Pathways of peritoneal tumour recurrence after abdominal surgical trauma

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PERITONEAAL TUMOR RECIDIEF NA
ABDOMINAAL CHIRURGISCH TRAUMA

PROEFSCHRIFT

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Promotor:	Prof. dr. J. Jeekel
Overige leden:	Prof. dr. Th. van der Kwast
	Prof. dr. S.W.J. Lamberts
	Prof. dr. H. Obertop
Co-promotores:	Dr. C.H.J. van Eijck
	Dr. L.J. Hofland

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Preface

Surgical treatment of malignancies within the abdominal cavity is regularly complicated by peritoneal tumour recurrence. Peritoneal tumour recurrence refers to the convalescence of tumour foci on the peritoneal membrane lining the abdominal cavity. The underlying mechanisms of tumour recurrence are not fully clear. To elucidate this, a profound understanding of the anatomy of the peritoneum and its elementary response to surgical trauma is essential.

The flaring up of an inflammatory reaction followed by the initial steps of wound healing are the first signs of regeneration after surgical trauma to the peritoneum. During these processes, Pandora's box of cytokines and growth factors is opened with a potentially ambiguous influence on tumour recurrence.

This thesis addresses the multi-factorial response of the peritoneum after surgical injury, clarifying different pathways of stimulated tumour recurrence.

Contents

To oma Lina

Aan Giedo

Part I

General Introduction and

Aim of the Thesis

Chapter I

GENERAL INTRODUCTION

The peritoneum

The peritoneum is the largest and the most complex arranged serous membrane in the body that lines both the intra-abdominal wall and the viscera contained within the peritoneal cavity. It is capable of walling off infections and has several functions such as the ability to synthesise, secrete or absorb. The peritoneum diminishes friction among abdominal viscera, thereby enabling their free movement.[1-4] With a surface area of some 10,000 cm^2 in adults[5], almost equal to that of the skin, this membrane may be considered among the largest organs in humans. The peritoneal cavity normally contains less than 100 ml of serous fluid that resembles an ultrafiltrate of plasma and contains less than 3 g/dl protein.[6] Taken together the surface area and the functional capacity of the peritoneum, this enables the peritoneal cavity to be used for continuous ambulatory dialysis (CAPD) as well as an internal reservoir during drainage procedures (i.e. ventriculoperitoneal shunts).

The peritoneum and the serosal surfaces of organs within the peritoneal cavity are composed of mesothelium and sub-mesothelial connective tissue. Highly differentiated mesothelial cells, resting on a basement membrane, overly the connective tissue. Embedded in this layer are numerous blood vessels and lymphatics. In terms of blood supply per mass, the peritoneum is one of the most richly vascularised organs.[3;4;7] Interspersed among the connective tissue are poorly differentiated, epithelioid-like cells similar to fibroblasts (figure 1.1).[4;8-10]

Injury of the peritoneum triggers a series of events aimed at mesothelial regeneration. Some of the principal elements in the regenerative process are leukocyte influx into the abdominal cavity, fibrin deposition and activation of both peritoneal macrophages and the normally quiescent mesothelial cells.[3;7;11]

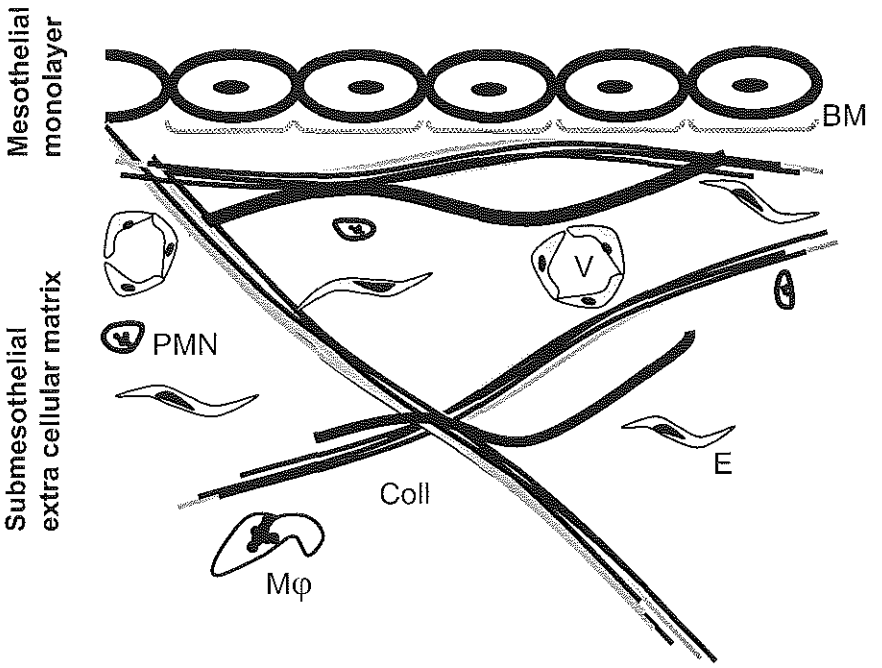

Figure 1.1

Schematic representation of the peritoneum. BM: basement membrane; PMN: leukocyte; Mφ:macrophage; E: epithelioid cells; Coll: collageneous fibers; V: vasculature.

Mesothelial cells

The mesothelium consists of a single layer of flattened cells with epithelioid morphology. The mesothelial cells produce a lubricating surfactant that allows easy gliding of opposing peritoneal surfaces. In addition, long microvilli project from the apical surface of the cells to minimise the shear between facing peritoneal surfaces.[8;9;12-16]

Electron microscopic studies show that mesothelial cells are joined peripherally through tight junctions while spot desmosomes provide mechanical stability.[9;10;14;15] Nonetheless, the mesothelium is very susceptible to damage. Any form of injury initiates acute changes of peritoneal morphology. Peritonitis is associated with a

transition in mesothelial morphology from flat to cuboidal, an increase in the intercellular space and cell shedding.[9] Drying, wetting or rubbing causes mainly denudation of the mesothelial surface.[16-18]

Minimal peritoneal tissue trauma already causes a reactive inflammatory response, which coincides with the appearance of numerous leukocytes between and on mesothelial cells.[19;20] Mesothelial cells are multipotential and capable of secreting pro- and potentially anti-inflammatory mediators and are thus capable of contributing significantly to a cytokine network operating to initiate, amplify and control peritoneal inflammation (table 1.1).[4;21;22]

Cytokines	Growth factors	Miscellaneous	Receptors
IL-1α[23;24]	TGF-β[7;25-27]	GM-CSF[7;24]	ICAM-I[28-33]
IL-1β[23;24;31;34]	bFGF[35;36]	RANTES[29]	VCAM-I[32;33]
IL-6[7;24;31;37-39]	PDGF-A[26]	MCP-1[11;29;37]	IL-1βR[24;34]
IL-8[7;11;29;40]	PDGF-B[26]	IL-1RA[22]	TNF-αR[37]
TNF-α[37;41]	IGF-I[42-44]	PG[11;21;45]	EGF-R[35;46;47]
	IGF-II[43]	PAI-I[7;48]	IGF-IR[44]

Table 1.1

Secretory products and receptors expressed by mesothelial cells.

Primary peritoneal defence mechanisms

Complement activation is an early component of the non-specific peritoneal defence mechanisms and involves opsonisation of micro-organisms, enhancement of inflammatory response, clearance of immune complexes and cell lysis.[49] The normal peritoneal cavity contains about 300 cells/mm^3, including mainly macrophages plus some lymphocytes and desquamated mesothelial cells. Peritoneal injuries induce the migration of leukocytes into the peritoneum. Inflammation can result in leukocyte counts of more than 3000 cells/mm^3.[50] Studies of kinetics of macrophage recruitment to the peritoneum after induction of acute inflammation show most of the resident macrophages disappear from the recoverable peritoneal cell population within the first hour. This coincides with the influx of leukocytes and is sustained for at least several

days.[51] Degranulation of peritoneal mast cells releases vasoactive substances with an outpouring of fluid rich in complement and opsins that coat bacteria and promote phagocytosis.[6] Mesothelial cells have the potential to contribute to the activation and control of the inflammatory process, i.e. by generating signals for the recruitment of leukocytes into the peritoneum.[11;22]

Peritonitis

Inflammation of the peritoneum can be caused by a multitude of factors like bacteria, starch and bile. Infectious peritonitis occurs by contamination of the peritoneal cavity with micro-organisms. Following surgical trauma of the peritoneum, sterile peritonitis generally plays a role. Regardless of the type of peritonitis, a common series of events follows.

When challenged by an inflammatory stimulus, resident peritoneal macrophages become activated and begin to secrete cytokines and chemoattractants, which initiate the inflammatory cascade. Leukocytes respond to the presence of specific chemoattractants and begin to appear in large numbers. Mesothelial cells amplify the inflammatory response by generating chemoattractants and cytokines and by becoming more adhesive.[11;22]

During inflammation morphological changes of the mesothelial cells are also evident. An increased number of peritoneal cells adhere to the mesothelium and the continuity of the mesothelium is interrupted by rounding of mesothelial cells and the infiltration of leukocytes.[9;19]

The scientific basis of this non-specific inflammatory reaction within the peritoneal cavity is also founded by the lack of difference in cytokine and reactive oxygen species production during infectious or sterile peritonitis.[7;52]

Surgical Peritoneal Trauma

Surgical trauma to the peritoneum embraces multiplex pathways of injury, including drying, wetting, rubbing and inflammation. Loss of mesothelial integrity after surgery may induce a sequence of events, which ultimately leads to fibrous adhesions between the visceral and parietal surfaces.[2;3;7;18;53;54] However, fibrosis is inhibited when the integrity of the mesothelial cell lining is maintained, the inhibition being attributed to the

mesothelium's capacity of fibrinolysis. Development of *modus operandi* to protect peritoneal surfaces from surgical injury and to foster fibrinolysis is among the avenues of adhesion prevention research.[18;54-56]

The pathophysiological sequence from peritoneal injury to permanent fibrous adhesions is controlled by the local inflammatory reaction.[7;54] The effect of inflammatory cells and cytokines has gained attention in the context of peritoneal inflammation and repair. Invading leukocytes have been shown to enhance peritoneal injury by adhering to and damaging mesothelial cells through the release of reactive oxygen species.[57-60] Experimental studies demonstrate a modulating role of leukocytes in post operative adhesion formation.[61;62] Cytokines reduce fibrinolytic activity of the mesothelium. It seems the inflammatory reaction after surgery may enhance mesothelial damage and prevent normal mesothelial regeneration.[3;7;54;56]

Minimising peritoneal surgical trauma through the use of minimal invasive techniques is a turning point in the development of post-surgical adhesions.[54;56] Laparoscopic surgical procedures with their minimal access to the abdominal cavity are probably associated with fewer postoperative adhesions compared to open surgery.[63;64] Additionally, laparoscopic surgery appears to impact less on the cellular components of the immune response.[65-67]

Acute Phase Response

Tissue injury induces a constellation of host responses, collectively referred to as the acute phase response, aimed at minimising damage and starting the healing process.[68;69] Inflammation is a multifunctional process, with both local and systemic features. The primary inflammatory response, the acute phase, is the migration of neutrophils, macrophages and other leukocytes to the site of inflammation.[6;11;20;69]

Cytokines are the key mediators of this inflammatory reaction. Tissue injury triggers the synthesis and release of several cytokines that act locally, but may also act systemically after entry to the blood stream. The cytokine response to operative trauma appears to be influenced by the degree of surgical trauma.[70-75] Post-surgical cytokine concentrations measured systemically and locally (in the peritoneal fluid) demonstrate the cytokine response after abdominal surgery originates largely from the peritoneal cavity.[74;76] Peritoneal lymphocytes, sub-mesothelial monocytes, leukocytes 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 (figure 1.2).[4;21;77;78]

The results of the inflammatory response include ultimate resolution of the inflammatory process, by triggering events that lead to cell regeneration and wound healing. On the other hand, progression of the inflammatory response may lead to progressive organ dysfunction.[68;69;79] Normal inflammatory defences become detrimental to the host if the balance between mediators of the inflammatory response is lost.[80;81]

Peritoneal wound healing after surgical trauma

The response of the peritoneal membrane to acute injuries highlights some of the inherent defence mechanisms and reparative properties of this tissue. Normally, the mesothelium is a quiescent population of cells with a low endogenous turnover rate.[11] Minimal damage to the mesothelium causes a detachment of many of these cells, denuding the surface and herewith exposing the underlying extra cellular matrix components.[16-18] Upon denudation, which occurs fairly easily through wetting[17], drying[16;17] or rubbing with surgical gauze[18], the percentage of mesothelial cells undergoing mitosis has been reported to increase from less than 3% to as much as 8%.[11;82]

Mesothelial surfaces regenerate in a different way than the skin. The two main differences are the way epithelialisation occurs and the consequences of fibrin deposition. In addition to centripetal migration as seen in skin injury, in mesothelial regeneration small islets of cells intersperse throughout the wound surface.[3;7-9;53;83;84] This implies that large defects require the same time to re-epithelialise as small ones, generally completed in seven days, and that peritoneal defects do not have to be re-approximated to ensure optimal healing. At present, there is considerable debate as to the origin of cells that colonise the exposed surface of the peritoneum. Hypotheses extend from differentiation of multipotential sub-mesothelial cells to exfoliation and proliferation of mesothelial cells from adjacent or opposing surfaces.[8;9;82-84]

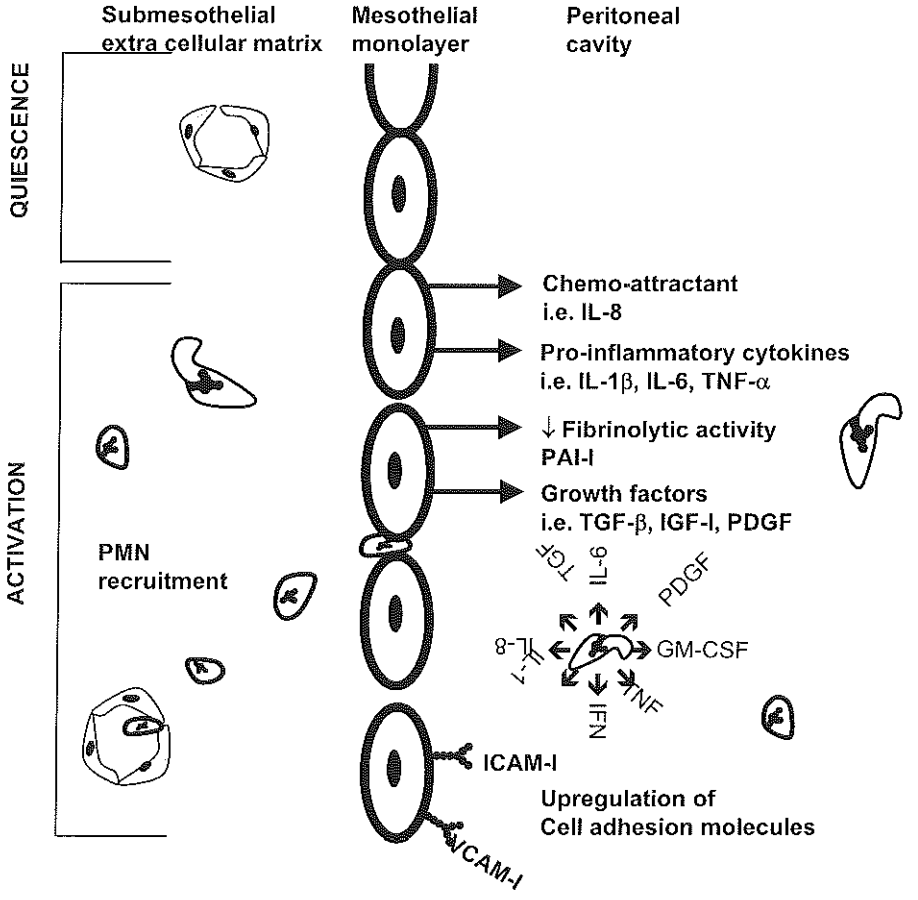

Figure 1.2

Schematic representation of the acute phase response after surgical peritoneal trauma. Production of chemo-attractants and inflammatory factors by mesothelial cells and macrophages leads to an upregulation of cell adhesion molecules and leukocyte recruitment from the sub-mesothelial vasculature. Production of growth factors by mesothelial cells and macrophages stimulates the wound healing process.

Cytokines, growth factors and peritoneal wound healing

Numerous cytokines and growth factors have a role in the stimulation of a particular facet of the healing process, such as angiogenesis, matrix protein deposition or cell proliferation.[85-87] Wound healing is based upon the synthesis and release of several growth factors and cytokines at the site of injury, which act through autocrine and paracrine pathways to regulate healing. Individual and synergistic interactions of growth factors and cytokines take place, as well as cross talking between cells.

In peritoneal wound healing the mesothelium plays an active role by producing chemoattractants, prostaglandins, cytokines and growth factors (table 1.1).[4;11;23-25] Peritoneal macrophages and leukocytes, attracted to the injured site contribute to the production of factors to enhance wound healing.[3;7;25] The microenvironment of the wound therefore, contains a plethora of growth factors and cytokines and is a prerequisite area for cell proliferation and differentiation. Although the exact mechanisms involved in mesothelial healing are controversial, it is certain that the healing process requires the activation, proliferation and migration of residual mesothelial cells.[7;82]

Cell adhesion molecules

Cell adhesion molecules provide the possibility for specific cell adhesion to tissue surfaces. A number of cell surface associated molecules have been characterised and are classified in four groups according to their biochemical structure: integrins, immunoglobulin-like proteins, cadherins and selectins.[88;89]

Adhesion molecules mediate specific cell-cell or cell-extra cellular matrix interactions. Besides a role in physiological cellular communications, an essential role in the pathophysiology of disease has been demonstrated. During peritoneal inflammation, leukocyte influx across mesothelial cells depends on specific adhesion molecules.[29] Lack of expression or dysfunction of these adhesion molecules causes leukocyte adhesion deficiency (LAD) characterised by frequent and overwhelming infections due to the inability of leukocytes to infiltrate pathogen-bearing tissues. In contrast, inflammatory diseases like psoriasis and arthritis are associated with upregulation or over-expression of adhesion molecules.[89]

In tumour progression and metastasis, adhesion molecules have also been demonstrated to play an essential role.[88;89] The initial attachment of tumour cells to the

intact or denuded peritoneal surface is a critical step in establishing tumour implantation. Cytokines are known to induce or upregulate adhesion molecules, hereby increasing the prospect of cell adhesion.[28;32;33;90;91] During wound healing enhanced concentrations of cytokines and growth factors in the peritoneal cavity may framework a prerequisite area for enhanced cell adhesion.

Surgery and peritoneal tumour recurrence

Peritoneal dissemination is a common cause for post surgical tumour recurrence and represents the terminal stage of disease.[92-95] Distribution patterns of first peritoneal recurrence show the resection site is preferential, and combined recurrence on peritoneal surfaces and resection site is common.[95] Peritoneal recurrence rates of up to 50% have been reported in patients with gastric cancer. The majority of patients with recurrent pancreatic cancer can be found to have peritoneal carcinomatosis. Nearly half of all patients who undergo reoperative surgery for recurrent colorectal cancer have implants on peritoneal surfaces.[96]

Early tumour cell seeding or per-operative spill are the cause of local recurrence or peritoneal carcinosis.[92;97-99] With current techniques, disseminated cancer cells are detectable in the peritoneal cavity in patients with gastric, colorectal and pancreatic cancer.[100 94;101] Previous experimental data has demonstrated that the proliferative and metastatic potential of these spilled cells are well preserved.[99;102] Furthermore, the occurrence of tumour cells in the peritoneal cavity has been shown to correlate with the postoperative survival rate. Positive peritoneal cytology in patients with gastric, colorectal and pancreatic cancer is associated with poor prognosis.[94;103-105]

The process of haemotogenic or lymphogenic cancer metastasis consists of a series of sequential, interrelated steps including invasion, embolism and transport, arrest in organs, adherence and growth.[106] In case of peritoneal carcinosis due to pre- or per-operatively seeded tumour cells, the metastatic process only depends on adherence and subsequent growth. Prevention of peritoneal carcinosis by impeding these steps offers a promising mode of improving disease free survival.

