recurrence pathway by culturing both mesothelial and tumour cells of the same patient and investigate the individual interactions, thereby studying the adherence and release of different cytokines, and expression of receptors and adhesion molecules on individual tumours. Furthermore, research into the chemokine network on immune cells, mesothelial cells and tumour cells can reveal parallels between the pathology of inflammation and cancer recurrence. Preliminary laboratory data show promising results with chemokine-receptor antagonists.[45] However their role in cancer and metastatic disease has not been unravelled yet, certainly caused by the enormous number of chemokines and chemokine-receptors.

Every pathway investigated *in vitro* must have a descendant *in vivo*, mimicking as much as possible the cascade of biological events leading to the formation of metastasis. The disadvantages of rodent models are the major differences in lifespan, body weight, gut microflora and intestinal morphology compared to humans. Another disadvantage is that human cancer cell lines only can be investigated in genetically manipulated models. To investigate the process of metastases of human cancer cells in an experimental model a nude mouse model is inevitable, however this model brings along enormous costs. This metastasis model will only succeed if the mice and tumours are specific pathogen free and maintained under barrier conditions.[46] The major advantage of animal models nowadays is that models can be genetically manipulated in such a way that hypotheses can be addressed more easily.[47] However, only proper use and choice of an animal model will allow us to investigate and bring us closer to the biologic behaviour of metastases.

'A good model is the model that works for you!' The *in vivo* experiments in our laboratory were all done in a rat model with the rat CC531 colon carcinoma cell line. Our *in vivo* rat model provided us outstanding results, in particular the outcomes of the tumour adhesion and promoting studies and the investigations on the use of scavengers.[48-50] Nonetheless this good reproducible model has its limitations in investigating, because no cancer cell lines of human origin can be used.

Using nude mice to study the invasion of human tumour cell lines is unavoidable. However, the usefulness of the athymic nude mouse as an *in vivo* model for studying the

biology of neoplasms has been limited by the fact that malignant tumours only rarely metastasize when ectopicly transplanted.[46] However implantation of human tumour cells into anatomically relevant organs (orthotopic implantation) results in superior growth and metastasis.[51, 52] Studies after the introduction of the green fluorescent protein (GFP) as a marker for tumour cells has changed the use of nude models radically. Studies with GFP offer great promise in detection single cell metastases *in vivo*.[53]
To enhance the knowledge of the tumour recurrence pathways, it might be essential to have an *in vivo* model investigating the cascade of pathways, studied previously *in vitro*.

Clinical applications

Notwithstanding that surgery remains the treatment of choice in colorectal and pancreatic cancer, local recurrence after curative surgical resection is an incessant drawback. This thesis unravelled different pathways of local tumour recurrence in *in vitro* studies with human cells, nonetheless in what way can our investigational results be implemented in clinical practice?
Per-operative diminishing surgical trauma, by using less traumatic tools, gauzes and techniques, seems to be an adequate option. The study done by Bouvy et al. showed in a rat model, that laparoscopic surgery is associated with less tumour growth stimulation compared to conventional surgery, due to reduced surgical trauma.[54] Additionally laparoscopic surgery is correlated with less immunological alterations and this may imply less local tumour recurrence as well.[55, 56] Lacy et al. showed that laparoscopic-assisted colectomy is associated with a significantly lower probability of tumour recurrence and a higher probability of overall and cancer-related survival in stage III cancer.[57] Although the recent publication of Law et al. is not a randomized controlled trial, this study put forward a significant survival benefit for patients, who underwent a laparoscopic resection in stage I to III colon cancer.[58] The two randomized controlled trials, in which laparoscopic-assisted colectomy was compared to open surgery, could not reveal this benefit concerning tumour recurrence in the laparoscopic group.[57, 59]
Since the expression pattern of adhesion molecules on tumour cells is tremendously divers, it is not feasible to use single monoclonal antibodies to confront spilled tumour cells. The development of cell adhesion peptides (i.e. RGD peptide) provide promising results by blocking the adherence of tumour cells to the components of the extracellular matrix. The use of RGD peptides has been expanded with preliminary results by using the RGD peptides for delivering drugs to tumour cells that express certain integrin types after which an internalization process takes place of the integrin adhesion complex.[60, 61]
Interfering with the inflammatory process during and after surgery is a reliable option to prevent tumour recurrence, however wound healing is depending on this process. This interference should be very selective, otherwise the healing process will be disturbed.