Mechanisms of action in peritoneal tumour recurrence

Several theories speculate on the fundamental mechanisms in recurrence of spilled tumour cells. Implantation strategies of tumour cells include the theory of metastatic efficiency and the fibrin entrapment hypothesis. According to the theory of metastatic efficiency, implantation of tumour cells onto raw tissue surfaces is an efficient process as opposed to inefficient implantation on intact surfaces.[95] The fibrin entrapment hypothesis proposes that peroperatively spilled tumour cells are trapped in fibrin that occurs in the resection site and on abraded peritoneal surfaces. These tumour cells will be protected from host defences and systemic chemotherapy causing a high incidence of intra-abdominal tumour recurrence.[107]

In peritoneal wound healing, inflammatory and mesothelial cells produce an abundance of cytokines and growth factors.[3;7;11;21;23-25] Surgical trauma to the peritoneum may therefore induce the release of substances that are also beneficial for tumour growth. This makes the surgical wound in particular, a fertile field for tumour growth.[106;108;109] Apart from stimulated cell growth, tumour cells may profit from the enriched wound microenvironment by enhancing the prospect of cell adhesion.[32;89;91] This would explain tumour recurrence at not directly traumatised peritoneal surfaces. Most likely, metastatic cells respond to physiologic signals produced when homeostasis is disturbed. Tumour cells that either originate from or have an affinity for growth in a particular organ can also respond to these signals.[106]

It seems surgical trauma to the peritoneum and the ensuing regeneration may affect tumour recurrence in various ways. The experiments described in this thesis aim at unravelling pathways of enhanced tumour cell adhesion and growth after surgical peritoneal trauma.

Chapter II

AIM OF THE THESIS

Pre- or per-operatively seeded tumour cells influence the prognosis of patients with gastro-intestinal malignancies dramatically. Tumour cells traumatically disseminated after surgical dissection cannot be treated using conventional routines. Intra-operative irrigation will remove some spillage, but sufficient irrigation of the peritoneal cavity is not possible.

New surgical techniques, like extended lymphadenectomy and wider margins of excision, minimise the surgery induced microscopic residual disease that may result from surgical trauma.[110;111] In addition, several experimental approaches to control the local recurrence of tumour cells have been initiated. Clinical trials are investigating the possibility of intra-peritoneal chemotherapy.[110;112;113] Lavaging the abdominal cavity with chemotherapeutic agents will deteriorate the micro milieu for residual tumour cells. However, adverse side-effects on wound healing and peritoneal adhesion formation obscure success.[114-117] Photodynamic therapy (PDT) is another promising approach to attack manifest peritoneal tumour recurrence. PDT is a surface oriented, locally cytotoxic intervention. Defined tumour foci are specifically eradicated, hereby only traumatising restricted areas of the peritoneum.[118-120]

The above mentioned therapies are mainly aimed at annulling manifest peritoneal tumour foci. A profound understanding of the pathophysiology of tumour recurrence may lead to more specific tools to confront the initial process of implantation. As mentioned in the previous section, recurrence patterns may be related to surgical trauma. The fact that the resection site and surgical wounds are predestined sites for recurrence[109], indicates there may be a mechanistic relation between surgical tissue trauma and tumour recurrence. Therefore, this thesis aims at identifying pathways of enhanced tumour recurrence and to elucidate surgery related factors.

***In vivo* studies**

Recent experimental studies elaborate on the correlation between peritoneal injury and fibrous adhesions. Diminishing the extent of peritoneal trauma minimises adhesion formation.[18;121] Clinical studies comparing minimal invasive surgery with conventional surgery support this notion.[63] Possibly, similar mechanisms are at play during tumour recurrence.

Operative skills seem to be a relevant factor in tumour prognosis. Excessive per-operative blood loss followed by transfusion has been associated with increased tumour recurrence.[122] Based on these clinical data, experiments are performed to explore the relation between the extent of peritoneal tumour recurrence and operative skills, like trauma-intensity and per-operative blood-loss. Different *in vivo* models are used to differentiate whether surgery related factors impact on tumour recurrence as a whole or specifically enhance tumour growth.

Furthermore, the consequence of surgical trauma to the peritoneal equilibrium is investigated. The inflammatory response to surgical trauma, its related cytokine production and cellular migration are taken into account. The effect of acute inflammatory mediators on peritoneal tumour recurrence is evaluated.

Supported by the results from the *in vivo* experiments described above, the generalised term 'tumour recurrence' is separated into the more elementary mechanisms tumour cell adhesion and tumour growth. Both components may contribute evenly to successful tumour recurrence (figure 2.1).

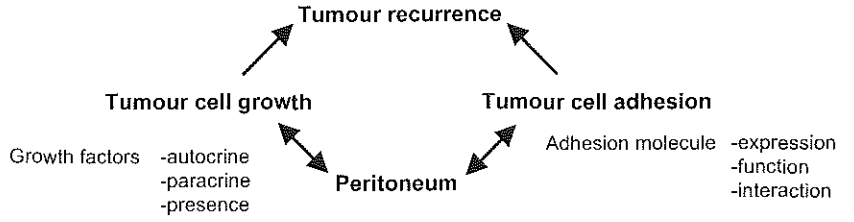

Figure 2.1

Elementary mechanisms of peritoneal tumour recurrence.

***In vitro* studies**

In the subsequent experiments the attention is focused on the influence of surgical trauma on these individual aspects. The peritoneal response to surgical trauma plays a pivotal role in these processes. Mesothelial cells of the peritoneum form the primary barrier for tumour cells. Therefore, an *in vitro* model is developed to study tumour cell adhesion to a monolayer of mesothelial cells in order to mimic *in vivo* tumour recurrence. Using this model, possible interactions between tumour cells and the peritoneum can be correlated with adhesion molecule expression and function.

Following adhesion, tumour cells must grow to form recurrent disease. Growth factors and cytokines, produced after surgical trauma to enhance peritoneal wound healing, may also stimulate tumour cell growth. Their role is investigated in peritoneal regeneration and, additionally, the direct (autocrine) and paracrine response of tumour cells to these factors will be studied.

The results obtained from these experiments will elucidate the influence of surgical peritoneal trauma on local tumour recurrence and on which processes recurrence is based. Furthermore, it will provide information which pathways may be used for specific therapeutical alternatives.

References

1. Bouchet Y VCYR. The peritoneum and peritoneal access. In: Bengmark S, ed. The peritoneum and its anatomy, London: Wright, 1989: 1-13.
2. diZerega GS. The cause and prevention of postsurgical adhesions: a contemporary update. **Prog Clin Biol Res** 1993; **381**: 1-18.
3. diZerega GS. Biochemical events in peritoneal tissue repair. **Eur J Surg Suppl** 1997; 10-16.
4. Nagy JA. Peritoneal membrane morphology and function. **Kidney Int Suppl** 1996; **56**: S2-11.
5. Esperanza M, Collins D. Peritoneal dialysis efficiency in relation to body weight. **J Pediatr Surg** 1966; **1**: 162-169.
6. Hall JC, Heel KA, Papadimitriou JM, Platell C. The pathobiology of peritonitis. **Gastroenterology** 1998; **114**: 185-196.
7. Holmdahl L, Ivarsson ML. The role of cytokines, coagulation, and fibrinolysis in peritoneal tissue repair. **Eur J Surg** 1999; **165**: 1012-1019.
8. Eskeland G, Kjaerheim A. Regeneration of parietal peritoneum in rats. 2. An electron microscopical study. **Acta Pathol Microbiol Scand** 1966; **68**: 379-395.
9. Whitaker D, Papadimitriou JM, Walters MN. The mesothelium and its reactions: a review. **Crit Rev Toxicol** 1982; **10**: 81-144.
10. Junqueira LC, Joazeiro PP, Toledo OM, Montes GS. A morphologic and histochemical study of the mesentery in the guinea pig. **Acta Anat (Basel)** 1987; **129**: 214-219.
11. Nagy JA, Jackman R. Response of the peritoneal membrane to injury. **Semin Dialysis** 1998; **11**: 242-248.
12. Ettarh RR, Carr KE. Ultrastructural observations on the peritoneum in the mouse. **J Anat** 1996; **188 (Pt 1)**: 211-215.
13. Koga S, Kudo H, Kiyasu Y, et al. A scanning electron microscopic study on the peritoneal implantation of ascites hepatoma AH100B cells in rats. **Gann** 1980; **71**: 8-13.
14. Michailova KN. A combined electron microscopic investigation of the peritoneal mesothelium in the rat. **Eur J Morphol** 1995; **33**: 265-277.
15. Slater NJ, Raftery AT, Cope GH. The ultrastructure of human abdominal mesothelium. **J Anat** 1989; **167**: 47-56.
16. Watters WB, Buck RC. Scanning electron microscopy of mesothelial regeneration in the rat. **Lab Invest** 1972; **26**: 604-609.
17. Ryan GB, Grobety J, Majno G. Mesothelial injury and recovery. **Am J Pathol** 1973; **71**: 93-112.

18. van den Tol MP, van S, I, Bonthuis F, Marquet RL, Jeekel J. Reduction of intraperitoneal adhesion formation by use of non-abrasive gauze. **Br J Surg** 1997; **84**: 1410-1415.
19. Leak LV. Interaction of mesothelium to intraperitoneal stimulation. I. Aggregation of peritoneal cells. **Lab Invest** 1983; **48**: 479-491.
20. Verger C, Luger A, Moore HL, Nolph KD. Acute changes in peritoneal morphology and transport properties with infectious peritonitis and mechanical injury. **Kidney Int** 1983; **23**: 823-831.
21. Topley N, Williams JD. Role of the peritoneal membrane in the control of inflammation in the peritoneal cavity. **Kidney Int Suppl** 1994; **48**: S71-S78
22. Topley N, Mackenzie RK, Williams JD. Macrophages and mesothelial cells in bacterial peritonitis. **Immunobiology** 1996; **195**: 563-573.
23. Douvdevani A, Rapoport J, Konforty A, Argov S, Ovnat A, Chaimovitz C. Human peritoneal mesothelial cells synthesize IL-1 alpha and beta. **Kidney Int** 1994; **46**: 993-1001.
24. Lanfrancone L, Boraschi D, Ghiara P, et al. Human peritoneal mesothelial cells produce many cytokines (granulocyte colony-stimulating factor [CSF], granulocyte-monocyte-CSF, macrophage- CSF, interleukin-1 [IL-1], and IL-6) and are activated and stimulated to grow by IL-1. **Blood** 1992; **80**: 2835-2842.
25. Chegini N. The role of growth factors in peritoneal healing: transforming growth factor beta (TGF-beta). **Eur J Surg Suppl** 1997; 17-23.
26. Gerwin BI, Lechner JF, Reddel RR, et al. Comparison of production of transforming growth factor-beta and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. **Cancer Res** 1987; **47**: 6180-6184.
27. Offner FA, Feichtinger H, Stadlmann S, et al. Transforming growth factor-beta synthesis by human peritoneal mesothelial cells. Induction by interleukin-1. **Am J Pathol** 1996; **148**: 1679-1688.
28. Klein CL, Bittinger F, Skarke CC, et al. Effects of cytokines on the expression of cell adhesion molecules by cultured human omental mesothelial cells. **Pathobiology** 1995; **63**: 204-212.
29. Li FK, Davenport A, Robson RL, et al. Leukocyte migration across human peritoneal mesothelial cells is dependent on directed chemokine secretion and ICAM-1 expression. **Kidney Int** 1998; **54**: 2170-2183.
30. Muller J, Yoshida T. Interaction of murine peritoneal leukocytes and mesothelial cells: in vitro model system to survey cellular events on serosal membranes during inflammation. **Clin Immunol Immunopathol** 1995; **75**: 231-238.

31. Sendt W, Amberg R, Schoffel U, Hassan A, von Specht BU, Farthmann EH. Local inflammatory peritoneal response to operative trauma: studies on cell activity, cytokine expression, and adhesion molecules. **Eur J Surg** 1999; **165**: 1024-1030.
32. Meager A. Cytokine regulation of cellular adhesion molecule expression in inflammation. **Cytokine Growth Factor Rev** 1999; **10**: 27-39.
33. Jonjic N, Peri G, Bernasconi S, et al. Expression of adhesion molecules and chemotactic cytokines in cultured human mesothelial cells. **J Exp Med** 1992; **176**: 1165-1174.
34. Yung S, Coles GA, Davies M. IL-1 beta, a major stimulator of hyaluronan synthesis in vitro of human peritoneal mesothelial cells: relevance to peritonitis in CAPD. **Kidney Int** 1996; **50**: 1337-1343.
35. Bermudez E, Everitt J, Walker C. Expression of growth factor and growth factor receptor RNA in rat pleural mesothelial cells in culture. **Exp Cell Res** 1990; **190**: 91-98.
36. Cronauer MV, Stadlmann S, Klocker H, et al. Basic fibroblast growth factor synthesis by human peritoneal mesothelial cells: induction by interleukin-1. **Am J Pathol** 1999; **155**: 1977-1984.
37. Riese J, Denzel C, Zowe M, Mehler C, Hohenberger W, Haupt W. Secretion of IL-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1alpha, and TNFalpha by cultured intact human peritoneum. **Eur Surg Res** 1999; **31**: 281-288.
38. Offner FA, Obrist P, Stadlmann S, et al. IL-6 secretion by human peritoneal mesothelial and ovarian cancer cells. **Cytokine** 1995; **7**: 542-547.
39. Topley N, Jorres A, Luttmann W, et al. Human peritoneal mesothelial cells synthesize interleukin-6: induction by IL-1 beta and TNF alpha. **Kidney Int** 1993; **43**: 226-233.
40. Topley N, Brown Z, Jorres A, et al. Human peritoneal mesothelial cells synthesize interleukin-8. Synergistic induction by interleukin-1 beta and tumor necrosis factor-alpha. **Am J Pathol** 1993; **142**: 1876-1886.
41. Douvdevani A, Einbinder T, Yulzari R, Rogachov B, Chaimovitz C. TNF-receptors on human peritoneal mesothelial cells: regulation of receptor levels and shedding by IL-1 alpha and TNF alpha. **Kidney Int** 1996; **50**: 219-228.
42. Lee TC, Zhang Y, Aston C, et al. Normal human mesothelial cells and mesothelioma cell lines express insulin-like growth factor I and associated molecules. **Cancer Res** 1993; **53**: 2858-2864.
43. Rutten AA, Bermudez E, Stewart W, Everitt JI, Walker CL. Expression of insulin-like growth factor II in spontaneously immortalized rat mesothelial and spontaneous mesothelioma cells: a potential autocrine role of insulin-like growth factor II. **Cancer Res** 1995; **55**: 3634-3639.

44. van der Wal BC, Hofland LJ, Marquet RL, van Koetsveld PM, van Rossen ME, van Eijck CH. Paracrine interactions between mesothelial and colon-carcinoma cells in a rat model. **Int J Cancer** 1997; **73**: 885-890.
45. Topley N, Petersen MM, Mackenzie R, et al. Human peritoneal mesothelial cell prostaglandin synthesis: induction of cyclooxygenase mRNA by peritoneal macrophage-derived cytokines. **Kidney Int** 1994; **46**: 900-909.
46. Franzen L, Ghassemifar R, Schultz G. Specific binding of EGF in connective tissue repair. **Eur J Cell Biol** 1993; **60**: 346-350.
47. Van der Meeren A, Levy F, Renier A, Katz A, Jaurand MC. Effect of epidermal growth factor on rat pleural mesothelial cell growth. **J Cell Physiol** 1990; **144**: 137-143.
48. Whawell SA, Thompson JN. Cytokine-induced release of plasminogen activator inhibitor-1 by human mesothelial cells. **Eur J Surg** 1995; **161**: 315-318.
49. Pascual M, French LE. Complement in human diseases: looking towards the 21st century. **Immunol Today** 1995; **16**: 58-61.
50. Caldwell MT, Watson RG. Peritoneal aspiration cytology as a diagnostic aid in acute appendicitis [see comments]. **Br J Surg** 1994; **81**: 276-278.
51. Melnicoff MJ, Horan PK, Morahan PS. Kinetics of changes in peritoneal cell populations following acute inflammation. **Cell Immunol** 1989; **118**: 178-191.
52. Heinzelmann M, Simmen HP, Battaglia H, Friedl HP, Trentz O. Inflammatory response after abdominal trauma, infection, or intestinal obstruction measured by oxygen radical production in peritoneal fluid. **Am J Surg** 1997; **174**: 445-447.
53. Ellis H. Wound repair. Reaction of the peritoneum to injury. **Ann R Coll Surg Engl** 1978; **60**: 219-221.
54. Thompson J. Pathogenesis and prevention of adhesion formation. **Dig Surg** 1998; **15**: 153-157.
55. Arnold PB, Green CW, Foresman PA, Rodeheaver GT. Evaluation of resorbable barriers for preventing surgical adhesions. **Fertil Steril** 2000; **73**: 157-161.
56. Holmdahl L, Risberg B, Beck DE, et al. Adhesions: pathogenesis and prevention-panel discussion and summary. **Eur J Surg Suppl** 1997; 56-62.
57. Anderson BO, Brown JM, Harken AH. Mechanisms of neutrophil-mediated tissue injury. **J Surg Res** 1991; **51**: 170-179.
58. Andreoli SP, Mallett C, Williams K, McAteer JA, Rothlein R, Doerschuk CM. Mechanisms of polymorphonuclear leukocyte mediated peritoneal mesothelial cell injury. **Kidney Int** 1994; **46**: 1100-1109.
59. Weiss SJ. Tissue destruction by neutrophils [see comments]. **N Engl J Med** 1989; **320**: 365-376.

60. Weiss SJ, Young J, LoBuglio AF, Slivka A, Nimeh NF. Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. **J Clin Invest** 1981; **68**: 714-721.
61. Ar'Rajab A, Mileski W, Sentementes JT, Sikes P, Harris RB, Dawidson IJ. The role of neutrophils in peritoneal adhesion formation. **J Surg Res** 1996; **61**: 143-146.
62. Vural B, Canturk NZ, Esen N, et al. The role of neutrophils in the formation of peritoneal adhesions. **Hum Reprod** 1999; **14**: 49-54.
63. Schafer M, Krahenb hL, Buchler MW. Comparison of adhesion formation in open and laparoscopic surgery. **Dig Surg** 1998; **15**: 148-152.
64. Duron JJ, Hay JM, Msika S, et al. Prevalence and mechanisms of small intestinal obstruction following laparoscopic abdominal surgery: a retrospective multicenter study. French Association for Surgical Research. **Arch Surg** 2000; **135**: 208-212.
65. Griffith JP, Everitt NJ, Lancaster F, et al. Influence of laparoscopic and conventional cholecystectomy upon cell- mediated immunity. **Br J Surg** 1995; **82**: 677-680.
66. Jacobi CA, Ordemann J, Zieren HU, et al. Increased systemic inflammation after laparotomy vs laparoscopy in an animal model of peritonitis. **Arch Surg** 1998; **133**: 258-262.
67. Vittimberga FJJ, Foley DP, Meyers WC, Callery MP. Laparoscopic surgery and the systemic immune response. **Ann Surg** 1998; **227**: 326-334.
68. Reber PU, Andren S, Schmied B, Buchler MW. Cytokines in surgical trauma: cholecystectomy as an example. **Dig Surg** 1998; **15**: 92-101.
69. Ward PA, Lentsch AB. The acute inflammatory response and its regulation. **Arch Surg** 1999; **134**: 666-669.
70. Baigrie RJ, Lamont PM, Kwiatkowski D, Dallman MJ, Morris PJ. Systemic cytokine response after major surgery. **Br J Surg** 1992; **79**: 757-760.
71. Shenkin A, Fraser WD, Series J, et al. The serum interleukin 6 response to elective surgery. **Lymphokine Res** 1989; **8**: 123-127.
72. Baxevanis CN, Papilas K, Dedoussis GV, Pavlis T, Papamichail M. Abnormal cytokine serum levels correlate with impaired cellular immune responses after surgery. **Clin Immunol Immunopathol** 1994; **71**: 82-88.
73. Bruce DM, Smith M, Walker CB, et al. Minimal access surgery for cholelithiasis induces an attenuated acute phase response. **Am J Surg** 1999; **178**: 232-234.
74. Tsukada K, Katoh H, Shiojima M, Suzuki T, Takenoshita S, Nagamachi Y. Concentrations of cytokines in peritoneal fluid after abdominal surgery. **Eur J Surg** 1993; **159**: 475-479.

75. Ozawa A, Konishi F, Nagai H, Okada M, Kanazawa K. Cytokine and Hormonal Responses in Laparoscopic-Assisted Colectomy and Conventional Open Colectomy. **Surg Today** 2000; **30**: 107-111.
76. Badia JM, Whawell SA, Scott-Coombes DM, Abel PD, Williamson RC, Thompson JN. Peritoneal and systemic cytokine response to laparotomy. **Br J Surg** 1996; **83**: 347-348.
77. Topley N. The cytokine network controlling peritoneal inflammation. **Perit Dial Int** 1995; **15**: S35-S39
78. Topley N, Liberek T, Davenport A, Li FK, Fear H, Williams JD. Activation of inflammation and leukocyte recruitment into the peritoneal cavity. **Kidney Int Suppl** 1996; **56** : S17-S21
79. van Deuren M, Dofferhoff AS, van der Meer JW. Cytokines and the response to infection. **J Pathol** 1992; **168**: 349-356.
80. Simpson KJ. Cytokines, for better or worse? **Eur J Gastroenterol Hepatol** 1999; **11**: 957-966.
81. van Berge H, van der Poll T, van Deventer SJ, Gouma DJ. Peritoneal cytokine release after elective gastrointestinal surgery and postoperative complications. **Am J Surg** 1998; **175**: 311-316.
82. Whitaker D, Papadimitriou J. Mesothelial healing: morphological and kinetic investigations. **J Pathol** 1985; **145**: 159-175.
83. Eskeland G. Regeneration of parietal peritoneum in rats. 1. A light microscopical study. **Acta Pathol Microbiol Scand** 1966; **68**: 355-378.
84. Mutsaers SE, Whitaker D, Papadimitriou JM. Mesothelial regeneration is not dependent on subserosal cells [In Process Citation]. **J Pathol** 2000; **190**: 86-92.
85. Martin P, Hopkinson-Woolley J, McCluskey J. Growth factors and cutaneous wound repair. **Prog Growth Factor Res** 1992; **4**: 25-44.
86. Slavin J. The role of cytokines in wound healing. **J Pathol** 1996; **178**: 5-10.
87. Bennett NT, Schultz GS. Growth factors and wound healing: biochemical properties of growth factors and their receptors. **Am J Surg** 1993; **165**: 728-737.
88. Frenette PS, Wagner DD. Adhesion molecules--Part 1. **N Engl J Med** 1996; **334**: 1526-1529.
89. Menger MD, Vollmar B. Adhesion molecules as determinants of disease: from molecular biology to surgical research. **Br J Surg** 1996; **83**: 588-601.
90. Bittinger F, Brochhausen C, Kohler H, et al. Differential expression of cell adhesion molecules in inflamed appendix: correlation with clinical stage. **J Pathol** 1998; **186**: 422-428.

91. Rice GE, Gimbrone MAJ, Bevilacqua MP. Tumor cell-endothelial interactions. Increased adhesion of human melanoma cells to activated vascular endothelium. **Am J Pathol** 1988; **133**: 204-210.
92. Abulafi AM, Williams NS. Local recurrence of colorectal cancer: the problem, mechanisms, management and adjuvant therapy [see comments]. **Br J Surg** 1994; **81**: 7-19.
93. Delpero JR, Pol B, Le Treut P, et al. Surgical resection of locally recurrent colorectal adenocarcinoma. **Br J Surg** 1998; **85**: 372-376.
94. Schott A, Vogel I, Krueger U, et al. Isolated tumor cells are frequently detectable in the peritoneal cavity of gastric and colorectal cancer patients and serve as a new prognostic marker. **Ann Surg** 1998; **227**: 372-379.
95. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. **World J Surg** 1991; **15**: 609-616.
96. Sugarbaker PH, Cunliffe WJ, Belliveau J, et al. Rationale for integrating early postoperative intraperitoneal chemotherapy into the surgical treatment of gastrointestinal cancer. **Semin Oncol** 1989; **16**: 83-97.
97. Catterall JB, Gardner MJ, Jones LM, Thompson GA, Turner GA. A precise, rapid and sensitive in vitro assay to measure the adhesion of ovarian tumour cells to peritoneal mesothelial cells. **Cancer Lett** 1994; **87**: 199-203.
98. Skipper D, Cooper AJ, Marston JE, Taylor I. Exfoliated cells and in vitro growth in colorectal cancer. **Br J Surg** 1987; **74**: 1049-1052.
99. Umpleby HC, Fermor B, Symes MO, Williamson RC. Viability of exfoliated colorectal carcinoma cells. **Br J Surg** 1984; **71**: 659-663.
100. Juhl H, Stritzel M, Wroblewski A, et al. Immunocytological detection of micrometastatic cells: comparative evaluation of findings in the peritoneal cavity and the bone marrow of gastric, colorectal and pancreatic cancer patients. **Int J Cancer** 1994; **57**: 330-335.
101. Leather AJ, Kocjan G, Savage F, et al. Detection of free malignant cells in the peritoneal cavity before and after resection of colorectal cancer [see comments]. **Dis Colon Rectum** 1994; **37**: 814-819.
102. Fermor B, Umpleby HC, Lever JV, Symes MO, Williamson RC. Proliferative and metastatic potential of exfoliated colorectal cancer cells. **J Natl Cancer Inst** 1986; **76**: 347-349.
103. Bonenkamp JJ, Songun I, Hermans J, van d, V. Prognostic value of positive cytology findings from abdominal washings in patients with gastric cancer. **Br J Surg** 1996; **83** : 672-674.

104. Vogel I, Kruger U, Marxsen J, et al. Disseminated tumor cells in pancreatic cancer patients detected by immunocytology: a new prognostic factor. **Clin Cancer Res** 1999; **5**: 593-599.
105. Burke EC, Karpeh MSJ, Conlon KC, Brennan MF. Peritoneal lavage cytology in gastric cancer: an independent predictor of outcome. **Ann Surg Oncol** 1998; **5**: 411-415.
106. Gutman M, Fidler IJ. Biology of human colon cancer metastasis. **World J Surg** 1995; **19**: 226-234.
107. Cunliffe WJ, Sugarbaker PH. Gastrointestinal malignancy: rationale for adjuvant therapy using early postoperative intraperitoneal chemotherapy. **Br J Surg** 1989; **76**: 1082-1090.
108. Baker DG, Masterson TM, Pace R, Constable WC, Wanebo H. The influence of the surgical wound on local tumor recurrence [see comments]. **Surgery** 1989; **106**: 525-532.
109. Hofer SO, Molema G, Hermens RA, Wanebo HJ, Reichner JS, Hoekstra HJ. The effect of surgical wounding on tumour development. **Eur J Surg Oncol** 1999; **25**: 231-243.
110. Sugarbaker PH. Successful management of microscopic residual disease in large bowel cancer. **Cancer Chemother Pharmacol** 1999; **43 Suppl**: S15-S25
111. Heald RJ, Karanjia ND. Results of radical surgery for rectal cancer. **World J Surg** 1992; **16**: 848-857.
112. Esquivel J, Vidal-Jove J, Steves MA, Sugarbaker PH. Morbidity and mortality of cytoreductive surgery and intraperitoneal chemotherapy [see comments]. **Surgery** 1993; **113** : 631-636.
113. Sugarbaker PH, Landy D, Pascal R. Intraperitoneal chemotherapy for peritoneal carcinomatosis from colonic or appendiceal cystadenocarcinoma: rationale and results of treatment. **Prog Clin Biol Res** 1990; **354B**: 141-170.
114. Adachi W, Koike S, Rafique M, et al. Preoperative intraperitoneal chemotherapy for gastric cancer, with special reference to delayed peritoneal complications. **Surg Today** 1995; **25**: 396-403.
115. Drake DB, Oishi SN. Wound healing considerations in chemotherapy and radiation therapy. **Clin Plast Surg** 1995; **22**: 31-37.
116. Howell SB, Pfeifle CE. Peritoneal access for intracavitary chemotherapy. **Cancer Drug Deliv** 1986; **3**: 157-161.
117. Jacquet P, Sugarbaker PH. Effects of postoperative intraperitoneal chemotherapy on peritoneal wound healing and adhesion formation. **Cancer Treat Res** 1996; **82**: 327-335.

118. Gahlen J, Prosst RL, Pietschmann M, Rheinwald M, Haase T, Herfarth C. Spectrometry supports fluorescence staging laparoscopy after intraperitoneal aminolaevulinic acid lavage for gastrointestinal tumours. **J Photochem Photobiol B** 1999; **52**: 131-135.
119. Pass HI, Donington JS. Use of photodynamic therapy for the management of pleural malignancies. **Semin Surg Oncol** 1995; **11**: 360-367.
120. Veenhuizen RB, Ruevekamp MC, Oppelaar H, Helmerhorst TJ, Kenemans P, Stewart FA. Foscan-mediated photodynamic therapy for a peritoneal-cancer model: drug distribution and efficacy studies. **Int J Cancer** 1997; **73**: 230-235.
121. Holmdahl L, al-Jabreen M, Risberg B. Experimental models for quantitative studies on adhesion formation in rats and rabbits. **Eur Surg Res** 1994; **26**: 248-256.
122. Busch OR, Hop WC, Hoynck vPM, Marquet RL, Jeekel J. Blood transfusions and prognosis in colorectal cancer [see comments]. **N Engl J Med** 1993; **328**: 1372-1376.

Part II

Surgical Peritoneal Trauma and Tumour Recurrence

in vivo

Chapter III

REDUCTION OF PERITONEAL TRAUMA BY USING NON-SURGICAL GAUZE LEADS TO LESS IMPLANTATION METASTASIS OF SPILLED TUMOUR CELLS

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Surgical abdominal trauma induces mechanical damage to the peritoneal lining of the abdominal cavity. The experiments described in this chapter were designed to evaluate whether surgical peritoneal trauma affects tumour recurrence.

The results of the presented in vivo experiments demonstrate that a significant correlation exists between the amount of peritoneal trauma and the degree of tumour recurrence at traumatised and not directly traumatised peritoneal surfaces. In addition, a significant correlation between the degree of peritoneal trauma and the growth of ectopic tumours, situated under the renal capsule, is demonstrated. Within a few hours after infliction of peritoneal trauma, factors can be captivated in a lavage fluid and enhance peritoneal tumour recurrence in naive recipients.

From these studies we conclude that surgical trauma promotes tumour recurrence. This effect is not restricted to the traumatised sites, but has a generalised character. Furthermore, surgery related factors produced in the early hours after surgical peritoneal trauma, are potent stimulators of tumour recurrence.

INTRODUCTION

Locoregional recurrence of gastro-intestinal carcinoma remains an important complication after potentially curative surgical resection. Its incidence varies from 0% to 45%.[1-4] Ways to prevent these locoregional recurrences are the subject of various clinical and experimental studies.[5-9] The most common site for colorectal adeno-carcinoma to recur is the site of the primary tumour; the second is the peritoneal surface.[3;5;10;11] The tumour cell entrapment hypothesis might explain this pattern of surgical treatment failure resulting in loco-regional recurrence. When a tumour is removed, tumour cells can leak from transected lymphatics into the abdominal cavity.[11] Experimental studies have shown that the proliferative and metastatic potentials of these spilled cells are very well preserved. Consequently, exfoliated carcinoma cells may undergo further division and give rise to implantation of metastases.[12;13] However, implantation of spilled tumour cells on surfaces with intact basement membranes is an inefficient process, whereas implantation on damaged surfaces, resulting from intra-abdominal manipulation, is very efficient.[11;14] The dynamic process of peritoneal healing after ischaemic damage of the peritoneal surfaces, sometimes leading to adhesion formation, also seems to be important in the process of adhesion and growth of spilled tumour cells on the peritoneum.[15] In clinical situations, it appears that peritoneal tumour implants may recur within a fibrous adhesion resulting from surgical trauma.[11]

In a rat model, we recently showed that surgical trauma evoked by standard surgical gauze led to marked adhesion formation, which could significantly be reduced by using a nonabrasive textile.[16] The present study was undertaken to evaluate whether the intra-abdominal use of this less traumatic non-surgical textile would also lead to less intra-peritoneal tumour cell adhesion and tumour growth of spilled carcinoma cells. In addition, experiments were performed to evaluate whether the relation between the degree of trauma and tumour growth was merely a local phenomenon, or whether systemic effects might also be involved.

MATERIALS AND METHODS

Animals

Female inbred rats of the WAG strain were obtained from Harlan-CPB, Zeist, The Netherlands. The rats were bred under specific pathogen free conditions, kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 hours light/ 12 hours dark), fed

with laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee in Animal Research of the Erasmus University Rotterdam, The Netherlands. Before performing any new experiments, the validity of the model used in the previous adhesion studies, employing Wistar rats,[16] was checked and found equally valid in WAG/Rij rats.

Tumour cell line

CC531 is a moderately differentiated, weakly immunogenic colon adeno-carcinoma induced in the WAG/Rij rat by 1,2-dimethylhydrazine.[17] A cell line was established from this carcinoma and maintained by serial passage after trypsinization in culture medium. CC531 tumour cells were cultured in RPMI 1640 medium supplemented with 5% foetal calf serum, L-glutamin (2 mM) and penicillin (10^5 U/L). Medium and all supplements were obtained from Life Technologicals BV, Breda, The Netherlands. Cells were passaged once a week using trypsin (0.05%) and EDTA (0.02%). Viability was measured by trypan blue exclusion and always exceeded 90 percent.
To grow solid tumour, $1x10^8$ tumour cells were injected into the right flank of a syngeneic WAG/Rij rat. After 6 weeks, a tumour mass with a volume of 2.5 cm^3 had grown and could be aseptically isolated form the outer membrane of the main lesion with a scalper. The harvested tumour was cut into 1 mm^3 cubes (weighing 5.8-7.2 mg) and immersed in a culture solution stored at 4°C. Within 1 to 4 hours after collection of the solid CC531 tumour, the cubes were implanted sub-renally in syngeneic WAG/Rij rats.

Gauzes

The gauzes used were surgical Medipres gauze, consisting of 100% cotton, commonly used in abdominal surgery (van Heek Medical, Losser, The Netherlands) and non-surgical Fastsorb clean-room wiper, used in the electronics industry on abrasion-sensitive surfaces (Berkshire Corp., Great Barrington, MA, USA). Fastsorb is a rayon-polyester blend with strength, softness, and a high absorbing capacity. In previous experiments, we demonstrated that non-surgical Fastsorb textile was less traumatic for the peritoneum and caused less adhesion formation after intra-abdominal manipulation than surgical Medipres gauze.[16]

Operative procedures

Under ether anaesthesia, the abdomen was shaved and cleansed with alcohol 70%. Laparatomy was performed using a lower midline incision of 5 cm. Both horns of the uterus were exposed, rubbed with either severely traumatising surgical gauze or less traumatising non-surgical Fastsorb textile, or not manipulated at all by any gauze. Rubbing took place using a device enabling the application of a constant pressure of 120 g/cm^2.[16] The uterus horn was

rubbed 10 times over its total length. Thus, three different peritoneal traumas could be inflicted. After performing one of these 3 procedures, the uterus horn was subsequently sutured to the lateral peritoneum, both proximally and distally, using single Surgilene 6-0 sutures (B Braun, Melsungen AG, Germany). The abdomen was closed in 2 layers with Dexon 5-0 and silk 2-0 sutures (B Braun).

Experimental design

Effect of uterus horn manipulation on intra-peritoneal tumour growth

Ten rats (group I) underwent an operation in which one uterus horn was rubbed with surgical gauze and the other was not manipulated. In 10 rats (group II), one uterus horn was rubbed with non-surgical textile and the other was not touched. In 9 rats (group III), one uterus horn was rubbed with surgical gauze and the other with non-surgical textile. Directly after manipulation of the peritoneum and before closing the abdomen, 0.5 million CC531 tumour cells in 1 ml RPMI 1640 medium were injected intra-peritoneally. Three weeks after surgery, the rats were killed and intra-peritoneal tumour load was scored the parietal peritoneum, omentum, liver, kidneys, retroperitoneum and mesentery. The scoring ranged from 0 to 5 per site according to the peritoneal cancer index as described by Steller. A score of 0 meant there was no tumour growth, a score of 1 indicated an estimated tumour diameter of less than 0.5 cm, a score of 2 a tumour diameter between 0.5 and 1 cm, a score of 3 a tumour diameter between 1 and 2 cm, a score of 4 a tumour diameter between 2 and 3 cm, and a score of 5 an estimated diameter of more than 3 cm.[18]

Effect of uterus horn manipulation on established ectopic tumour growth

On day 1, 30 rats underwent a laparotomy using a midline incision of 2.5 cm. A solid cube of CC531 colon tumour weighing about 6 mg was placed under the capsule of both exposed kidneys under microscopic vision. Thereafter, the abdomen was closed in one layer. On day 3, all 30 rats were operated on again. Both uterus horns and 5 cm of the small bowel were rubbed, in 10 rats (group IV) with surgical gauze and in 10 rats (group V) with non-surgical textile. Group VI (n=10) underwent a laparotomy only; the uterus horns nor the small bowel were touched. Ten days after tumour implantation, the rats were killed and the growth of the sub-capsular tumours was measured by weighing the 60 e-nucleated lumps (for each rat, the 2 individual data were averaged; 10 data per group were used for statistical analysis).

Passive transfer experiments

To evaluate whether the tumour promoting effect of surgical trauma of the peritoneum could be passively transferred to naive non-traumatised rats, the following procedure was employed. Three rats were operated on. Two animals underwent a laparotomy, during which both uterus

horns and a 5 cm long part of the small intestine were rubbed with either surgical gauze (rat 1) or non-surgical textile (rat 2). Rat 3 only underwent a laparotomy. The abdomen was closed in one layer. After 5 hours, these rats underwent a second laparotomy, during which the abdominal cavity was rinsed 5 times per rat with 5 ml RPMI 1640. Each time, 1 ml of the injected irrigant was collected.

Subsequently, 15 naive rats were treated. In 5 rats (group VII), 1 ml of irrigant collected from rat 1 (peritoneal manipulation with surgical gauze) and 0.5 million CC531 tumour cells in 1 ml RPMI 1640 were injected intra-peritoneally. In 5 rats (group VIII), the same procedure was performed with the irrigants collected from rat 2 (peritoneal manipulation with non-surgical textile). In the last group (group IX), irrigants collected from rat 3 (no peritoneal manipulation) were used.

After 3 weeks, the rats were killed after which tumour load and growth were scored.

Statistical analysis

The median and range of intra-peritoneal tumour load and the means and standard deviations of the sub-renal tumour weights were calculated. Statistical analysis was performed using the Wilcoxon matched pairs test if two groups were compared and the non-parametric Kruskal-Wallis test if three groups were compared. If the latter overall test indicated significance, comparisons between groups were made using the Mann-Whitney *U* test. Statistical significance was defined as $p<0.05$.

Uterus horns	Tumour load	p-value
I. No touch	0 (0-0)	
I. Medipres manipulation	4.5 (3-5)	0.0001
II. No touch	0 (0-0)	
II. Fastsorb manipulation	2 (0-3)	0.0001
III. Medipres manipulation	5 (4-5)	
III. Fastsorb manipulation	1 (0-3)	0.0002

Table 3.1

Median tumour load (range) at uterus horns severely traumatised by rubbing with surgical Medipres gauze, mildly traumatised by rubbing with non-surgical Fastsorb textile and not directly traumatised uterus horns (no touch).

RESULTS

Effect of uterus horn manipulation on intra-peritoneal tumour growth

Table 3.1 summarises the results observed at the site of the uterus horns. In three different experiments, a significant correlation was found between tumour load and the degree of peritoneal trauma imposed by rubbing. Rubbing with severely traumatising surgical gauze produced the highest tumour load, whereas no rubbing resulted in the lowest (group I; p=0.005). Rubbing with mildly traumatising non-surgical textile raised a low degree of tumour load, but it was still significantly more than if no rubbing had taken place (group II; p=0.0018). At the site of the uterus horns, the tumours were often located in adhesions. Table 3.2 shows the tumour load at the non-manipulated remote peritoneal sites in groups I, II and III. It shows significant differences at two abdominal sites (the retroperitoneum ($p \leq 0.01$) and the omentum ($p \leq 0.01$) between group II (non-surgical textile) and groups I and III (surgical gauze). A significant difference in total tumour load between the same groups (II *versus* I and II *versus* III) was found ($p \leq 0.005$). Differences in tumour load at the other three peritoneal sites were not significant.

Abdominal Sites	**I Medipres**	**II Fastsorb**	**III Medipres & Fastsorb**	p1	p2	p3
Par. Peritoneum	0 (1-3)	2 (0-3)	1 (0-4)	ns	ns	ns
Mesentery	0 (0-2)	0 (0-1)	0 (0-1)	ns	ns	ns
Kidney	1 (0-2)	1 (0-2)	1 (0-2)	ns	ns	ns
Liver	2 (0-3)	1 (0-2)	2 (1-3)	ns	ns	ns
Retroperitoneum	2 (1-3)	1 (0-2)	2 (1-3)	0.007	ns	0.01
Omentum	2 (0-3)	1 (0-1)	2 (2-4)	0.01	ns	<0.0001
Total	1.5 (0-3)	1 (0-3)	1.5 (0-4)	0.005	ns	0.001

Table 3.2

Median tumour load (range) at different not directly traumatised peritoneal sites in rats having been intra-abdominally manipulated by severely traumatising surgical gauze (group I), mildly traumatising non-surgical textile (group II) or a combination of both materials (group III).