Interference with the inflammatory response might be accomplished by inhibiting the influx of PMN into the peritoneal cavity with anti-neutrophil serum (ANS) after curative resections of gastro-intestinal tumours. An *in vivo* study showed a significantly decrease of peritoneal tumour recurrence after intra-peritoneal injection of ANS.[62]

Another pathway of interest is the influence of ROS on tumour cell adhesion. Accumulation inhibitors of ROS are superoxide dismutase, catalases, gluthatione peroxidases, vitamins C and E, these antioxidant enzymes and non-enzymatic systems are engineered during stress. Since the release of ROS is enormous following surgical trauma, additional exogenous administration of antioxidants might be a therapeutical option.

An antioxidant with potential is melatonin.[63] Melatonin, produced mainly in the pineal gland, possesses a wide spectrum of biologic activities, including its function as a naturally occurring oncostatic neurohormone by inhibiting cell proliferation, inducing apoptosis and reducin metastatic spread.[64-66] Regarding the scavenger function of melatonin, a synthetic form might be of interest in prevention of tumour recurrence.

Since the discovery of NF-κB in the mid 1980s, this transcription factor has been a subject of intense investigation.[67] The NF-κB transcription factor complex is a pleiotropic activator that participates in the induction of a wide variety of cellular and viral genes. Binding sites for NF-κB are present in the promoter region of many cell adhesion molecules, cytokines and growth factors. Antisense inhibition of NF-κB activity causes a block of cellular adhesion to the extracellular matrix, inhibition of *in vivo* growth of adherent cells, and inhibition of *in vivo* tumourigenicity in nude mouse models.[68-70] Recent investigations also showed that ROS is engaged in a unique reciprocal cross talk with NF-κB.[71] The exact mechanism has not been unravelled yet, however remarkable is that the induction of NF-κB is abrogated by overexpression of ROS scavenging enzymes.[72]

A promising NF-κB inhibitor is pentoxifylline (trental), the inhibition of the transcription of NF-κB might cause a suppression of cell adhesion molecules and in that way a potential decrease of lung metastasis.[73] In addition pentoxyfilline may reduce the TNF-α induced oxidative burst by reduced binding of FMLP to PMN surface receptors.[74]

Interference with the invasion of tumour cells through the extracellular matrix is another therapeutic option. Matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases, play an important role in the growth and invasion of colorectal and pancreatic cancers by degradation of the extracellular matrix. The levels of certain MMPs can be used to estimate the metastatic capacity and recurrence of disease as well as prognosis of patients.[75, 76] However, for effective therapy using MMP inhibitors, highly selective administration may be required. Since MMP-7 is the most critical MMP for colorectal cancer progression, developing selective inhibitors against this protease and their administration in the early stage of disease may be worth trying.[77]

Interference with the inflammatory sequelae (i.c. cytokines, PMN, ROS, adhesion molecules, NF-κB) produced per-operatively must be well balanced, without disturbing the

wound healing process and the systemic immune response. Developing an elixir (containing e.g. ANS, scavengers and/or NF-κB antibodies) to lavage the intra-abdominal cavity after surgery, might be the tool to prevent intra-abdominal tumour cell recurrence after curative surgical resections.