The p-value 1 represents differences between group I and II; p-value 2 represents differences between group I and III; p-value 3 represents differences between group II and III.

Effect of uterus horn manipulation on established ectopic tumour growth

The mean weight of the sub-renal tumours was measured 10 days after tumour implantation, 7 days after manipulation with surgical gauze and non-surgical textile. Significant differences in mean tumour weight between the three groups were found (Figure 3.1). Again, a significant correlation between the degree of peritoneal trauma and tumour growth was observed. The mean weight of the ectopic tumour was the highest in rats rubbed with surgical gauze (IV) and significantly lower in rats rubbed with non-surgical textile (V) (p=0.009). When the peritoneum was not touched (VI), the lowest mean tumour weight was found, significantly lower than rubbing with non-surgical textile (p=0.002) or surgical gauze (p<0.0001).

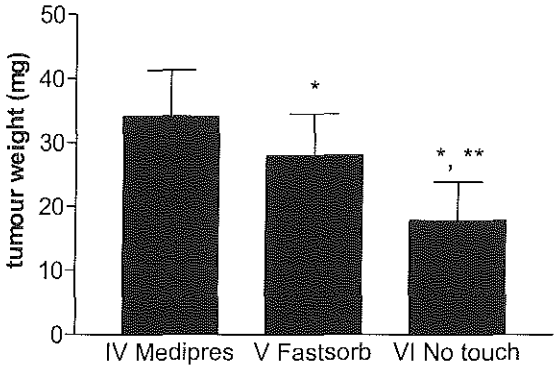

Figure 3.1

Tumour weight (mean (SD) in mg) of the sub-renal tumours after intra-abdominal rubbing with severely traumatising surgical gauze (group IV), with mildly traumatising non-surgical textile (group V) or after no rubbing of the peritoneum (group VI). * p<0.01 *vs* Medipres, ** p<0.001 *vs* Fastsorb.

Passive transfer experiments

The median total peritoneal tumour load in rats injected with irrigants collected from abdominal cavities, manipulated with surgical gauze or non-surgical textile or not, differed significantly (table 3.3, $p \leq 0.016$). These differences were mainly due to differences at the site of the omentum and the kidney. As in the previous experiments, a decreasing gradient of tumour load was found, from surgical (VII) to non-surgical (VIII) to non-traumatised abdominal cavities (IX).

Abdominal Sites	VII Medipres	VII Fastsorb	IX No touch	p1	p2	p3
Uterus A	0	0	0	ns	ns	ns
Uterus B	0	0	0	ns	ns	ns
Mesentery	0	0	0	ns	ns	ns
Par. peritoneum	0	0	0	ns	ns	ns
Kidney	2 (0-3)	1 (0-2)	0	ns	0.03	0.03
Liver	2 (1-3)	1 (0-2)	0 (0-2)	ns	ns	ns
Retroperitoneum	1 (0-3)	1 (0-2)	1 (0-2)	ns	ns	ns
Omentum	3 (2-5)	1 (0-3)	0 (0-2)	0.03	0.008	ns
Total	0 (0-5)	0 (0-3)	0 (0-2)	0.02	0.008	0.002

Table 3.3

Median tumour load (range) at different peritoneal sites in rats after intra-abdominal injection with irrigant obtained form rats that underwent rubbing of the peritoneum with severely traumatising surgical gauze (group III), mildly traumatising non-surgical textile (group VIII) or no rubbing (group IX).

The p-value 1 represents differences between group VII and VIII; p-value 2 represents differences between group VII and IX; p-value 3 represents differences between group VIII and IX.

DISCUSSION

Experimental and clinical studies suggest that surgical trauma promotes tumour cell adhesion and tumour growth.[15;19-22] The mechanism by which surgical trauma promotes these processes is not completely understood but is probably multi-factorial, because tumour cell adhesion as well as local and regional tumour growth can be enhanced. It seems that trauma leads to a process during which locally and regionally active tumour promoting agents are produced.[15;22] We recently demonstrated that surgical Medipres gauze was more traumatising to the peritoneum than non-surgical Fastsorb textile, leading to significantly more adhesion formation.[16] Our current data suggest that the factors responsible for the formation of post-surgical adhesions also play a role in the adhesion and growth of tumour cells to the peritoneum. The most impressive tumour growth was observed at sites where abrasion of the mesothelium was most severe. The degree of tumour growth at traumatised sites was highly correlated with the degree of trauma. Abrasion with surgical gauze produced the highest tumour load, whereas untouched peritoneum showed the lowest tumour burden and surfaces traumatised by non-surgical textile presented intermediate tumour growth. The finding that traumatised surfaces are privileged sites for tumour cells has been demonstrated before.[15;23;24]

It is conceivable that the process of enhanced tumour growth in traumatised tissue is biphasic. First, trauma of the peritoneum and the ensuing inflammatory response leads to upregulation of adhesion molecules, thus promoting the anchoring of tumour cells. Second, the subsequent healing of the peritoneum leads to growth promotion of the adhered tumour cells through the action of locally produced growth factors.

Using the same tumour model as in the present study, we recently demonstrated that the phenomenon of enhanced tumour growth as it relates to trauma and healing also occurs in the experimental settings. It was found that laparoscopic removal of a bowel segment led to less adherence of intra-peritoneal tumour cells than when conventional surgery was performed, again indicating that the degree of surgical trauma was proportional tot the extent of tumour growth.[19] In addition, we observed that the growth of a regenerating liver after partial hepatectomy led to a marked propagation of intra-hepatic tumour growth.[25;26]

Interestingly, our present results indicate that the sequelae of peritoneal trauma with regard to tumour growth are not confined to the inflicted site itself, but appear to have a generalised character. We showed that trauma led to more tumour at the traumatised site and also at non-traumatised peritoneum. Again, the amount of tumour at these

loco-regional sites correlated with the severity of the inflicted trauma. This clear correlation was also found in the experiment in which we studied the effect of peritoneal trauma on tumour growth under the renal capsule. Even in this ectopic tumour model, the consequences of the intra-abdominal trauma were demonstrable. Because promotion of adherence was irrelevant in this model, this experiment also revealed that trauma could evoke enhancement of the growth of an established tumour. Gutman *et al.*[27] made comparable observations, finding that a regenerating liver induced enhanced tumour growth not only in the liver but also at distant sites.

Our final experiment, in which we demonstrated that within a few hours after infliction of peritoneal trauma, the effects on tumour growth could be passively transferred to naive recipients, supports the notion that trauma *per se* has a marked effect, most likely on tumour cell adhesion.

Taken together, the current experiments suggest that both tumour cell adherence and growth are modified by surgical trauma. It is clear that the present model provides unique possibilities to unravel further the similarities and differences between the processes of adhesion formation and tumour cell adhesion and tumour growth. Variables such as kinetics of adhesion molecule expression with regard to inflammatory cytokines and growth factors and the role of mesothelial hyaluronic acid are currently being investigated. These studies may lead to sophisticated tools to prevent the unwanted side effects of surgery. The present study clearly indicates that these unwanted side effects can partly be omitted by the use of delicate surgery and non-abrasive gauze material.

REFERENCES

1. Brodsky JT, Cohen AM. Peritoneal seeding following potentially curative resection of colonic carcinoma: implications for adjuvant therapy. **Dis Colon Rectum** 1991; **34**: 723-727.
2. Long RT, Edwards RH. Implantation metastasis as a cause of local recurrence of colorectal carcinoma [see comments]. **Am J Surg** 1989; **157**: 194-201.
3. McDermott FT, Hughes ES, Pihl E, Johnson WR, Price AB. Local recurrence after potentially curative resection for rectal cancer in a series of 1008 patients. **Br J Surg** 1985; **72**: 34-37.
4. Rich T, Gunderson LL, Lew R, Galdibini JJ, Cohen AM, Donaldson G. Patterns of recurrence of rectal cancer after potentially curative surgery. **Cancer** 1983; **52**: 1317-1329.
5. Cunliffe WJ, Sugarbaker PH. Gastrointestinal malignancy: rationale for adjuvant therapy using early postoperative intraperitoneal chemotherapy. **Br J Surg** 1989; **76**: 1082-1090.
6. Eggermont AM, Steller EP, Marquet RL, Jeekel J, Sugarbaker PH. Local regional promotion of tumor growth after abdominal surgery is dominant over immunotherapy with interleukin-2 and lymphokine activated killer cells. **Cancer Detect Prev** 1988; **12**: 421-429.
7. Eggermont AM, Sugarbaker PH. Immunogenicity of the tumor determines the outcome of immunotherapy with interleukin-2, ABPP, and cyclophosphamide of micro- and macrometastatic intraperitoneal tumor. **Cancer Detect Prev** 1990; **14**: 483-490.
8. Hubens G, Willems G. Factors influencing the implantation of colon cancer cells on the colonic suture line in rats. **Eur Surg Res** 1990; **22**: 356-364.
9. Reinbach D, McGregor JR, O'Dwyer PJ. Effect of suture material on tumour cell adherence at sites of colonic injury [see comments]. **Br J Surg** 1993; **80**: 774-776.
10. Galandiuk S, Wieand HS, Moertel CG, et al. Patterns of recurrence after curative resection of carcinoma of the colon and rectum. **Surg Gynecol Obstet** 1992; **174**: 27-32.
11. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. **World J Surg** 1991; **15**: 609-616.
12. Fermor B, Umpleby HC, Lever JV, Symes MO, Williamson RC. Proliferative and metastatic potential of exfoliated colorectal cancer cells. **J Natl Cancer Inst** 1986; **76**: 347-349.
13. Umpleby HC, Fermor B, Symes MO, Williamson RC. Viability of exfoliated colorectal carcinoma cells. **Br J Surg** 1984; **71**: 659-663.
14. Fisher B, Fisher ER, Feduska N. Trauma and the localization of tumor cells. **Cancer** 1967; **20**: 23-30.

15. Eggermont AM, Steller EP, Sugarbaker PH. Laparotomy enhances intraperitoneal tumor growth and abrogates the antitumor effects of interleukin-2 and lymphokine-activated killer cells. **Surgery** 1987; **102**: 71-78.
16. van den Tol MP, van S, I, Bonthuis F, Marquet RL, Jeekel J. Reduction of intraperitoneal adhesion formation by use of non-abrasive gauze. **Br J Surg** 1997; **84**: 1410-1415.
17. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. **Int J Cancer** 1984; **33**: 689-692.
18. Steller EP. Enhancement and abrogation; modifications of host immune status influence IL-2 and LAK cell immunotherapy. 1988. (GENERIC) Ref Type: Thesis/Dissertation
19. Bouvy ND, Marquet RL, Hamming JF, Jeekel J, Bonjer HJ. Laparoscopic surgery in the rat. Beneficial effect on body weight and tumor take. **Surg Endosc** 1996; **10**: 490-494.
20. Busch OR, Hop WC, Hoynck vPM, Marquet RL, Jeekel J. Blood transfusions and prognosis in colorectal cancer [see comments]. **N Engl J Med** 1993; **328**: 1372-1376.
21. Ratajczak HV, Lange RW, Sothern RB, et al. Surgical influence on murine immunity and tumor growth: relationship of body temperature and hormones with splenocytes [published erratum appears in Proc Soc Exp Biol Med 1992 Jul;200(3):448-51]. **Proc Soc Exp Biol Med** 1992; **199**: 432-440.
22. Weese JL, Ottery FD, Emoto SE. Do operations facilitate tumor growth? An experimental model in rats. **Surgery** 1986; **100**: 273-277.
23. Lee JY, Murphy SM, Scanlon EF. Effect of trauma on implantation of metastatic tumor in bone in mice. **J Surg Oncol** 1994; **56**: 178-184.
24. Murthy SM, Goldschmidt RA, Rao LN, Ammirati M, Buchmann T, Scanlon EF. The influence of surgical trauma on experimental metastasis. **Cancer** 1989; **64**: 2035-2044.
25. de Jong KP, Lont HE, Bijma AM, et al. The effect of partial hepatectomy on tumor growth in rats: in vivo and in vitro studies. **Hepatology** 1995; **22**: 1263-1272.
26. Slooter GD, Marquet RL, Jeekel J, Ijzermans JN. Tumour growth stimulation after partial hepatectomy can be reduced by treatment with tumour necrosis factor alpha. **Br J Surg** 1995; **82**: 129-132.
27. Gutman M, Fidler IJ. Biology of human colon cancer metastasis. **World J Surg** 1995; **19**: 226-234.

Chapter IV

THE INFLAMMATORY SEQUELAE OF SURGERY PROVOKE ENHANCED TUMOUR RECURRENCE; A CRUCIAL ROLE FOR NEUTROPHILS AND CYTOKINES

Submitted for publication

In the previous chapter we demonstrated that within a few hours after peritoneal trauma, surgery related factors in the abdominal cavity could be captivated in a lavage fluid and enhance peritoneal tumour recurrence in naive recipients.

Using the same experimental model, in this chapter, the collected post trauma lavage fluid was separated in a cellular and supernatant component, the latter containing soluble factors. Intra-peritoneal injection of naive recipients with both components of the collected lavage fluid, without inflicting additional surgical trauma, resulted in statistically significant more tumour recurrence. The cellular component produced the highest tumour load.

Analysis of the lavage fluid, gathered immediately after laparotomy (mild trauma) or five hours after laparotomy combined with additional peritoneal trauma (severe trauma), demonstrated a significant influx of neutrophils after severe trauma. The acute phase cytokines IL-1β, IL-6 and TNF-α were present irrespective of trauma-intensity. Statistically significant more IGF-I was detected in the severely traumatised rats.

It seems in vivo tumour recurrence is mainly promoted by the cellular component of the post-surgical inflammatory process. Cytokines also enhance in vivo recurrence, but play an inferior role. IGF-I may facilitate the growth of lodged tumour cells.

INTRODUCTION

Surgical treatment of gastro-intestinal malignancies is often complicated by loco-regional recurrence.[1] Regardless this detrimental adversity, surgery remains the best treatment option. In a clinical trial, Busch *et al* put forward an association between recurrent disease 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]

In the previous chapter we demonstrated that within a few hours after infliction of peritoneal trauma, surgically related factors in the abdominal cavity could be captivated in a lavage fluid and enhance tumour recurrence in naive, non-operated recipients.[7]

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 traumatised site is mediated through chemotactic factors like IL-8 and pro-inflammatory cytokines such as IL-1β or TNF-α and is the first line of defence.[8-10]

Insights in the host defence mechanisms of the peritoneum have demonstrated that peritoneal lymphocytes, sub-mesothelial 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.[11;12]

Inflammatory products produced after abdominal surgery might participate directly or indirectly in effective tumour recurrence. In this study, we focus our attention on the individual capacity of inflammatory peritoneal cells and soluble factors to ascertain which element is responsible for enhanced tumour recurrence. In an experimental model post surgically produced tumour enhancing factors are captivated in a lavage fluid, and passively transferred to naive recipients.

The *in vivo* and *in vitro* studies presented in this paper investigate a possible correlation between the inflammatory cells present in the abdominal cavity after surgical trauma and the cell free soluble factors on tumour cell adhesion and growth.

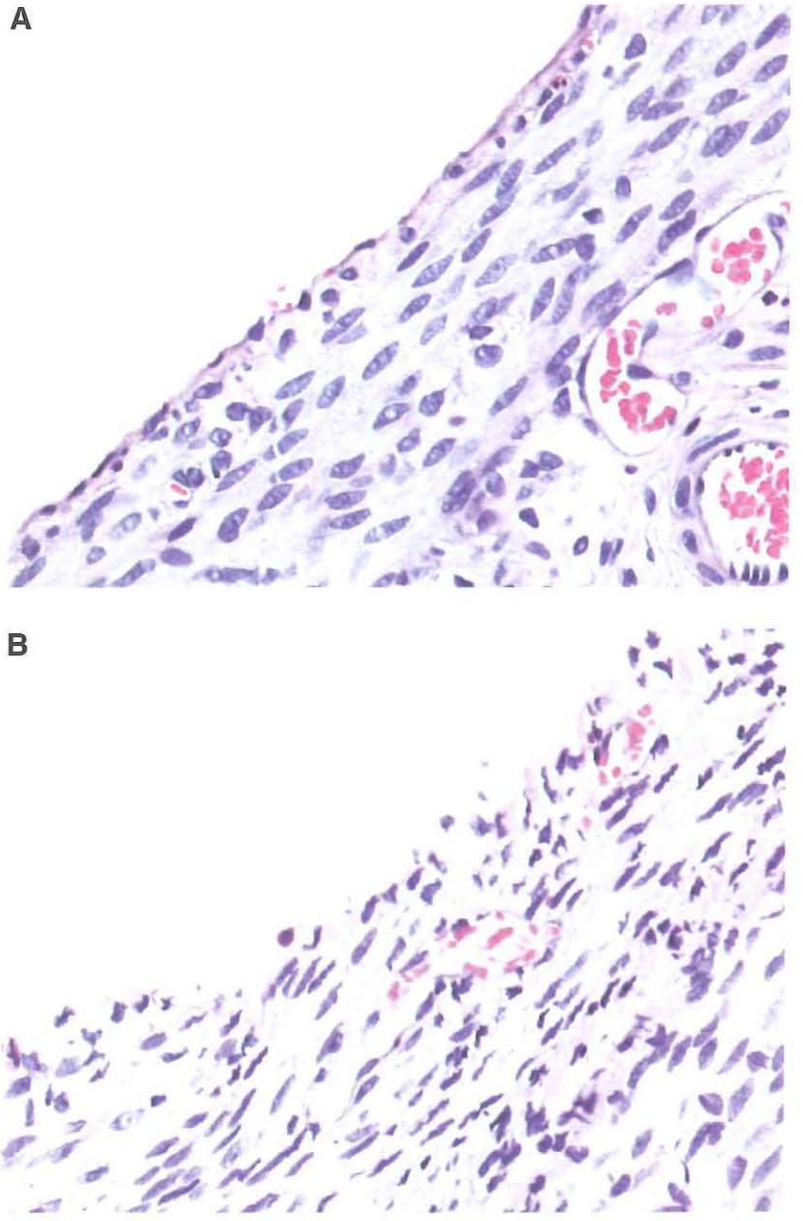

Figure 4.1

Surface of the uterine horn before (1A) and after (1B) standardised rubbing with surgical gauze. The peritoneum has been traumatised by exfoliation of the covering mesothelial cells and disruption of the underlying layers. Magnification 40x.

MATERIALS AND METHODS

Animals

Female inbred rats of the WAG strain were obtained from Harlan-CPB, Zeist, The Netherlands. The rats were bred under specific pathogen free conditions, kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 hours light/ 12 hours dark), fed with laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee in Animal Research of the Erasmus University Rotterdam.

Tumour cell line

CC531 is a moderately differentiated, weakly immunogenic colon adenocarcinoma induced in the WAG/Rij rat by 1,2-dimethylhydrazine. A cell line was established from this carcinoma and maintained by serial passage after trypsinization in culture medium.[13] CC531 tumour cells were cultured in RPMI 1640 medium supplemented with 5% foetal calf serum, L-glutamin (2 mM) and penicillin (10^5 U/L). Medium and all supplements were obtained from Life Technologicals BV, Breda, The Netherlands. Cells were passaged once a week using trypsin (0.05%) and EDTA (0.02%). Viability was measured by trypan blue exclusion and always exceeded 90 percent.

IN VIVO EXPERIMENTS

Gathering of lavage fluid for passive transfer

Under ether anaesthesia, of 14 rats the abdomen was shaved and cleaned with alcohol 70%. Laparotomy was performed using a midline incision; exposure and rubbing of the uterus horns and a 5-cm long part of the small intestine with surgical Medipres gauze inflicted subsequent trauma to the peritoneum. Rubbing was performed with a device enabling the application of a constant pressure of 120 g/cm^2.[7] In this way, a standardised amount of peritoneal trauma can be inflicted. Figure 4.1 shows the extent of peritoneal damage after gauze rubbing. The abdomen was closed in one layer with silk 2-0 sutures (Braun, The Netherlands). After 5 hours, a second laparotomy was performed during which the abdominal cavity was lavaged with 5 ml RPMI 1640 medium. After massaging the abdomen the remaining fluid was aspirated, pooled and kept on ice until further processing.

Experimental design passive transfer

The collected post trauma lavage fluid was centrifuged, the cell pellet resuspended to original volume with RPMI and thus divided into a "supernatant" containing soluble components produced after surgical trauma and a "cellular" component containing the different cell types present in the abdominal cavity after surgical trauma.