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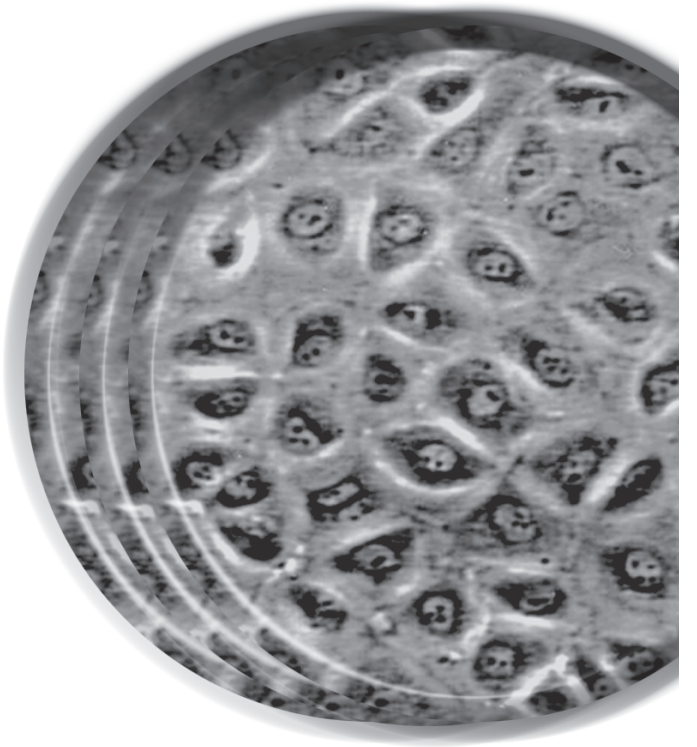
CHAPTER 10

Summary and conclusions



SUMMARY AND CONCLUSIONS

Chapter 1

The general introduction gives an overview of our previous studies, the incidence of gastro-intestinal malignancies and the pathophysiology of local tumour recurrence. This chapter outlines the basics of the peritoneum, inflammatory reaction, cytokines, adhesion molecules, and the principles of surgery and tumour recurrence. The aim of our study was to unravel several pathways of tumour cell adhesion in an *in vitro* model, in which human mesothelial and cancer cells were studied.

Chapter 2

Colorectal cancer accounts for more than 50 percent of the cases of gastro-intestinal malignancies. The overall 5 year survival has improved, caused by diverse new adjuvant treatment modalities. Nevertheless 40% of the patients develop recurrent disease and the 5 year survival rate is approximately 35% for patients with local recurrence and 25% for patients with distant metastasis.

In this chapter the *in vitro* model is described, in which we studied the adhesion of colon carcinoma cells, Caco-2 and HT-29, to primary cultures of omental derived human mesothelial cells. The adhesion of colon carcinoma cells was investigated after incubation of the mesothelial cells with inflammatory cytokines IL-1β, IL-6 and TNF-α. Tumour cell adhesion after incubation with IL-1β and TNF-α was significantly enhanced for both cell lines. In comparison with the results of the adhesion experiments, we investigated the upregulation of expression of adhesion molecules on the mesothelial cells after cytokine incubation. IL-1β and TNF-α significantly enhanced the expression of ICAM-1, VCAM-1 and CD44 *in vitro*. These results prove that IL-1β and TNF-α are potential stimulating factors of tumour cell adhesion by upregulation the adhesion molecules and in that way may account for tumour recurrence *in vivo*.

Chapter 3

Pancreatic cancer has a tremendously poor prognosis and peritoneal dissemination frequently occurs after surgical resection. The loco-regional recurrence rate ranges between 38 and 86%. The mechanism of local recurrence has not been unravelled yet. We investigated the adhesion of pancreatic cancer cells (Panc-1, Mia-PaCa-2, BxPC-3) to mesothelial monolayers, using the *in vitro* model as described in chapter 3. The adhesion of Mia-PaCa-2 and BxPC-3 to mesothelial monolayers is enhanced after incubation the mesothelial cells with IL-1β or TNF-α. According to the immunocytochemical staining of the pancreatic cancer cells and mesothelial cells and the results of the adhesion assay, this study reveals that adhesion molecules ICAM-1 and CD44 are of the utmost impor-

tance in pancreatic tumour cell adhesion *in vitro*, and suggests that interference with these adhesion molecules may decrease tumour recurrence *in vivo*.

Chapter 4

Surgery induces an acute phase response, aimed at minimizing damage and starting the healing process. During this response specific chemoattractants induce the appearance of a great number of polymorphonuclear leukocytes (PMN). These PMN are known to assemble reactive oxygen species (ROS) to destroy invading organisms. Beside this beneficial effect, the oxidative potential can result in changes of cell function and additional tissue destruction. The influence of PMN, stimulated PMN and ROS on tumour cell adhesion has been studied in this chapter. PMN were stimulated with FMLP and O_2^- production was determined by measuring the superoxide dismutase inhibitable reduction of cytochrome c. The reactive oxygen species (ROS) superoxide and hydrogen peroxide were generated by Xanthine Oxidase (XO) upon addition of Xanthine (X). Adhesion of colon carcinoma cells, Caco-2, to mesothelial monolayers was significantly enhanced after incubation the mesothelial cells with stimulated PMN. Incubation of the mesothelial cells with the ROS generating system resulted in a 70% enhancement of tumour cell adhesion.