Subsequently 24 rats were divided in three groups. Group I served as control group receiving RPMI 1640 medium. Group II was acceptor for the supernatant of the post trauma lavage fluid and group III was acceptor for the cellular component. Of all three components, 3 ml was injected intraperitoneally together with 0.5 million CC531 cells (in 0.5 ml RPMI) without opening the abdominal cavity. In this way, the factors contained by the different components represented the mediators after surgical abdominal trauma, without inflicting additional trauma.

Tumour score

Tumour scoring took place three weeks after tumour injection. The rats were sacrificed and intraperitoneal tumour take was scored at the parietal peritoneum, omentum, liver, kidneys, retro peritoneum and mesentery. The scoring ranged from 0 to 5 per site according to the peritoneal cancer index as described by Steller. A score of 0 meant there was no tumour growth, a score of 1 indicated an estimated tumour diameter of less than 0.5 cm, a score of 2 a tumour diameter between 0.5 and 1 cm, a score of 3 a tumour diameter between 1 and 2 cm, a score of 4 a tumour diameter between 2 and 3 cm, and a score of 5 an estimated diameter of more than 3 cm.[14] For each rat the scores were summarised and defined as total tumour load.

Gathering of lavage fluid for ELISA and cell differentiation

Additional lavage fluid was collected directly after performing a midline laparotomy (mild trauma, n=10) and 5 hours after standardised trauma by gauze rubbing (severe trauma, n=14). The collected lavage fluid samples were kept individually on ice and were separated in a supernatant and cellular component. The supernatants were filtered over a low binding 0.45 μm filter and stored at –80°C for further analysis. The presence of the acute phase cytokines IL-1β, IL-6 and TNF-α in addition to the growth factor IGF-I were determined by ELISA (Biomedical Diagnostics, Brugge, Belgium) and RIA (Biosource Europe, Fleurus, Belgium).

The cellular component was resuspended in RPMI medium, total cell amount was determined and HE stained cytocentrifuge slides were made for cell differentiation. Under a light microscope at a magnification of 100x, 100 cells were counted and classified into granulocytes (eosinophils, neutrophils and mast cells) and lymphoid cells (mononuclear phagocytes and lymphocytes). Cell classification was done on duplicate slides.

IN VITRO EXPERIMENTS

Mesothelial cell culture

Mesothelial cells (MC) were isolated from the small bowel mesentery of rats as described before.[15] Mesothelial monolayers were established in 96 well plates (Packard, The Netherlands) precoated with collagen type I (15 μg/cm^2, Boehringer Mannheim, Mannheim, Germany). The

plates were incubated at 37°C, in a humidified atmosphere of 5% CO_2 in air. Medium consisted of RPMI enriched with 10% FCS, glutamin (2 mM), penicillin (10^5 U/L) and fungizone (1.25 mg/L), and was replaced daily with fresh medium. Monolayers reached confluence in 2 days as determined by microscopic evaluation.

Tumour cell labelling

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 and adding this solution to 5 ml of RPMI medium supplemented with 0.5% bovine serum albumin (RPMI/0.5%BSA). Trypsinized CC531 cells ($1*10^6$ cells/ml) were incubated in this solution at 37°C for 45 minutes with occasional mixing. Before adding to the mesothelial monolayers, the labelled cells were washed twice with RPMI/0.5%BSA to remove free dye.

Tumour cell adhesion assay

To demonstrate an effect of surgery related soluble factors on tumour cell adhesion to the mesothelial cells of the peritoneum, a standardised tumour cell adhesion model was used.[16] After confluence, overnight pre-incubation of the mesothelial monolayers took place with 200 µl of the collected supernatant samples or RPMI medium enriched with 10% FCS. Non pre-incubated monolayers in mesothelial cell culture medium served as standardised control.

Medium was removed from the monolayers and 200 µl RPMI/0.5%BSA containing 30,000 calcein labelled tumour cells was added. Plates were centrifuged for 1 minute at 80 g on a Heraeus centrifuge and incubated for 1 hour at 37°C to allow cell adhesion. After this, the medium of each well was removed and washed twice with 200 µl RPMI/0.5%BSA. Fluorescence of adherent cells was measured on a Perkin Elmer plate reader using 485 excitation and 530 emission filters. On each plate a standard curve 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

In case of more than two groups, statistical analysis was performed using the non-parametric Kruskall Wallis analysis of variance to determine overall differences. Differences between groups were analysed using the non-parametric Mann Whitney *U*-test.

RESULTS

Effect of post trauma lavage fluid on peritoneal tumour load

After intra peritoneal injection of the lavage fluid samples collected after surgical trauma, diffuse peritoneal tumour load was found in all groups. However, injection with the cellular component of the lavage fluid resulted in the highest amount of recurrence. When compared to the control group (RPMI) both the supernatant and cellular factors caused significantly enhanced tumour recurrence. Injection of tumour cells with RPMI alone resulted in a total of 31% tumour load whereas injection with the supernatant or cells resulted in 67% and 79% total tumour load (figure 4.2).

Impressive differences in peritoneal tumour deposits were observed between the groups receiving the supernatant and cellular part of the lavage fluid. When taking the tumour size into account an obvious shift towards larger tumours is evident in the latter group. Whereas no tumours larger than 2 cm are found in the control or supernatant group, 38% of the cellular group was scored in this range (figure 4.3). Table 4.1 shows the distribution of tumour deposits at the different peritoneal sites. Statistically significant differences in tumour load were scored in omentum, liver, mesentery and retroperitoneum between the supernatant and cellular fraction.

FACS analysis of the cellular solution showed 75% PMN, 10% lymphocytes and 5% macrophages in the cell differentiation (data not shown). Therefore, additional lavage fluid was collected to investigate what effect surgical trauma has on the cellular distribution of inflammatory cells in the abdominal cavity.

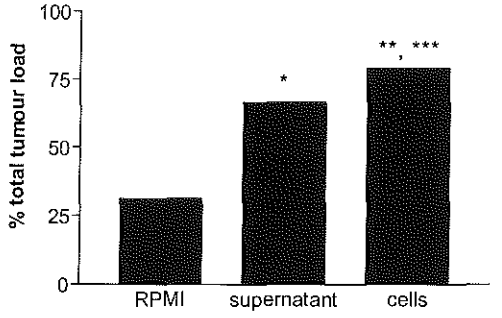

Figure 4.2

Differences in peritoneal tumour load after passive transfer of RPMI only, supernatant fraction or cellular fraction of lavage fluid. * p=0.01 and ** p=0.001 *versus* RPMI; *** p=0.003 *versus* supernatant. The percentage total tumour load was defined after scoring the presence or absence of a tumour irrespective of tumour size.

	I RPMI	II Supernatant	III Cells	p-value		
				p1	p2	p3
Omentum	0 (0-2)	3 (0-5)	4.5 (4-5)	0.004	0.000	0.005
Liver	1 (0-1)	2.5 (0-3)	4 (2-5)	0.01	0.001	0.01
Kidney	0 (0-3)	1 (0-2)	2.5 (0-4)	ns	ns	ns
Parietal peritoneum	0 (0-0)	0 (0-0)	0 (0-0)	ns	ns	ns
Mesentery	0 (0-2)	0.5 (0-2)	2 (1-3)	ns	0.007	0.03
Retroperitoneum	0 (0-2)	2.5 (0-3)	3.5 (3-5)	0.007	0.000	0.007
Total	**0 (0-3)**	**1 (0-5)**	**3 (0-5)**	**0.01**	**0.001**	**0.003**

Table 4.1

Median tumour load and range at different abdominal sites in rats having been injected with RPMI medium (group I), supernatant fraction of lavage fluid (group II) and the cellular fraction of lavage fluid (group III). The p-value 1 represents differences between group I and II; p-value 2 represents differences between group I and III; p-value 3 represents differences between group II and III.

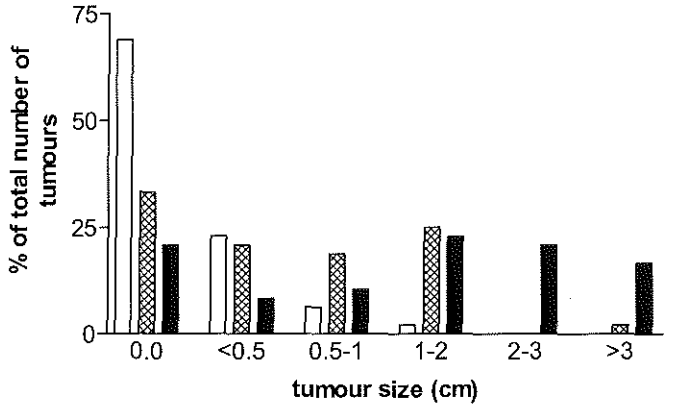

Figure 4.3

Comparison of tumour size after passive transfer of RPMI only, supernatant fraction or cellular fraction of lavage fluid. Open bars represent RPMI, cross hatched bars the soluble fraction, filled bars the cellular fraction.

Effect of surgical trauma on peritoneal cell compilation

There was no change in total cell amount between the mildly (laparotomy only) and severely (laparotomy and rubbing) traumatised rats (figure 4.4). In the cell differentiation however, a reversal was seen regarding the ratio of lymphoid cells and granulocytes, with a 22-78% ratio of granulocytes-lymphoid cells in the mildly traumatised group *versus* an 78-22% ratio in the severely traumatised group (figure 4.5, $p<0.0001$).

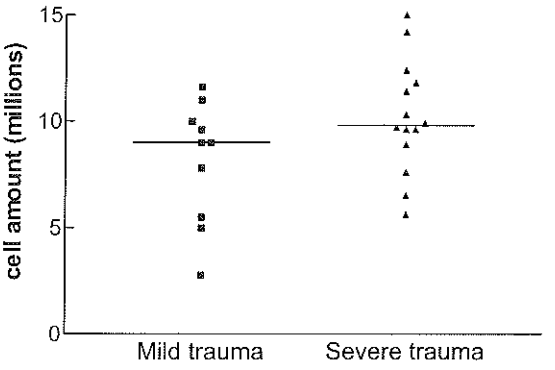

Figure 4.4

Differences in total cell amount between mildly and severely traumatised rats. Median and range are shown; mild trauma 9.0 (2.8-11.6) and severe trauma 9.8 (5.6-15.0), ns.

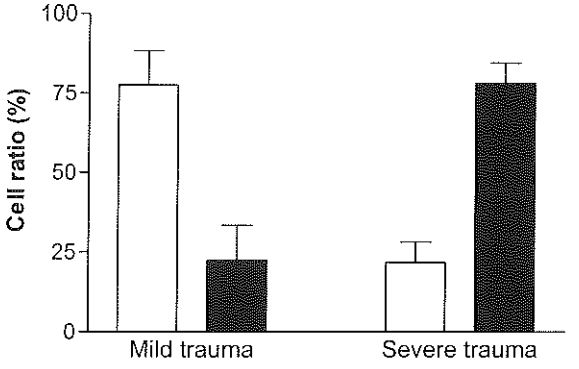

Figure 4.5

Differentiation of cellular fraction of lavage fluid taken from mildly and severely traumatised rats. Open bars represent lymphoid cells, filled bars granulocytes. There was a significant shift towards granulocyte ratio after severe trauma from 22% to 78% ($p=0.0001$).

Effect of surgical trauma on cytokine and growth factor production

IL-1β was detected in 7 out of 14 lavage fluid samples of the severely traumatised rats ranging from 20 to 72 pg/ml and 2 out of 10 of the mildly traumatised rats (37 and 69 pg/ml). IL-6 was present in 13 samples of the severely traumatised (range 54-848 pg/ml) and all samples of the mildly traumatised groups (range 72-1194 pg/ml), TNF-α in 2 and 5 samples of the mildly and severely traumatised rats (67 and 170 pg/ml and range 35-110 pg/ml) respectively. IGF-I was present in all samples, however statistically significant more IGF-I was detected in lavage fluid from the severely traumatised rats (figure 4.6, $p<0.0001$).

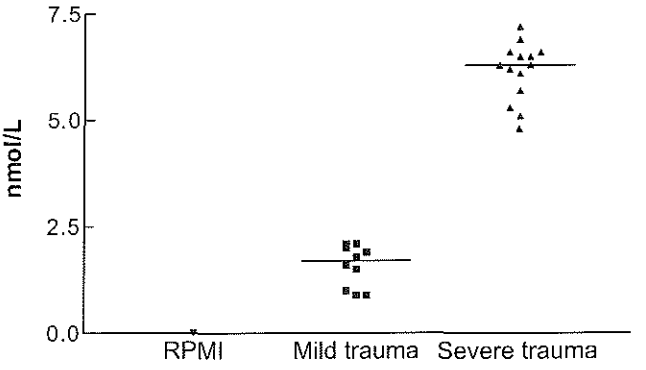

Figure 4.6

Median and range of IGF-I concentration in supernatant fraction of lavage fluid. Statistically significant more IGF-I was found in severely traumatised rats, $p<0.0001$.

Mild trauma: median (range) 1.7 (0.9-2.1). Severe trauma: median (range) 6.3 (4.8-7.2). No IGF-I was detected in RPMI.

Tumour cell adhesion assay

Figure 4.7 shows the percentage of tumour cell adhesion *versus* the standardised control of the adhesion assay, consisting of mesothelial cell culture medium. Control cell adhesion consisted of 23% from the total amount of cells added (data not shown). RPMI did not affect tumour cell adhesion, nor did lavage fluid samples from both surgically traumatised groups.

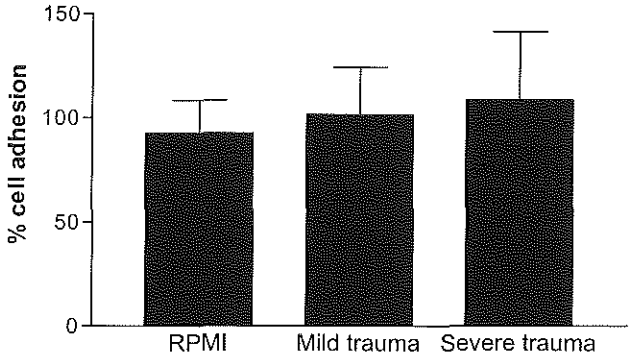

Figure 4.7

Percentage tumour cell adhesion *versus* control after pre-incubation of mesothelial cells with lavage fluid gathered after different intensities of surgical trauma. Mean values are shown +SD (n=6 per fluid sample). There was no difference in cell adhesion between groups.

DISCUSSION

The success of surgical treatment in patients with gastro intestinal cancer is often limited due to local recurrence or peritoneal carcinosis by peroperatively seeded tumour cells.[1] In this study, a cell seeding model was used to mimic the clinical situation of free intra peritoneal tumour cells and associates the combination of cell adhesion and growth ultimately leading to manifest tumour recurrence. The presented results give evidence that the inflammatory sequelae of surgery enhance peritoneal tumour recurrence. Using the same experimental rat model, we demonstrated in chapter 3 that components produced after surgical abdominal trauma, combined in a lavage fluid, could enhance tumour recurrence in naive recipients.[7] The current study demonstrates that components of the lavage fluid collected after inflicting surgical

trauma i.e. inflammatory cells and soluble factors, each lead to enhanced tumour recurrence.

The cellular fraction however, led to the greatest tumour load manifested by large tumours. More detailed analysis of the cellular fraction reveals a trauma related influx of PMN into the abdominal cavity. Similar shifts in cell differentiation have also been demonstrated in other animal models following peritoneal trauma.[9;10;17] The shift of primarily monocytes to PMN will evidently affect the homeostatic milieu of the peritoneum. PMN generate reactive oxygen metabolites and discharge contents of granular organelles into either phagocytic vacuoles or the local environment to ingest foreign particles or microorganisms. Both oxygen-dependent and oxygen-independent processes participate in the killing of bacteria and also in damage to host tissue.[10;18;19] *In vitro*, increased adhesion of activated PMN to a mesothelial monolayer has been shown to induce retraction, gap formation and detachment ending with substantial mesothelial cell injury.[20] Mesothelial cell injury leads to exposure of the underlying extra cellular matrix components. Experimental studies have demonstrated a preferential adhesion of tumour cells to these denuded areas.[21] Effective inhibition of tissue injury by PMN has been achieved by blocking of cell adhesion molecules used to enter the inflamed tissue[20;22] and scavenging of reactive oxygen species.[23;24]

A relation has been demonstrated between the extent of tissue trauma and tumour recurrence. In order to diminish tissue trauma and tumour recurrence, minimal invasive surgery is promoted.[3;7;21] Diminished tumour recurrence after laparoscopic surgery as compared to conventional surgery has been attributed to this phenomenon.[3;21] In addition, laparoscopic surgery appears to impact on the cellular components of the immune response less than laparotomy.[5;25] Carbon dioxide pneumoperitoneum has been shown to impair peritoneal macrophage cytokine production (Il-1, TNF-α) and coincides with diminished neutrophil superoxide anion release and chemotaxis.[26]

In a peritonitis model, Pruimboom *et al* also showed a non-significant increase in cells in the peritoneal cavity. In the peritoneal cell compilation a pronounced increase of PMN was seen on the first day followed by an influx of macrophages from day 1 until seven days after induction of peritonitis. PMN produced inflammatory mediators, however the capacity of these cells was very low in comparison with peritoneal macrophages.[9;10] In this study, the supernatant fraction of the lavage fluid, containing inflammatory factors produced after surgical trauma also enhanced tumour recurrence

in vivo. *In vitro*, no difference was seen in cell adhesion between lavage fluid taken from differently traumatised rats *versus* the control.

The presence of the acute phase cytokines IL-1β, IL-6 and TNF-α was detected in the fluid although no significant differences in concentration were observed between mild and severely traumatised rats. IL-1β and TNF-α have been shown to upregulate cell adhesion molecules on mesothelial cells, which are used by PMN for adhesion.[20] In addition, cytokine activated mesothelial cells produce chemoattractant cytokines such as IL-8, required for PMN recruitment.[12;27] 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. However, when omitting inflammatory cells, as done *in vitro*, the sole effect of cytokines present in the peritoneal fluid does not affect cell adhesion. The *in vivo* effect may therefore be indirect, based on the additional trauma inflicted by inflammatory cells. High concentrations of IL-1β and TNF-α have been demonstrated to upregulate adhesion molecule expression and therefore enhance the possibility of cell adhesion.[12;27] Taking this into account a direct cytokine effect may occur locally, at the site where cytokines are produced. In this study this effect may be missed as the absolute concentration of cytokines is diluted by lavaging with excess fluid.

Growth factors play a vital role in post surgical wound healing. Insulin like growth factor-I (IGF-I) is released during the first stage of wound healing.[28] The *in vitro* experiments described in chapters 7 and 10 demonstrate that IGF-I does not affect tumour cell adhesion but is a potent growth stimulant for tumour and mesothelial cells. Clinical and experimental studies have demonstrated that surgery is followed by a rapid decrease of IGF-I in serum.[29;30] This apparent suppression of the IGF-I system is thought to be caused by an increased efflux of IGF-I from the blood to the peripheral tissues.[29] In this study, significantly higher IGF-I concentrations were found in the abdominal lavage fluids from severely traumatised rats. The origin of IGF-I could be the proposed efflux from the blood or could be the result of local production by mesothelial cells.[15] IGF-I stimulation and over-production has been shown in several types of carcinoma and may support an autocrine growth loop of the tumour cells. IGF-I is also potently capable of priming PMN for an enhanced respiratory burst that may lead to additional peritoneal tissue damage.[31] In this way tumour cells can use the hosts immune response in wound healing for their own benefit.

In conclusion, the studies brought forward in this paper give evidence that the inflammatory sequelae after surgery promote tumour recurrence and that this effect is mainly based on the cellular component of the inflammatory process. Extrapolation of the presented results to the clinical situation is limited as the inflammatory response in animals deviates from humans. There is however limited species variation in the first line of defence after surgical trauma when taking PMN influx and macrophage activation into account.[10] 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. Tackling of reactive oxygen products however seems a feasible way of preventing cellular tissue damage ultimately resulting in diminished tumour recurrence.

REFERENCES

1. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. **World J Surg** 1991; **15**: 609-616.
2. Busch OR, Hop WC, Hoynck vPM, Marquet RL, Jeekel J. Blood transfusions and prognosis in colorectal cancer [see comments]. **N Engl J Med** 1993; **328**: 1372-1376.
3. Bouvy ND, Marquet RL, Jeekel J, Bonjer HJ. Laparoscopic surgery is associated with less tumour growth stimulation than conventional surgery: an experimental study [see comments]. **Br J Surg** 1997; **84**: 358-361.
4. Eggermont AM, Steller EP, Sugarbaker PH. Laparotomy enhances intraperitoneal tumor growth and abrogates the antitumor effects of interleukin-2 and lymphokine-activated killer cells. **Surgery** 1987; **102**: 71-78.
5. Jacobi CA, Ordemann J, Zieren HU, et al. Increased systemic inflammation after laparotomy vs laparoscopy in an animal model of peritonitis. **Arch Surg** 1998; **133**: 258-262.
6. Lee YT. Local and regional recurrence of carcinoma of the colon and rectum: II. Factors relating to operative technique. **Surg Oncol** 1996; **5**: 1-13.
7. van den Tol PM, van Rossen EE, van Eijck CH, Bonthuis F, Marquet RL, Jeekel H. Reduction of peritoneal trauma by using nonsurgical gauze leads to less implantation metastasis of spilled tumor cells. **Ann Surg** 1998; **227**: 242-248.
8. Williams MA, Cave CM, Quaid G, Solomkin JS. Chemokine regulation of neutrophil function in surgical inflammation. **Arch Surg** 1999; **134**: 1360-1366.
9. Pruimboom W, Verdoold A, Tak C, et al. Changes in eicosanoid and tumor necrosis factor a production by rat peritoneal macrophages during carrageenin-induced peritonitis. **Mediators Inflamm** 1994; **3**: 335-340.
10. Styrt B. Species variation in neutrophil biochemistry and function. **J Leukoc Biol** 1989; **46**: 63-74.
11. Douvdevani A, Rapoport J, Konforty A, Argov S, Ovnat A, Chaimovitz C. Human peritoneal mesothelial cells synthesize IL-1 alpha and beta. **Kidney Int** 1994; **46**: 993-1001.
12. Topley N, Williams JD. Effect of peritoneal dialysis on cytokine production by peritoneal cells. **Blood Purif** 1996; **14**: 188-197.
13. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. **Int J Cancer** 1984; **33**: 689-692.
14. Steller EP. Enhancement and abrogation; modifications of host immune status influence IL-2 and LAK cell immunotherapy. 1988. (GENERIC) Ref Type: Thesis/Dissertation

15. van der Wal BC, Hofland LJ, Marquet RL, van Koetsveld PM, van Rossen ME, van Eijck CH. Paracrine interactions between mesothelial and colon-carcinoma cells in a rat model. **Int J Cancer** 1997; **73**: 885-890.
16. Catterall JB, Gardner MJ, Jones LM, Thompson GA, Turner GA. A precise, rapid and sensitive in vitro assay to measure the adhesion of ovarian tumour cells to peritoneal mesothelial cells. **Cancer Lett** 1994; **87**: 199-203.
17. Ho DPM, Krediet RT. Inflammatory changes in vivo during CAPD: what can the effluent tell us? **Kidney Int Suppl** 1996; **56**: S12-S16
18. Heinzelmann M, Simmen HP, Battaglia H, Friedl HP, Trentz O. Inflammatory response after abdominal trauma, infection, or intestinal obstruction measured by oxygen radical production in peritoneal fluid. **Am J Surg** 1997; **174**: 445-447.
19. Weiss SJ. Tissue destruction by neutrophils [see comments]. **N Engl J Med** 1989; **320**: 365-376.
20. Andreoli SP, Mallett C, Williams K, McAteer JA, Rothlein R, Doerschuk CM. Mechanisms of polymorphonuclear leukocyte mediated peritoneal mesothelial cell injury. **Kidney Int** 1994; **46**: 1100-1109.
21. Bouvy ND, Marquet RL, Jeekel H, Bonjer HJ. Impact of gas(less) laparoscopy and laparotomy on peritoneal tumor growth and abdominal wall metastases [see comments]. **Ann Surg** 1996; **224**: 694-700.
22. Walzog B, Scharffetter-Kochanek K, Gaehtgens P. Impairment of neutrophil emigration in CD18-null mice. **Am J Physiol** 1999; **276**: G1125-G1130
23. Shandall AA, Williams GT, Hallett MB, Young HL. Colonic healing: a role for polymorphonuclear leucocytes and oxygen radical production. **Br J Surg** 1986; **73**: 225-228.
24. Wang P, Chen H, Qin H, et al. Overexpression of human copper, zinc-superoxide dismutase (SOD1) prevents postischemic injury. **Proc Natl Acad Sci U S A** 1998; **95**: 4556-4560.
25. Griffith JP, Everitt NJ, Lancaster F, et al. Influence of laparoscopic and conventional cholecystectomy upon cell- mediated immunity. **Br J Surg** 1995; **82**: 677-680.
26. Vittimberga FJJ, Foley DP, Meyers WC, Callery MP. Laparoscopic surgery and the systemic immune response. **Ann Surg** 1998; **227**: 326-334.
27. Meager A. Cytokine regulation of cellular adhesion molecule expression in inflammation. **Cytokine Growth Factor Rev** 1999; **10**: 27-39.
28. Hofer SO, Molema G, Hermens RA, Wanebo HJ, Reichner JS, Hoekstra HJ. The effect of surgical wounding on tumour development. **Eur J Surg Oncol** 1999; **25**: 231-243.

29. Bouvy ND, Marquet RL, Tseng LN, et al. Laparoscopic vs conventional bowel resection in the rat. Earlier restoration of serum insulin-like growth factor 1 levels. **Surg Endosc** 1998; **12**: 412-415.

30. Skjaerbaek C, Frystyk J, Orskov H, et al. Differential changes in free and total insulin-like growth factor I after major, elective abdominal surgery: the possible role of insulin- like growth factor-binding protein-3 proteolysis. **J Clin Endocrinol Metab** 1998; **83** : 2445-2449.

31. Fu YK, Arkins S, Wang BS, Kelley KW. A novel role of growth hormone and insulin-like growth factor-I. Priming neutrophils for superoxide anion secretion. **J Immunol** 1991; **146**: 1602-1608.

Part III

Intra-Abdominal Blood Loss and Peritoneal Tumour Recurrence *in vivo*

Chapter V

RED BLOOD CELLS INHIBIT TUMOUR CELL ADHESION TO THE PERITONEUM

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Operative skills seem to be a relevant factor in tumour prognosis. Excessive per-operative blood loss followed by transfusion has been associated with increased tumour recurrence and poor prognosis. Particularly blood loss in the peritoneal cavity might be a tumour promoting factor when local recurrence is concerned. The aim of the present studies was to investigate whether the presence of blood in the peritoneal cavity affects local tumour recurrence.

Contrary to our hypothesis, intra-peritoneal (ip) injection of blood or red blood cell (RBC) concentrate prevented recurrence of per-operatively spilled tumour cells in vivo. This tumour inhibitive effect only took place if blood was injected in the peritoneal cavity within 24 hours after surgical trauma (laparatomy). RBC concentrate and intra RBC substances had a comparable inhibitive effect on tumour recurrence. Additional in vitro studies demonstrated that RBC inhibit tumour cell adhesion but do not affect tumour growth.

The presence of blood in the peritoneal cavity after surgical peritoneal trauma leads to reduced adhesion of per-operatively spilled tumour cells. The tumour inhibitive effect by blood takes place within 24 hours after surgical trauma and is mediated by intra-RBC substances.

INTRODUCTION

Peroperative blood transfusion, independent of its type -allogeneic or autologous- is associated with enhanced tumour recurrence and poor prognosis in patients with colorectal cancer. This was concluded in more than one clinical prospective randomised trial.[1-3]

In the study by Busch *et al*, the association between recurrent disease and blood transfusion appeared to exist only for the incidence of local recurrence and not for the incidence of distant metastases. Furthermore it was concluded that not so much the transfusion itself but the circumstances necessitating transfusion are the real determinants for prognosis.[1] Conditions leading to blood transfusion are the location and extent of the primary tumour, skill of the surgeon and length of operation. Blood loss in the per-operative period however, is the most common reason for transfusion.

The effect of blood loss on tumour recurrence has been studied extensively in experimental animal models. Studies performed in our laboratory demonstrated that blood loss with or without subsequent administration of blood transfusion had no significant effect on the formation of tumour colonies. However, blood loss promoted growth of established tumour metastases, irrespective of whether transfusion was given or not.[4] This tumour growth promoting effect could be prevented by an immediate plasma transfusion, but not by evoking a normal haemoglobin level after blood loss by pre-treatment with erythropoietin. In addition, NK activity was shown to be significantly depressed as a consequence of blood loss indicating that modulation of the immune response could play a role.[5] When different types of tumour cell lines were employed in the same experimental model, transfusion had both an inhibitory and stimulating effect on tumour growth and metastases, depending on the cell line. The authors concluded that several mechanisms, not only immunological, may be responsible.[6]

Based on the clinical observation that conditions leading to transfusion are the real determinants for prognosis, as well as the experimental results indicating that blood loss itself is an important factor influencing tumour growth, we hypothesised that local blood loss, i.e. in the peritoneal cavity, might be an overlooked factor influencing local tumour recurrence. This hypothesis was evaluated in a rat model by investigating the effect of blood and blood components on tumour cell adhesion and growth *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals

Inbred rats of the WAG strain were obtained from Harlan-CPB, Zeist, The Netherlands. The rats were bred under specific pathogen free conditions, kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 hours light/ 12 hours dark), fed with laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee in Animal Research of the Erasmus University Rotterdam.

Tumour cell line

CC531 is a moderately differentiated, weakly immunogenic colon adenocarcinoma induced in the WAG/Rij rat by 1,2-dimethylhydrazine. A cell line was established from this carcinoma and maintained by serial passage after trypsinization in culture medium.[7] CC531 tumour cells were cultured in RPMI 1640 medium supplemented with 5% foetal calf serum, L-glutamin (200 mmol/L), penicillin (5000 U/ml) and streptomycin (5000 U/ml). All supplements were obtained from Gibco, Paisley, UK. Before use, cells were trypsinized (5 minutes at 37°C), centrifuged (5 minutes at 700 g), resuspended in RPMI 1640 and counted. Viability was measured by trypan blue exclusion and always exceeded 90 percent.

Blood products

Syngeneic WAG rats were exsanguinated through heart punction. The blood was collected in a previously heparinized vial (heparin Leo, Leo Pharmaceutical products, Weesp, The Netherlands). RBC concentrate was obtained by centrifugation of blood at 3000 rpm during ten minutes (Mistral 2000i). The buffy coat and plasma were removed and the pelleted RBC resuspended in RPMI medium to original volume. Using this suspension, the RBC were lysed by freezing in liquid nitrogen. The resulting homogenate was divided in 1.5 ml aliquots and centrifuged at 10,000 rpm for 15 minutes to pellet cell fractions. The supernatants were used to determine the effect of RBC content on tumour load.

IN VIVO EXPERIMENTS

Operative procedure and tumour scoring

Under ether anaesthesia the abdomen of rats was shaved and cleaned with alcohol 70%. Laparotomy was performed using a midline incision followed by peritoneal inoculation of one million CC531 tumour cells. The abdomen was closed in one layer with silk 2-0 sutures (Braun, The Netherlands).

Three weeks postoperatively the rats were sacrificed and intra-peritoneal tumour load was scored at the following sites: sub-cutaneously (at the site of the incision), omentum, liver, kidneys, retroperitoneum and mesentery. The scoring ranged from 0 to 5 per site according to the peritoneal cancer index as described by Steller.[8] A score of 0 meant there was no tumour growth, a score of 1 indicated an estimated tumour diameter of less than 0.5 cm, a score of 2 a tumour diameter between 0.5 and 1 cm, a score of 3 a tumour diameter between 1 and 2 cm, a score of 4 a tumour diameter between 2 and 3 cm, and a score of 5 an estimated diameter of more than 3 cm. For each rat the scores were summarised and defined as total tumour load.

Experimental design

In the first experiment (I) the effect of syngeneic whole blood was evaluated on intra-peritoneal tumour load. After laparotomy, the experimental group (n=10) received 1.5 ml whole blood together with 1 million CC531 tumour cells (this amount was used for all experiments). The control group (n=10) received 1.5 ml PBS.

To distinguish between impairment of tumour cell adhesion or tumour growth, an additional experiment (II) was performed in which tumour cells were allowed to adhere before performing a re-laparotomy after 24 hours and adding 1.5 ml whole blood. The control group received 1 ml PBS. Both groups consisted of 8 rats.

In the third experiment (III) the effect of 1.5 ml RBC concentrate on tumour load was evaluated. The control group received 1.5 ml PBS; both groups consisted of 10 rats.

In the last experiment (IV) a distinction was made between RBC and RBC content using the supernatants of 1.5 ml lysed RBC concentrate. Both groups consisted of 9 rats.

IN VITRO EXPERIMENTS

Mesothelial cell culture

Mesothelial cells (MC) were isolated from the small bowel mesentery of rats and identified immunohistochemically as described before.[9]

Mesothelial monolayers were established in 24 well plates (Costar, The Netherlands), pre-coated with collagen type I (Boehringer Mannheim, Mannheim, Germany). To do so, confluent MC were washed with phosphate buffered saline (PBS) and harvested with trypsin/EDTA (0.05 % / 0.02 %) and 90-95,000 MC in 1 ml of culture medium were added 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 4 days as determined by microscopic evaluation.

Experimental design

RBC ranging in concentration from 10-250 x 10^6 were added to the wells with confluent monolayers mesothelium or to wells precoated with collagen type I. In the first experiment RBC and 40 000 CC531 tumour cells per well were added simultaneously to the plates.

In the second experiment the effect of RBC on tumour growth was evaluated. 40 000 CC531 cells were left to adhere to the multiwell plate overnight, before adding the RBC in different concentrations. To determine a potential direct toxic effect, RBC were added to a monolayer mesothelium.

After 48 hours, plates were collected by washing away superfluous non-adherent cells and kept at -20°C for DNA analysis to measure total amount of cells.

DNA analysis

The total DNA content of the wells was measured using the bisbenzimide fluorescent dye (Boehring Diagnostics, La Jolla, USA) as previously described by Hofland *et al.*[10] The DNA measured represents the total amount of cells/well.

Statistical analysis

Statistical analysis was performed using the non-parametric Kruskall Wallis analysis of variance to determine overall differences followed by the non-parametric Mann Whitney *U* test to compare differences between groups.

RESULTS

In vivo experiments

Effect of whole blood on peritoneal tumour recurrence

Statistically significant differences in tumour load at all scored peritoneal sites were found between the control group and experimental group. The results indicate that adding 1.5 ml blood to the peritoneal cavity simultaneously with tumour cells, prevents recurrence of the seeded tumour cells. Range with median tumour load together with p-values are shown in table 5.1.

When blood was injected after the CC531 tumour cells had adhered however, no statistically significant differences in tumour load could be detected (results not shown). This indicates that the protective effect of blood is based on warding off tumour cell adhesion rather than affecting tumour growth.

	1.5 ml PBS	1.5 ml blood	p-value
Subcutis	2-5 (3)	0-3 (1)	0.001
Mesentery	1-3 (2)	0-1 (0)	<0.0001
Kidney	1-5 (3)	0-1 (1)	0.0001
Liver	1-3 (2)	0-1 (0)	0.0001
Omentum	2-5 (3)	0-1 (0)	<0.0001
Retroperitoneum	0-2 (1.5)	0 (0)	0.0002

Table 5.1

Effect of blood in the peritoneal cavity on peritoneal tumour recurrence. Range with median tumour load (values between brackets) together with p-value are shown.

Effect of RBC on peritoneal tumour recurrence

The results obtained after adding 1.5 ml of pure RBC concentrate together with tumour cells were similar to those when whole blood was used. At all peritoneal sites statistically significant less tumour load was scored when RBC concentrate was added simultaneously with 1 million CC531 tumour cells (table 5.2).

When comparing the effect of RBC with RBC content, no differences in tumour load could be scored. These results indicate the evaded tumour cell adhesion to the

peritoneum can be attributed to the content of RBC rather than the RBC as a whole. Table 5.3 shows the range with median score.

	1.5 ml PBS	1.5 ml RBC	p-value
Subcutis	4-5 (5)	0-3 (1)	<0.001
Mesentery	1-4 (2.5)	0-4 (0)	=0.002
Kidney	2-5 (3)	0-5 (1)	=0.004
Liver	2-4 (2.5)	0-2 (0)	<0.001
Omentum	4-5 (5)	0-5 (0)	<0.001
Retroperitoneum	0-3 (1)	0-4 (0)	=0.05

Table 5.2

Effect of RBC in the peritoneal cavity on peritoneal tumour recurrence. Range with median tumour load (values between brackets) together with p-value are shown.

	1.5 ml RBC	1.5 ml RBC content	p-value
Subcutis	1-4 (2)	1-5 (1)	0.81
Mesentery	0-4 (1)	0-1 (1)	0.95
Kidney	0-2 (1)	0-1 (1)	0.33
Liver	0-3 (1)	0-3 (1)	0.70
Omentum	1-4 (1)	1-5 (3)	0.37
Retroperitoneum	0-3 (0)	0-1 (1)	0.55

Table 5.3

Effect of RBC *versus* RBC content in the peritoneal cavity on peritoneal tumour recurrence. Range with median tumour load (values between brackets) together with p-value are shown.

***In vitro* experiments**

RBC in high amounts did not have a direct toxic effect on the mesothelial monolayer. A statistically significant inhibition of tumour cell adhesion was observed, irrespective of adherent underground, after adding 50 million RBC or more ($p<0.05$). No increment of the inhibitive effect was seen after adding >100 million RBC. Values are shown with standard deviation in figure 5.1. When CC531 cells were left to adhere before adding the RBC no difference in adherent cells could be measured (data not shown). These *in vitro* results indicate, as the *in vivo* results, that the observed inhibition of tumour load by RBC is based on impeding tumour cell adhesion rather than affecting tumour growth.

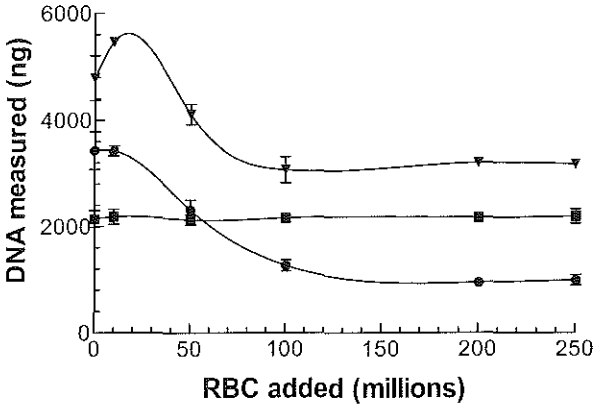


Figure 5.1

Tumour cell adhesion represented by total DNA measured. ■ monolayer mesothelium, ● CC531, ▼ monolayer mesothelium and CC531. Values ± SD (n=4).

DISCUSSION

Tumour recurrence is the result of a combination of tumour cell adhesion, cell survival and subsequent tumour growth. Per-operatively spilled tumour cells that fail to adhere, may not survive to form a local recurrence. Our observation that the presence of RBC in the peritoneal cavity could ward off tumour recurrence was reason enough to explore this phenomenon. There were primarily three hypotheses proposed for the mechanism of this effect, the first being a growth obstructing effect. Can RBC directly inhibit tumour

growth and consequently diminish tumour recurrence? The *in vitro* studies demonstrated that once the tumour cells had adhered, there was no decline in total DNA after 48 hours caused by RBC, i.e. no decline in tumour cell number. After adhesion of CC531 cells *in vivo* a growth inhibiting effect could not be detected either. Both experiments prove that RBC do not inhibit tumour growth, and that the inhibition of tumour load by RBC takes place at the point of tumour cell adhesion.

The second hypothesis proposed that tumour cell adhesion could be impeded by mechanical inhibition. Although in the *in vivo* studies this hypothesis was less likely to be relevant, in the *in vitro* studies a statistically significant inhibition of tumour cell adhesion was seen after adding $50x10^6$ RBC or more. This is at least 1250x more RBC than tumour cells which may lead to impeded tumour cell adhesion through mechanical inhibition by large amounts of RBC.

The third hypothesis was accredited to an unknown function of the RBC that could be executed by either the RBC-membrane or RBC-content. The results from experiment IV demonstrated that the latter resulted in a similar tumour inhibitory effect as the intact RBC, indicating that mechanical inhibition by an excess of RBC is of no relevance. In addition, these results demonstrate the evaded tumour cell adhesion to the peritoneum can be attributed to the content of RBC rather than the intact RBC.

Other than oxygen-transporters, RBC are well known for carrying several anti-oxidant protectors. These anti-oxidant scavengers catalyse or accelerate the dismantlement of oxygen free radicals which are well known to play a fundamental role in cellular injury in a wide variety of pathophysiologic processes. Following abdominal surgery, the most likely source of these free radicals is a rapid influx of neutrophils and macrophages, recruited to the abdominal cavity due to surgical trauma or inflammation.[11;12] The purpose of these reactive cell products is to destroy invading organisms and damaged tissue.