This study showed a significant increased expression of ICAM-1, VCAM-1 and CD44 on mesothelial cells after incubation the mesothelium with ROS. The results of this study give evidence that the inflammatory response, i.c. PMN influx after surgery promotes tumour adherence.

Chapter 5

Pancreatic tumour cell adhesion after incubation of the mesothelium with ROS has been studied in this chapter. Panc-1 tumour cell adhesion increases after pre-incubating the mesothelium with the X/XO generating system. This stimulatory effect of ROS could be inhibited almost completely with the anti-oxidant enzymes superoxide dismutase (SOD) and catalase. The enhancement of expression of adhesion molecules ICAM-1, VCAM-1 and CD44 on mesothelial cells was suppressed by adding SOD and catalase to the X/XO generating system. These findings may be suggestive for using anti-oxidants as a treatment modality to inactivate the mesothelium after surgical trauma and in that way prevent tumour recurrence.

Chapter 6

Surgical handling of the peritoneum induces an inflammatory reaction during which a cocktail of active mediators are produced. In this study the influence of systemically produced factors on tumour cell adhesion was investigated. Serum samples were obtained during surgery and the mesothelium was incubated with these samples overnight.

Hereafter the adhesion of Caco-2 colon carcinoma cells was studied. No statistically significant changes in adhesion were observed after incubation the mesothelium with the different serum samples taken a day before surgery, at the start of the surgical procedure and 2, 4, 8, 12, 24 and 48 hours after surgery. Although this is a very simplified model, we might conclude that local tumour recurrence is mainly influenced by locally produced mediators.

Chapter 7

The presence of foreign material in the abdominal cavity may aggravate the peritoneal lining leading to an inflammatory reaction of the peritoneum. This defensive mechanism can provoke adhesion formation and, according to previous studies done *in vivo*, also enhanced tumour recurrence.

The influence of four different type of gloves, powdered, non powdered and non-latex, on tumour cell adhesion *in vitro* was analysed. The non-powdered and/or non-latex gloves are not influencing tumour cell adhesion. Powdered gloves induces a significant enhancement of tumour cell adhesion.

Despite the evidence of starch induced complications, in some countries still a considerable number of surgeons are using starch powdered gloves. Furthermore, beside the usage of starch, one should abandon all substances causing enhanced trauma to the peritoneum from the operating room.