Excessive or inappropriate production of free radicals is associated with morbidity and mortality after peritoneal trauma and inflammatory diseases.[13;14] The role of oxidant damage to tissues has already been demonstrated in rheumatoid arthritis, reperfusion injury, ischemia and many other diseases. Diminution of peritoneal trauma by anti-oxidant scavengers affecting free radical mediated injury, might add to the prevention of implantation of spilled tumour cells.

The results of the present paper point in the direction of a possible relation between scavengers carried by RBC and prevention of free radical mediated injury. In chapter 3

we demonstrated a significant correlation between the extent of peritoneal trauma and the degree of tumour recurrence.[15] The evidence to date supports an effector role of the RBC in actively preventing tumour cell adhesion to the peritoneum resulting in less tumour recurrence. Additional studies need to be performed to illustrate which of the three anti-oxidant scavengers, that RBC transport in their cytoplasm (glutathion peroxidase, catalase and superoxide dismutase), bring about this effect.

The potential for clinical application of anti-oxidant scavengers to modify free radical injury, and hence inhibit tumour recurrence of per-operatively spilled tumour cells, sounds a promising indication for new therapeutic approaches for loco-regional recurrence.

REFERENCES

1. Busch OR, Hop WC, Hoynck vPM, Marquet RL, Jeekel J. Blood transfusions and prognosis in colorectal cancer [see comments]. **N Engl J Med** 1993; **328**: 1372-1376.
2. Heiss MM, Mempel W, Delanoff C, et al. Blood transfusion-modulated tumor recurrence: first results of a randomized study of autologous versus allogeneic blood transfusion in colorectal cancer surgery [see comments]. **J Clin Oncol** 1994; **12**: 1859-1867.
3. Houbiers JG, Brand A, van de Watering LM, et al. Randomised controlled trial comparing transfusion of leucocyte-depleted or buffy-coat-depleted blood in surgery for colorectal cancer [see comments]. **Lancet** 1994; **344**: 573-578.
4. Singh SK, Marquet RL, de Bruin RW, Hop WC, Westbroek DL, Jeekel J. Consequences of blood loss on growth of artificial metastases. **Br J Surg** 1988; **75**: 377-379.
5. Hoynk van Papendrecht M, usch O, JJ, Marquet R. The influence of blood loss on tumour growth: effect and mechanism in an experimental model. **The Neth J Surg** 1991; **43-4**: 85-88.
6. Marquet RL, de Bruin RW, Dallinga RJ, Singh SK, Jeekel J. Modulation of tumor growth by allogeneic blood transfusion. **J Cancer Res Clin Oncol** 1986; **111**: 50-53.
7. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. **Int J Cancer** 1984; **33**: 689-692.
8. Steller EP. Enhancement and abrogation; modifications of host immune status influence IL-2 and LAK cell immunotherapy. 1988. (GENERIC) Ref Type: Thesis/Dissertation
9. van der Wal BC, Hofland LJ, Marquet RL, van Koetsveld PM, van Rossen ME, van Eijck CH. Paracrine interactions between mesothelial and colon-carcinoma cells in a rat model. **Int J Cancer** 1997; **73**: 885-890.
10. Hofland LJ, van Koetsveld PM, Lamberts SW. Percoll density gradient centrifugation of rat pituitary tumor cells: a study of functional heterogeneity within and between tumors with respect to growth rates, prolactin production and responsiveness to the somatostatin analog SMS 201-995. **Eur J Cancer** 1990; **26**: 37-44.
11. Goldstein CS, Bomalaski JS, Zurier RB, Neilson EG, Douglas SD. Analysis of peritoneal macrophages in continuous ambulatory peritoneal dialysis patients. **Kidney Int** 1984; **26**: 733-740.
12. Kuraoka S, Campeau JD, Nakamura RM, diZerega GS. Modulation of postsurgical macrophage function by early postsurgical polymorphonuclear leukocytes. **J Surg Res** 1992; **53**: 245-250.
13. O'Leary JP, Wickbom G, Cha SO, Wickbom A. The role of feces, necrotic tissue, and various blocking agents in the prevention of adhesions. **Ann Surg** 1988; **207**: 693-698.

14. Tsimoyiannis EC, Tsimoyiannis JC, Sarros CJ, et al. The role of oxygen-derived free radicals in peritoneal adhesion formation induced by ileal ischaemia/reperfusion. **Acta Chir Scand** 1989; **155**: 171-174.
15. van den Tol PM, van Rossen EE, van Eijck CH, Bonthuis F, Marquet RL, Jeekel H. Reduction of peritoneal trauma by using nonsurgical gauze leads to less implantation metastasis of spilled tumor cells. **Ann Surg** 1998; **227**: 242-248.

Chapter VI

SCAVENGING OF REACTIVE OXYGEN SPECIES LEADS TO DIMINISHED PERITONEAL TUMOUR RECURRENCE

Submitted for publication

In the previous chapter we demonstrated that red blood cells (RBC) inhibit the recurrence of per-operatively spilled tumour cells. The aim of this study was to identify on which RBC component(s) the inhibitory effect is based. Employing a cell-seeding model in rats, the effect of RBC related anti-oxidant scavengers (haemoglobin, catalase and superoxide dismutase (SOD)) on peritoneal tumour recurrence was investigated.

Intra-peritoneal injection of haemoglobin caused 45% more tumour load. At least 40% inhibition of tumour recurrence was achieved with the use of catalase or SOD. Combining SOD and catalase did not lead to additional inhibition of tumour recurrence. Inhibition of the overwhelming oxidative potential after surgical peritoneal trauma with the use of scavengers may lead to interesting new approaches for diminishing peritoneal tumour recurrence.

INTRODUCTION

Local or regional tumour recurrence of gastro-intestinal carcinoma remains an important complication after potentially curative surgical resection.[1] Prevention of this affliction remains the goal of many clinical and experimental studies.[1-3] The results of these studies show a correlation between surgical peritoneal trauma and loco-regional tumour recurrence.[2;3] Minimising surgical trauma, through the use of laparoscopic surgery or other minimal invasive techniques, was shown to reduce peritoneal trauma and tumour recurrence.[2;3]

Excessive production of reactive oxygen species (ROS) and related tissue injury plays a fundamental role in a wide variety of disease processes.[4;5] Besides by chronic inflammatory diseases, ROS are also produced after surgical trauma.[4;6] The main producers of ROS are inflammatory cells entering damaged tissue after surgical trauma. The purpose of these cell products is to destroy invading organisms and damaged tissue. Despite this beneficial effect, the overwhelming oxidative potential can result in additional tissue destruction.[4;6]

After peritoneal surgery an acute inflammatory reaction occurs, even in the absence of any apparent bacterial contamination.[7] In chapters 3 and 4 we demonstrated that the inflammatory sequelae after abdominal surgery promote tumour recurrence and that this effect is mainly based on the cellular component of the inflammatory process.[3;8] Using the same experimental model we showed in chapter 5 that red blood cells (RBC), introduced in the peritoneal cavity after surgical trauma, effectively inhibited loco-regional tumour recurrence.[9] These studies were performed in a cell-seeding model and demonstrated that the inhibitory function of RBC was primarily based on preventing adhesion of per-operatively spilled tumour cells. Moreover, we demonstrated that RBC homogenates were equally effective excluding the possibility of a mere steric hindrance of tumour cell adhesion.

The aim of this study was to identify which component(s) of the RBC homogenate could underlie the inhibitory effect of RBC on tumour take. For this purpose, the effect of catalase (peroxide decomposition), haemoglobin (nitric oxide scavenger) and superoxide dismutase (SOD, superoxide decomposition) were compared to the inhibitory effect of RBC on peritoneal tumour recurrence.

MATERIALS AND METHODS

Animals

Inbred rats of the WAG strain were obtained from Harlan-CPB, Zeist, The Netherlands. The rats were bred under specific pathogen free conditions, kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 hours light/ 12 hours dark), fed with laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee for Animal Research of the Erasmus University Rotterdam.

Tumour cell line

CC531 is a moderately differentiated, weakly immunogenic colon adenocarcinoma induced in the WAG/Rij rat by 1,2-dimethylhydrazine. A cell line was established from this carcinoma and maintained by serial passage after trypsinization in culture medium.[10] CC531 tumour cells were cultured in RPMI 1640 medium supplemented with 5% foetal calf serum, L-glutamin (200 mM) and penicillin (10^5 U/L). All supplements were obtained from Life Technologicals BV, Breda, The Netherlands. Before use, cells were trypsinized (10 minutes at 37°C), centrifuged (5 minutes at 700 g), resuspended in RPMI 1640, counted and brought to a concentration of 0.5 million cells/ml. Viability was measured by trypan blue exclusion and always exceeded 90 percent.

RBC, Catalase, SOD and haemoglobin

To obtain RBC concentrate, the same procedure was followed as previously described.[9] In short, WAG/Rij rats were exsanguinated by heartpunction. The blood was collected in a heparinized vial (heparin Leo, Leo Pharmaceutical products, Weesp, The Netherlands). RBC concentrate was obtained by centrifugation of blood at 1000 g for ten minutes (Mistral 2000i), removing the buffy coat and plasma. The pelleted RBC were resuspended in RPMI medium to the original volume.

Catalase (3000 U/mg) and haemoglobin were purchased from SIGMA-Aldrich Chemie BV, Zwijndrecht, The Netherlands. Superoxide dismutase (SOD) (5000 U/mg) was purchased from Roche Diagnostics BV, Almere, The Netherlands. Before use, solutions containing the different scavengers in the desired concentration were prepared in phosphate buffered saline (PBS) and kept on ice.

IN VIVO EXPERIMENTS

Catalase and SOD determination in RBC

Catalase. Pelleted RBC were resuspended in 50 mM phosphate buffer (pH 7.0) to the original volume, and diluted 10 times in distilled water to lyse the RBC at 37°C for 45 min. Next, the hemolysate was further diluted in phosphate buffer, added in a volume of 0.02 ml to 3 ml 10.5 mM H_2O_2, and the time (sec) recorded to obtain a decrease in optical density at 240 nm from 0.450 to 0.400. This value was used to calculate the amount of International Units of catalase per ml sample according to the formula: 17 x 13.1/(time x 0.02).[11] We found per ml of pelleted RBC 13563 (± 1252) IU of catalase (n=8), thus 1.5 ml RBC concentrate contained 9155 IU.

SOD. The RBC hemolysate was serially diluted in 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.01 mM ferricytochrome *c*, 0.05 mM xanthine, 0.025 U xanthine oxidase/ml. The SOD was assayed on the basis of its ability to inhibit the reduction of ferricytochrome *c* at 550 nm by superoxide anions generated by the xanthine-xanthine oxidase system.[12] At pH 10.0 the SOD appeared about 9 times more active, and could be completely inhibited by 1 mM KCN. Pelleted RBC contained 1096 U SOD per ml, which meant that the RBC concentrate contained 750 U SOD per 1.5 ml.

Operative procedure and tumour scoring

Under ether anaesthesia the abdomen of rats was shaved and cleaned with ethanol 70%. Laparotomy was performed using a midline incision followed by intra peritoneal (ip) inoculation of 0.5 million CC531 tumour cells. The abdomen was closed in one layer with silk 2-0 sutures (Braun, The Netherlands).

Three weeks postoperatively the rats were sacrificed and intra-peritoneal tumour load was scored as described previously[9] at the following sites: parietal peritoneum (at the site of the incision), omentum, liver, kidneys, retro peritoneum and mesentery. The scoring ranged from 0 to 5 per site. A score of 0 meant there was no tumour growth, a score of 1 indicated an estimated tumour diameter of less than 0.5 cm, a score of 2 a tumour diameter between 0.5 and 1 cm, a score of 3 a tumour diameter between 1 and 2 cm, a score of 4 a tumour diameter between 2 and 3 cm, and a score of 5 an estimated diameter of more than 3 cm. For each rat the scores were summarised and defined as total tumour load.

Effect of catalase, SOD and haemoglobin on peritoneal tumour recurrence

In this experiment the inhibitory effect of RBC on peritoneal tumour recurrence was compared to the effect of two major components carried by RBC, i.e. catalase and haemoglobin. After laparotomy, 0.5 million CC531 cells were injected into the peritoneal cavity together with 1.5 ml PBS (control group, n=10), with 1.5 ml RBC concentrate (n=10), with 3000 U catalase (in 1.5 ml PBS, n=10) or 0.5 mg haemoglobin (in 1.5 ml PBS, n=10).

Next, the effect of a higher concentration of catalase was investigated. After laparotomy, CC531 cells were injected into the peritoneal cavity together with 1 ml PBS (control group, n=10), with 3000 U catalase (in 1 ml PBS, n=9) or with 6000 U catalase (in 1 ml PBS, n=9).

Finally, the effect of SOD alone and in combination with catalase was studied. The experimental groups received 0.5 million tumour cells together with 6000 U catalase (n=8), with 2000 U SOD (n=8) or with 6000 U catalase and 2000 U SOD (n=8). To make up for total volume, 1ml PBS was added. The control group (n=5) received 2 ml PBS and 0.5 million of tumour cells.

Statistical analysis

Statistical analysis was performed using the non-parametric Kruskall Wallis analysis of variance to determine overall differences followed by the non-parametric Mann Whitney *U* test to compare differences between groups.

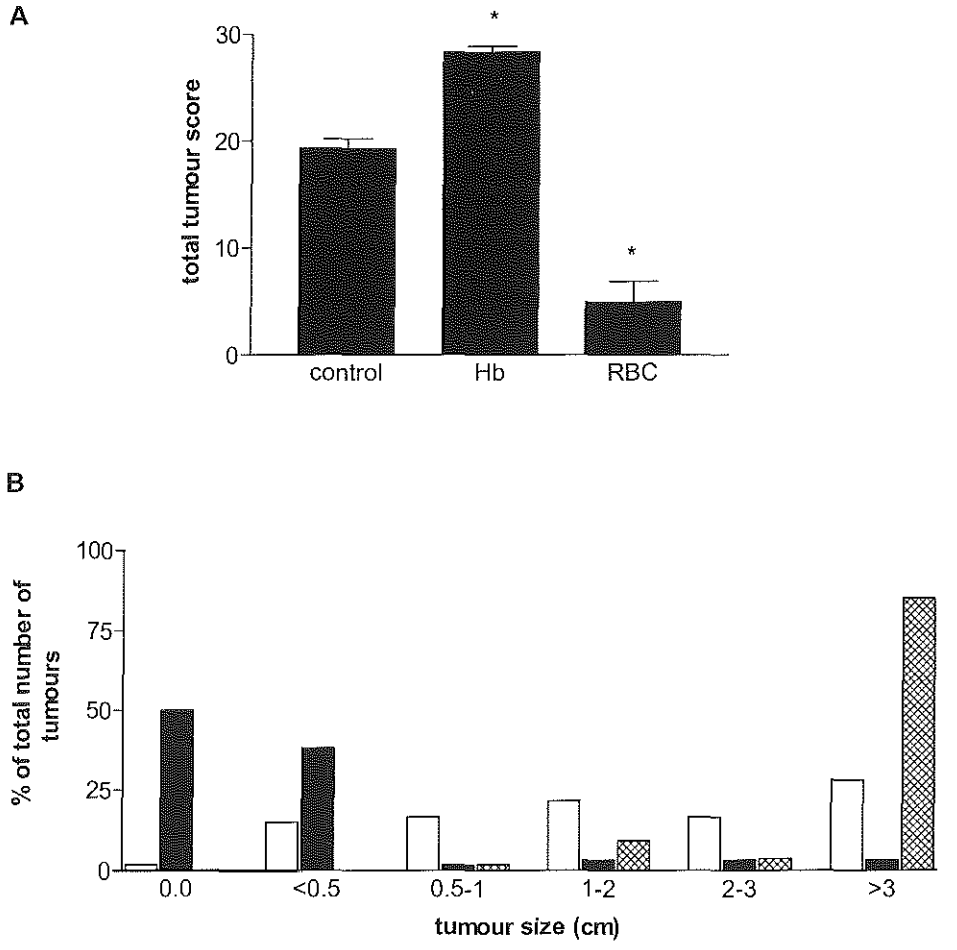


Figure 6.1

A: Effect of RBC and Hb on peritoneal tumour load. The mean of the total tumour score is shown with SEM. * $p \leq 0.001$ *vs* control. B: Distribution of treatment dependent tumour size. Open bars: control group; filled bars: RBC; crosshatched bars: haemoglobin.

RESULTS

Effect of two major components of RBC *versus* intact RBC on peritoneal tumour recurrence

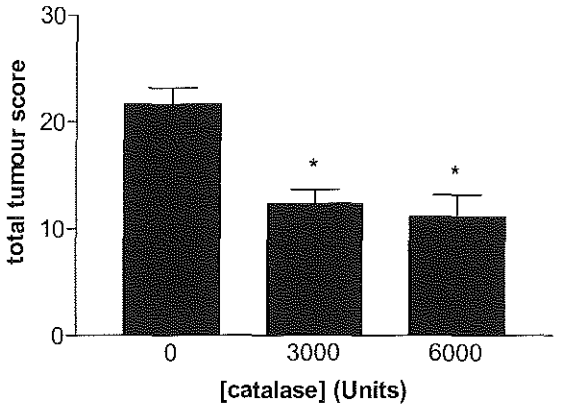
After intra-peritoneal injection of tumour cells with RBC, the inhibition of tumour recurrence was comparable (mean tumour load ± SEM is 4.9 ± 2.0 in RBC *vs* 19.3 ± 0.9 in control group, p=0.001; table 6.1, fig 6.1) to that as found previously.[9] Administration of haemoglobin led to more tumour recurrence (28.3 ± 0.6, p<0.0001 *vs* control), with statistically significant higher tumour diameters at 4 of 6 scored peritoneal sites. In the haemoglobin group, one rat suffered from overload peritoneal tumour load and was sacrificed before 3 weeks (this rat was excluded for statistical purposes). Significant differences in peritoneal tumour size were observed. In the haemoglobin group 85% of the tumours were larger than 3 cm, compared to 28% in the control and 3% in the RBC group (fig 6.1B).

When compared to the control, 3000 U of catalase inhibited tumour recurrence (10.5 ± 2.0 catalase, p=0.002). Although this was a statistically significant inhibition, the effect of catalase on peritoneal tumour load was less than the inhibitory effect of RBC (p=0.008). In the catalase group the majority of the tumours (40%) were between <0.5 and 2 cm in diameter while in the RBC group 50% was tumour free. Only 5% of the tumours in the catalase group were larger than 3 cm in diameter.

To study if increasing the amount of catalase would improve the inhibitory effect on tumour recurrence, 6000 U were administered ip. Total tumour load in the control group of this experiment was similar to the control in the previous section (mean tumour load ± SEM is 21.6 ± 1.5; fig 6.2A). However, in this experiment the tumours were relatively large, i.e. 42% of the tumours were larger than 3 cm (fig 6.2B), whereas in the previous experiment the control tumours were more heterogeneous in size (fig 6.1B). After intra peritoneal injection of 3000 U catalase, mean tumour load amounted to 12.3 ± 1.3 (p=0.004 *vs* control). Tumour load did not decrease significantly using 6000 U catalase (12.6 ± 2.4, p=0.003 *vs* control).

Using more catalase also did not lead to a further decrease in tumour size. With 3000 U catalase 41% tumours were smaller than 0.5 cm, with 6000 U catalase 43% (not significant).