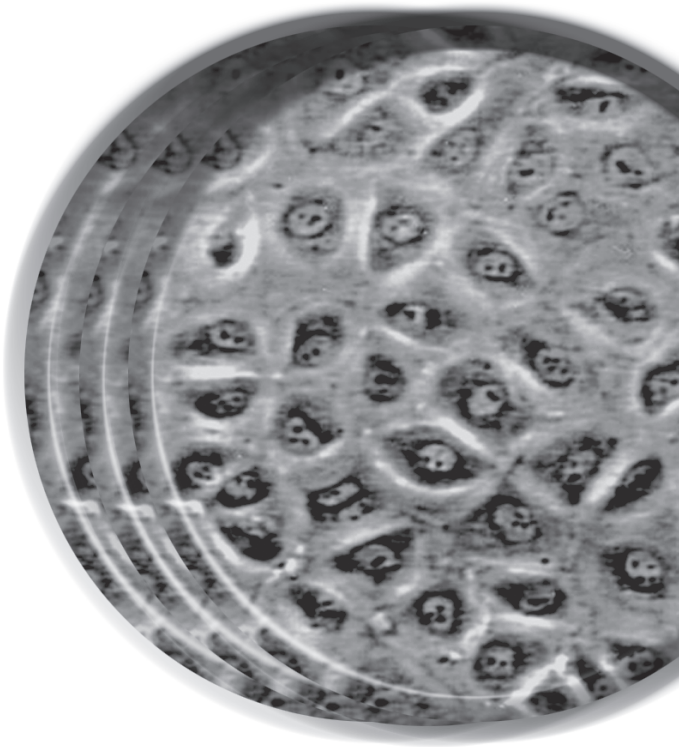
Chapter 8

Adhesion of tumour cells to the mesothelium can cause a discontinuity of the cell layer, thereby exposing the extracellular matrix (ECM). In this way tumour cells can enter the underlying tissue, proliferate and grow out to form a metastasis. This study investigated the adhesion of Caco-2 and HT 29 cells to different components of the ECM, i.c. collagen type I and IV, fibronectin and laminin. The adhesion of the tumour cell lines to the components of the ECM was significantly higher than to the mesothelium. Antibodies against the adhesion molecules ICAM-1, VCAM-1 and the VLA-integrins were studied. These inhibition assays clearly display that the α2- and α6-unit play a major role in the adhesive interactions between different tumour cells and components of the extracellular matrix, although both tumour cell lines display different adhesion patterns to the components of this matrix. However, caused by the complexity of the ECM and the heterogeneity between the tumour cells, the exact mechanism of local tumour recurrence has not been unravelled yet.

Conclusions

- IL-1β and TNF-α enhance the adhesion of colon carcinoma cells to human mesothelial cells *in vitro*.
- The enhancement of adhesion of colon carcinoma cells to mesothelial monolayers is based on a cytokine induced upregulation of adhesion molecules, i.c. ICAM-1, VCAM-1 and CD44.
- Caco-2 and HT-29 can benefit this upregulation of adhesion molecules, because they express the counterparts of ICAM-1 and VCAM-1, i.c. LFA-1 and VLA-4.
- IL-1β and TNF-α stimulate the adhesion of BxpC-3 and MiaPaCa-2 cells, both pancreatic cancer cell lines, to monolayers mesothelium.
- ROS produced by PMN, enhance tumour cell adhesion to mesothelial monolayers. The ROS producing system X/XO resulted in the highest enhancement of tumour cell adhesion.
- The expression of ICAM-1, VCAM-1 and CD44 on mesothelial cells is enhanced by the ROS producing system X/XO.
- Serum produced per-operatively does not influence the adhesion of tumour cells to mesothelial monolayers.
- The adhesion of tumour cells to mesothelial monolayers is significantly enhanced after incubating the monolayers with powdered gloves solutions.
- Colon carcinoma cells display a strong adhesion to components of the ECM.
- Interference with the local inflammatory response after surgery may prevent intra-abdominal tumour recurrence.

Appendices



SAMENVATTING VOOR DE LEEK

De incidentie van colon carcinomen (kwaadaardige tumoren van de dikke darm) neemt toe in de westerse wereld. In Europa wordt jaarlijks bij 400 000 patiënten dikke darm kanker vastgesteld en overlijden er jaarlijks 200 000 patiënten met deze vorm van kanker. Van alle vormen van kanker staan colon carcinomen met een incidentie van 14% op de derde plaats na long- en prostaatkanker. De overall 5-jaarsoverleving is ongeveer 60-70%, indien er sprake is van lokale uitzaaiing (lokaal recidief) daalt de 5-jaarsoverleving naar 35% en bij uitzaaiingen in andere organen (metastasen) naar 25%.
Kwaadaardige tumoren van de alvleesklier (pancreas) hebben een zeer slechte prognose. Na chirurgische resectie zal meer dan 50% van de patiënten een metastase ontwikkelen. Minder dan 15% van de patiënten met een pancreas carcinoom overleeft het eerste jaar en de 5-jaarsoverleving is kleiner dan 5%.
De behandeling van keuze bij colon of pancreas carcinomen is chirurgische resectie. Het nadeel van deze chirurgische behandeling is dat tijdens de operatie tumorcellen kunnen los raken door manipulatie van de primaire tumor ('spilled tumour cells'). Deze tumorcellen kunnen aanhechten op het resetievlak of op een andere plaats in de buik en op deze manier uitgroeien tot een recidief tumor. Bij zowel colon als pancreas carcinomen komt lokaal tumor recidief vaak voor. Daarom is kennis van het ontstaan van lokaal tumor recidief zeer belangrijk. Dit proces kan uitgesplitst worden in tumorcel aanhechting en tumor groei. Het proces van aanhechting van een tumorcel is afhankelijk van een groot aantal factoren. De experimenten beschreven in dit proefschrift bestuderen het proces van aanhechting van de tumorcel en de invloed van factoren, die postoperatief intra-abdominaal (in de buik) worden geproduceerd op deze aanhechting.

Experimenten

Alle organen in de buik zijn bedekt door het peritoneum (buikvlies), hetgeen is opgebouwd uit mesotheelcellen van slechts een cellaag dik met daaronder de extra cellulaire matrix (ECM), opgebouwd uit eiwitten. In voorgaande experimenten in ons laboratorium is aangetoond, dat er een correlatie bestaat tussen chirurgische beschadiging van het peritoneum en postoperatief tumor recidief. De ontstekingsreactie die na chirurgisch peritoneaal trauma ontstaat is van grote invloed op het ontstaan van lokaal tumor recidief. Deze voorgaande studies zijn verricht in een rattenmodel. Om het gedrag van humane cellen te bestuderen, is er voor de experimenten beschreven in dit proefschrift een *in vitro* model ontwikkeld waarin de interactie tussen humane mesotheel- en tumorcellen is bestudeerd. De term *'in vitro'* verwijst naar de observatie van fenomenen 'onder glas', ook wel een celkweekmodel genoemd. In dit model hebben wij de invloed van ontstekingsmediatoren, zoals leukocyten en cytokinen op de aanhechting van tumorcellen op mesotheelcellen bestudeerd.

Mesotheelcellen werden geïsoleerd uit omentum (buikschort), verkregen tijdens een operatie met toestemming van de patiënt. De mesotheelcellen werden gekweekt met toevoeging van cytokinen, IL-1β, IL-6 en TNF-α, in het kweekmedium gedurende 12 uur. Hierna werden de tumorcellen bij de mesotheel cellen gevoegd en de aanhechting van de tumorcellen gemeten. Na incubatie van de mesotheelcellen met de cytokinen IL-1β en TNF-α was er een evidente toename van aanhechting van tumorcellen waarneembaar. Er was sprake van een verhoogde expressie van adhesiemoleculen (aanhechtingsmoleculen) op de mesotheelcellen na incubatie van de mesotheelcellen met cytokinen, hetgeen de toename van aanhechting van tumorcellen op de mesotheelcellen verklaart. Dit fenomeen is waargenomen bij zowel colon carcinoom cellijnen als pancreas carcinoom cellijnen.

Tijdens en na een chirurgische ingreep is er sprake van een toename van witte bloedcellen intra-abdominaal, ook wel polymorphe neutrophiele leukocyten (PMN) genoemd. Deze PMN zorgen voor een uitstoot van zuurstofradicalen. Deze zuurstofradicalen dragen bij aan de vernietiging van ongewenste organismen intra-abdominaal en brengen omliggende cellen in een verhoogde staat van activiteit. De aanhechting van tumorcellen was verhoogd, nadat de mesotheelcellen samen met de PMN werden gekweekt. Deze zuurstofradicaal productie werd tevens nagebootst door de mesotheelcellen te kweken met een zuurstofradicaal productie systeem, xanthine/xanthine oxidase (X/XO). Na incubatie van de mesotheelcellen met dit systeem was de aanhechting van tumorcellen significant verhoogd. Er werd ook een verhoogde expressie van adhesiemoleculen waargenomen na incubatie van de mesotheelcellen met zuurstofradicalen.

Een daaropvolgende vraag was of het mechanisme van lokale aanhechting alleen verklaard kan worden door een toename van lokaal geproduceerde factoren, of dat factoren in bloedcirculatie ook een invloed hebben op de aanhechting. Daartoe werden bloed samples van patiënten verzameld voor, tijdens en na de operatie en uit deze samples werd het serum geëxtraheerd. Mesotheelcellen van dezelfde patiënt werden geincubeerd met deze samples waarna de aanhechting werd bestudeerd. Er werd geen toename gezien in aanhechting van tumorcellen na pre-incubatie van de mesotheelcellen met de serum samples.

Uit voorgaande experimenten weten wij dat poeder, hetgeen gebruikt wordt in de 'chirurgische' handschoenindustrie, zeer nadeling is voor tumor recidief. In ons humane model hebben we de invloed van 4 soorten handschoenextracties (i.c extracties van handschoenen met poeder en zonder poeder) op tumorcel aanhechting bestudeerd. Het extract van de handschoenen met poeder zorgde voor een significante toename van aanhechting van tumor cellen op mesotheelcellen.

Naast de aanhechting van tumorcellen op mesotheelcellen is ook de aanhechting van tumorcellen op de extra cellulaire matrix (ECM, de laag waar mesotheelcellen op rusten en opgebouwd is uit eiwitten) van belang in tumor metastasering. De aanhechting van tumor

werd bestudeerd op verschillende substanties van de ECM. De colon carcinoom cellijnen hechten zeer sterk aan de verschillende componenten van de ECM en de aanhechting kan worden geremd door de tumorcellen te incuberen met specifieke antilichamen tegen bepaalde adhesiemoleculen.

Toekomst

Concluderend kan gesteld worden dat de ontstekingsfactoren, die geproduceerd worden tijdens en na een operatie, invloed hebben op de aanhechting van tumorcellen in dit humane *in vitro* model. Toch blijft chirurgie de therapie van keuze bij colon en pancreas carcinomen, maar het is belangrijk om tijdens de operatie de chirurgische schade zoveel mogelijk te beperken. Tevens is interferentie met de ontstekingsmediatoren mogelijk ook een optie om het lokaal tumor recidief te voorkomen. De genezing komt tot stand, doordat er verschillende ontstekingsmechanismen werkzaam zijn tijdens en na een operatie. Al deze pathways stop zetten is geen optie, omdat dan het genezingsproces wordt stil gezet en de patiënt na de operatie in een shock terecht kan komen. Wellicht is de ontwikkeling van een multifunctioneel 'elixer' een alternatief. Dit elixer kan postoperatief in de buik achter gelaten worden om bepaalde ontstekingsfactoren weg te vangen en tevens een barrière vormen tegen de aanhechting van 'spilled tumour cells'.

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In the classical antiquity it was wise to be escorted, as a PhD student, with two powerful and muscular men, because the ancient academically disputes could end up with a fight and the paranymphs served as body-guards. I do not think my two paranymphs, Arend Aalbers and Hans Smeenk are that muscular. Nevertheless, I am proud that both of you are willing to escort me as a paranymph. Thanks for your friendship and for acting as two BIG-brothers, during my research and resident years. I will never forget our trip to London.

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CURRICULUM VITAE AUCTORIS

Wilhelmina Margaretha Ursinus van Grevenstein was born on July 3rd 1973 in Krimpen aan den IJssel, The Netherlands. Although she adored James Herriot and horse back riding was her favourite sport, she already decided during her elementary years to become a surgeon. After finishing high school she continued her studies at the Faculty of Medicine of the Erasmus University in Rotterdam, The Netherlands. Her interest in research aroused when she worked as a student in the Laboratory of Genetics and Paediatric Surgery, where she finished her graduating project. After her bachelor degree she worked as a research fellow for 6 months at the Division of Human Genetics and Molecular Biology, Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, USA.
In 2000 she obtained her medical degree and in the same year she was offered a position as PhD student at the Laboratory of Experimental Surgery and Oncology, Erasmus MC, Rotterdam (Professor J Jeekel, Dr CHJ van Eijck). During these 'lab' years the basis of this thesis was created. Her work was awarded by the European Society of Surgical Research (ESSR Students' Award) and by the Collegium Chirurgicum Neerlandicum (CCN Travel Award). In 2003 she started her training in surgery at the Surgical Department of the Zuiderziekenhuis (dr. K Brouwer). In 2004 the Zuiderziekenhuis and the Clara Ziekenhuis merged as the Medical Center Rijnmond Zuid (MCRZ, Prof.dr. JF Lange, dr. E van der Harst, dr. PPLO Coene). In the MCRZ she worked in an outstanding environment to develop herself in general surgery and become a devoted and enthusiastic resident. In January 2007 she started her last two years of her surgical training at the Surgical Department of the Erasmus MC (Prof.dr. JNM IJzermans), during her last year she will differentiate in GI surgery. Together with Hans Smeenk and Niels Schep she wrote and edited the Dutch Surgical Handbook 'Leidraad Chirurgie'.