A



B

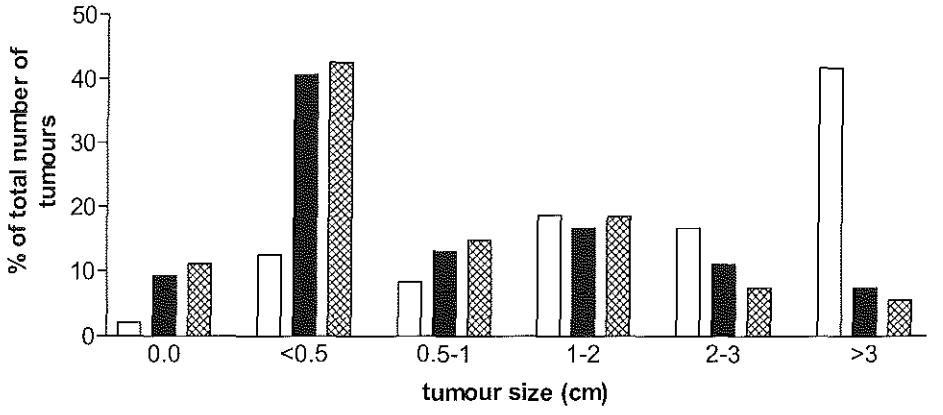
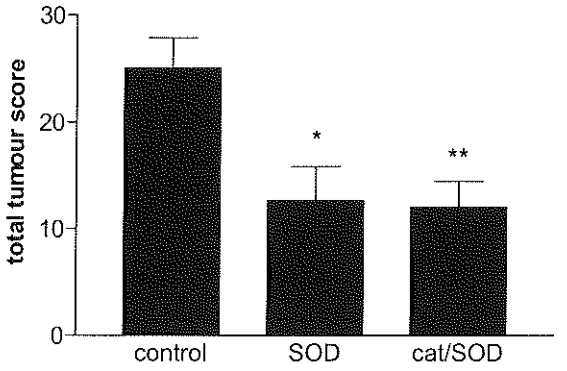


Figure 6.2

A: Effect of different concentrations of catalase on peritoneal tumour load. The mean of the total tumour score is shown with SEM. * $p \leq 0.01$ *vs* control. B: Distribution of treatment dependent tumour size. Open bars: control group; filled bars: 3000 U catalase; crosshatched bars: 6000 U catalase.

A



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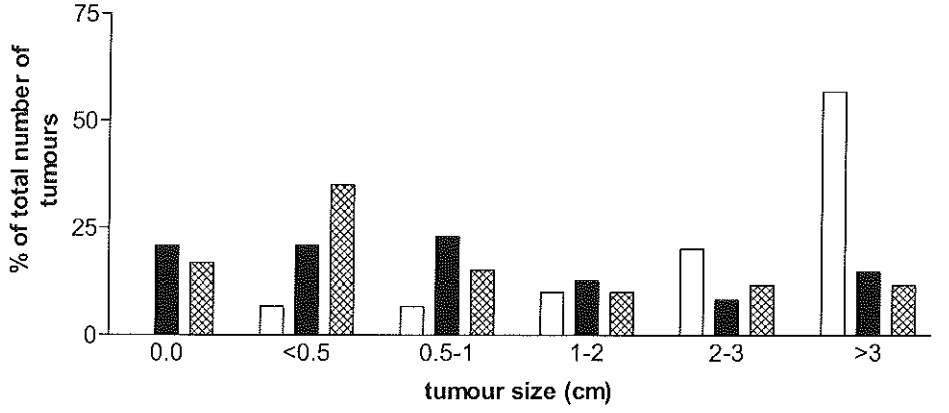


Figure 6.3

A: Differences in peritoneal tumour load in rats receiving SOD (2000 U) and a combination of catalase (6000 U) and SOD (cat/SOD) compared to a control. The mean of the total tumour score is shown with SEM. * $p \leq 0.05$ and ** $p < 0.01$ *vs* control. B: Distribution of treatment dependent tumour size. Open bars: control group; filled bars: SOD; crosshatched bars: cat/SOD.

Effect of catalase and SOD on peritoneal tumour recurrence

To investigate if SOD could affect tumour recurrence as well, 2000 U of SOD were administered ip at the time of tumour seeding. SOD led to a significant reduction in tumour recurrence in comparison with the control, respectively 12.6 ± 3.2 and 25 ± 2.9 ($p<0.05$). The inhibitory effect of 6000 U catalase was similar as described in the previous section (table 6.2). The combined administration of SOD (2000 U) and catalase (6000 U) showed a minor but not significant stronger inhibition of tumour recurrence (mean tumour score 10.6 ± 2.3 in the cat/SOD group; fig 6.3A, table 6.2).

The sizes of tumour deposits in the antioxidant enzyme groups varied greatly, whereas in the control group mostly large tumours were scored (57% larger than 3 cm). In the control group 6.7% scored between 0 and < 0.5 cm tumour size, in the SOD group 42% and in the combined group 57% (fig 6.3B).

DISCUSSION

The major finding of the present study is that the antioxidant enzymes SOD and catalase have been identified as the components that are (partially) responsible for the tumour inhibiting function of RBC.

In a previous experimental study 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.[3;8] Analysis of the cell population demonstrated the presence of more than 75% PMN. In ischaemia reperfusion injury, where related inflammatory mechanisms are at play, most tissue trauma is inflicted by ROS.[13-15] Scavenging of, or blocking its production demonstrated that ROS significantly impede wound healing and graft survival after transplantation.[14;16;17] Since PMN are a major source of ROS, similar PMN related injury might play a role in surgical peritoneal trauma and tumour recurrence and likewise antioxidants may reduce tumour recurrence.

Based on previous results of a tumour impeding effect of RBC, we questioned which RBC component could underlie this effect. RBC convey three major scavenging enzymes to diminish the toxic effects which can be caused by uncontrolled levels of superoxide radical anions ($O_2^{-\bullet}$) or hydrogen peroxide (H_2O_2). These are superoxide dismutase (SOD), catalase and glutathion peroxidase.[18] While haemoglobin is the principal oxygen carrier, it can scavenge nitric oxide radical as well.[19]

First we investigated the effect of haemoglobin and found tumour recurrence was in

fact increased compared to the control. Catalase inhibited tumour recurrence more than 40% in all experiments, showing the consistency of this effect *in vivo*. However, the peritoneal tumour load remaining after catalase injection was significantly higher than after RBC injection. Although we detected 9000 U catalase in RBC concentrate, we did not see a decrease in tumour recurrence when using a higher amount of catalase in the *in vivo* experiments. This indicates an additional tumour impeding effect brought about by RBC that is not attained with catalase.

The affinity of catalase for its substrate hydrogen peroxide is low, in other words, catalase works efficiently only when the concentration of hydrogen peroxide is high. The finding that catalase is a potent inhibitor of tumour recurrence indicates that hydrogen peroxide is an important factor in enhancing peritoneal tumour recurrence. However, apparently the concentration of this ROS is low and therefore glutathion peroxidase, another component of RBC that reduces low concentrations of hydrogen peroxide in the presence of reduced glutathion with high efficiency[18], may be a better choice.

SOD, another antioxidant enzyme contained by RBC, dismutates superoxide to peroxide and oxygen ($O_2^-\bullet+O_2^-\bullet+2H^+ \rightarrow H_2O_2+O_2$). Experiments investigating ischaemia reperfusion injury use SOD to inactivate the tissue traumatising effect of superoxide.[13-15] The results of these experiments show that neutralising superoxide reduces tissue injury[15], enhances post operative wound healing[14], increases bursting pressure of sutured small intestine[14] and increases graft survival after transplantation.[13] In our experiment 50% inhibition of tumour recurrence was observed by using this enzyme in excess (500 U present in RBC concentrate and 2000 U used in experiments). Apparently the superoxide anion also promotes tumour recurrence. However, SOD alone and in combination with catalase was not more efficient than catalase alone.

The above mentioned results illustrate that intra peritoneal injection of haemoglobin stimulates tumour recurrence and that inhibition of hydrogen peroxide and superoxide related tissue trauma diminishes tumour recurrence. Taken together, these results provide evidence for the role of another ROS, namely the hydroxyl radical ($OH\bullet$) as the real effector molecule in promoting tumour recurrence. Hydroxyl radicals are extremely reactive, unstable and powerful free radicals compared to the relatively unreactive peroxide and superoxide.[4;5;15] Catalysed by iron, peroxide is converted to the hydroxyl radical in the Fenton reaction ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH^-+OH\bullet$). On its turn ferric iron is

reduced to ferrous iron by superoxide in the Haber-Weiss reaction ($O2^{-}+H_2O_2 \rightarrow O_2+OH^{-}+OH\bullet$). This may explain why the combination of SOD and catalase did not lead to a better outcome than each antioxidant enzyme alone, and why haemoglobin, given as a source of iron, increased tumour recurrence. The production of the hydroxyl radical will ultimately lead to enhanced peritoneal tissue damage and hence, enhanced tumour recurrence. Eliminating superoxide or hydrogen peroxide with SOD and catalase respectively prevents the production of the hydroxyl radical. Although glutathion peroxidase (together with reduced glutathion), based on its high efficiency to neutralise low hydrogen peroxide concentrations, may improve the inhibitory effect on tumour recurrence, a promising alternative approach may be the use of iron chelators.

In conclusion, we have shown that inhibition of ROS mediated peritoneal damage with the use of scavengers leads to diminished tumour recurrence. A great deal of insight exists about ROS mediated ischaemia reperfusion damage and the prevention of this mechanism by scavengers. Recent studies proclaim improved results against tissue damage using chemically and biologically stable mimics of SOD and catalase.[20] Inhibition of the overwhelming oxidative potential after surgical peritoneal trauma (or any other form of surgical trauma) with the use of scavengers may also lead to interesting new approaches for diminishing tumour recurrence.

MEDIAN TUMOUR LOAD (RANGE) AT DIFFERENT PERITONEAL SITES

Abdominal Sites	PBS (n=10)	RBC (n=10)	Haemoglobin (n=9)	catalase (n=10)
Omentum	5 (4-5)	0 (0-5)	5 (5)	1.5 (0-4)
Liver	2.5 (2-4)	0 (0-2)	5 (5)	1.5 (0-3)
Kidney	3 (2-5)	1 (0-5)	5 (4-5)	3 (1-5)
Par. peritoneum	5 (4-5)	1 (0-3)	5 (5)	1.5 (1-5)
Mesentery	2.5 (1-4)	0 (0-4)	3 (2-5)	1.5 (0-4)
Retro peritoneum	1 (0-3)	0 (0-4)	5 (3-5)	1 (0-2)
Total	19.5 (15-24)	2.5 (1-21)	28 (25-30)	12 (3-21)

A

Abdominal Sites	p1	p2	p3	p4
Omentum	<0.0001	ns	<0.0001	0.04
Liver	<0.0001	<0.0001	0.008	0.03
Kidney	0.004	0.002	ns	ns
Par. peritoneum	<0.0001	ns	0.001	0.05
Mesentery	0.002	0.05	ns	ns
Retro peritoneum	0.05	<0.0001	ns	0.03
Total	0.001	<0.0001	0.002	0.008

B

Table 6.1

A: Median tumour load and range at different abdominal sites in rats having undergone a laparotomy followed by ip injection of tumour cells with PBS, RBC, haemoglobin (0.5 g) or catalase (3000 U). B: The p-value 1 represents differences between rats receiving PBS (control) and RBC, p-value 2 represents differences between PBS and haemoglobin; p-value 3 between PBS and catalase and p-value 4 between RBC and catalase.

MEDIAN TUMOUR LOAD (RANGE) AT DIFFERENT PERITONEAL SITES

Abdominal Sites	control (n=5)	catalase (n=8)	SOD (n=8)	Cat/SOD (n=8)	p1	p2	p3
Omentum	5 (3-5)	2.5 (0-5)	2 (0-5)	1 (1-3)	ns	ns	0.003
Liver	4 (1-5)	1.5 (0-4)	1 (0-3)	1 (0-2)	ns	0.03	0.01
Kidney	4 (2-5)	0.5 (0-4)	2 (0-4)	1 (0-4)	0.007	0.02	0.01
Par. perit	5 (1-5)	0.5 (0-5)	2.5 (0-5)	4 (0-5)	ns	0.03	0.02
Mesentery	5 (1-5)	1.5 (0-4)	2 (0-5)	1 (0-5)	0.03	ns	0.03
Retro perit	5 (2-5)	1 (0-5)	1.5 (0-5)	1 (0-5)	0.02	ns	0.04
Total	28 (14-30)	13 (1-25)	11.5 (1-26)	9 (2-24)	0.03	0.02	0.007

Table 6.2

Median tumour load and range at different abdominal sites in rats having undergone a laparotomy followed by ip injection of tumour cells with PBS, catalase (6000 U), SOD (2000 U) or a combination of catalase and SOD (cat/SOD). The p-value 1 represents differences between rats receiving PBS (control) and catalase; p-value 2 represents differences between PBS and SOD, p-value 3 between PBS and cat/SOD.

REFERENCES

1. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. **World J Surg** 1991; **15**: 609-616.
2. Bouvy ND, Marquet RL, Jeekel J, Bonjer HJ. Laparoscopic surgery is associated with less tumour growth stimulation than conventional surgery: an experimental study [see comments]. **Br J Surg** 1997; **84**: 358-361.
3. van den Tol PM, van Rossen EE, van Eijck CH, Bonthuis F, Marquet RL, Jeekel H. Reduction of peritoneal trauma by using nonsurgical gauze leads to less implantation metastasis of spilled tumor cells. **Ann Surg** 1998; **227**: 242-248.
4. Bulkley GB. The role of oxygen free radicals in human disease processes. **Surgery** 1983; **94** : 407-411.
5. Weiss SJ. Tissue destruction by neutrophils [see comments]. **N Engl J Med** 1989; **320**: 365-376.
6. Billing AG, Jochum M, Frohlich D, Cheronis JC, Fritz H. Oxidative autoaggression by phagocytes in human peritonitis. **Eur J Clin Invest** 1997; **27**: 1030-1037.
7. Kuraoka S, Campeau JD, Nakamura RM, diZerega GS. Modulation of postsurgical macrophage function by early postsurgical polymorphonuclear leukocytes. **J Surg Res** 1992; **53**: 245-250.
8. van Rossen M, van den Tol MP, Aalbers AGJ, et al. 34th Congress of the European Society for Surgical Research (ESSR). Bern, Switzerland, April 22-24, 1999. Abstracts. **Eur Surg Res** 1999; **31 Suppl 1**: 1-244.
9. van Rossen ME, Stoop MP, Hofland LJ, et al. Red blood cells inhibit tumour cell adhesion to the peritoneum [published erratum appears in Br J Surg 1999 Oct;86(10):1353]. **Br J Surg** 1999; **86**: 509-513.
10. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. **Int J Cancer** 1984; **33**: 689-692.
11. Bergmeyer HU. Zur Messung von Katalase-Aktivitäten. **Biochem Zeitschr** 1955; **327**: 255-258.
12. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). **J Biol Chem** 1969; **244**: 6049-6055.
13. Baker GL, Corry RJ, Autor AP. Oxygen free radical induced damage in kidneys subjected to warm ischemia and reperfusion. Protective effect of superoxide dismutase. **Ann Surg** 1985; **202**: 628-641.
14. Foschi D, Trabucchi E, Musazzi M, et al. The effects of oxygen free radicals on wound healing. **Int J Tissue React** 1988; **10**: 373-379.
15. Grace PA. Ischaemia-reperfusion injury. **Br J Surg** 1994; **81**: 637-647.

16. Horton JW, White DJ. Free radical scavengers prevent intestinal ischemia-reperfusion-mediated cardiac dysfunction. **J Surg Res** 1993; **55**: 282-289.
17. White MJ, Heckler FR. Oxygen free radicals and wound healing. **Clin Plast Surg** 1990; **17**: 473-484.
18. Maral J, Puget K, Michelson AM. Comparative study of superoxide dismutase, catalase and glutathione peroxidase levels in erythrocytes of different animals. **Biochem Biophys Res Commun** 1977; **77**: 1525-1535.
19. Winslow RM. New transfusion strategies: red cell substitutes. **Annu Rev Med** 1999; **50**: 337-353.
20. Salvemini D, Wang ZQ, Zweier JL, et al. A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats [see comments]. **Science** 1999; **286**: 304-306.

Part IV

Mesothelial and Tumour Cell Adhesive Interactions

in vitro

Chapter VII

EFFECT OF INFLAMMATORY CYTOKINES AND GROWTH FACTORS ON TUMOUR CELL ADHESION TO THE PERITONEUM

Accepted for publication in the **Journal of Pathology**

In this experimental study the effect of inflammatory cytokines and growth factors on tumour cell adhesion to the peritoneum was investigated. A reproducible in vitro assay to study the adhesion of CC531 colon carcinoma cells to an autologous monolayer of rat mesothelial cells was developed.

Tumour cell adhesion to mesothelium pre-incubated with IL-1β and EGF resulted in at least 60% more tumour cell adhesion at maximal stimulation. TGF-β pre-incubation resulted in minor though significant stimulation of cell adhesion (maximal 16%). The effect of IL-1β was time and dose dependent. No mesothelial cell proliferation took place after pre-treatment with IL-1β, indicating enhanced adhesion was not based on an increase in the number of mesothelial cells. Pre-treatment with EGF stimulated mesothelial cell growth as measured by DNA analysis. This effect on cell growth and adhesion was dose dependent.

Additional blocking experiments with anti-IL-1β resulted in statistically significant inhibition of IL-1β stimulated tumour cell adhesion, demonstrating the specificity of this effect. IFN-γ, TNF-α, IL-6 and IGF-I pre-incubation had no effect on tumour cell adhesion.

The presented results prove IL-1β and EGF are significant promoting factors in tumour cell adhesion to mesothelium in vitro and may therefore account for tumour recurrence to the peritoneum in vivo.

INTRODUCTION

Despite intentionally curative resection for gastro-intestinal carcinoma, peritoneal dissemination is a common cause for post-surgical recurrence.[1] Several theories suggest that exfoliation of tumour cells at the time of operation, microscopic deposits in lymphatics and presence of malignant cells at the anastomosis play an important role in the aetiology of intra-abdominal tumour recurrence.[2] Patterns of first recurrence show the resection site is preferential, and combined recurrence on peritoneal surfaces and resection site is common.[1-3]

The tumour cell entrapment theory proposes that the fibrinous exudate, formed as initial response to surgical trauma, facilitates the implantation of cancer cells onto raw tissue. Tumour growth is subsequently encouraged by growth factors produced by inflammatory cells participating in the repair process.[2] Using an ectopic tumour growth model, we recently demonstrated enhanced growth of extra peritoneal tumour metastases after inflicting surgical trauma to the peritoneum (see also chapter 3).[4;5] In an experimental model, Hofer *et al* demonstrated enhanced tumour growth *in vivo* after "direct" surgical wounding and "artificial" wounding with wound fluid or growth factors.[6] These studies indicate that factors produced after surgical trauma enhance local and distant tumour growth.

Apart from stimulated cell growth, residual tumour cells may profit from the enriched wound micro-environment by enhancing the prospect of cell adhesion. This would explain tumour recurrence at not directly traumatised peritoneal surfaces. Although tumour cell adhesion is the first step for successful recurrence, few studies have been performed to elucidate the mechanisms behind this process. *In vitro* upregulation or *de novo* synthesis of cell adhesion molecules in a wide range of cell types after pre-treatment with inflammatory cytokines has been reported.[7-10] These studies however, do not foresee the effect on tumour cell adhesion.

In the present study, we developed an *in vitro* cell adhesion model in order to quantify cell adhesion and evaluate the effect of inflammatory cytokines and growth factors on the number of adhered tumour cells. A reproducible assay was designed to study the adhesion of CC531 colon carcinoma cells to an autologous monolayer of rat mesothelial cells. This study was undertaken to demonstrate that cytokines and growth factors present in wound fluid after surgical trauma not only enhance tumour growth, but also affect tumour cell adhesion. Expression of cell adhesion molecules was assessed to verify elementary interactions between the investigated cell types.

MATERIALS AND METHODS

Animals

Inbred WAG/Rij rats, obtained from Harlan-CPB, Zeist, The Netherlands were used. The rats were bred under specific pathogen-free conditions, kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 hours light/ 12 hours dark), fed with laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee in Animal Research of the Erasmus University Rotterdam.

Isolation and identification of mesothelial cells

Mesothelial cells (MC) were isolated from the small bowel mesentery of a male rat, according to techniques modified from Akedo et al[11] as described previously.[12] In brief, under ether anaesthesia the abdomen of a rat was shaved, cleaned with alcohol 70% and subsequently opened. Window like transparent triangular sheets of mesentery were isolated and collected in Hank's Balanced Salt Solution (HBSS) containing 5% human serum albumin (CLB, Amsterdam, The Netherlands) penicillin (10^5 U/L), glutamin (2 mM) and fungizone (1.25 mg/L). After washing the sheets twice in this medium, they were incubated in a mixture of collagenase (1 g/L) and dispase (2.4 x 10^3 U/L) (Roche Diagnostics, Almere, The Netherlands). Following incubation during 15 minutes at 37°C and continuous gentle shaking, the detached mesothelial cells were pelleted by centrifugation at 300 g for 5 minutes. Cell viability was determined by trypan blue and always exceeded 95%. The pelleted mesothelial cells were resuspended in culture medium. Culture medium consisted of RPMI 1640 supplemented with 10% foetal calf serum, glutamin (2 mM), penicillin (10^5 U/L) and fungizone (1.25 mg/L). Medium and all supplements were obtained from Life Technologicals BV (Breda, The Netherlands). The mesentery derived cells grew forming a monolayer in a 37°C, fully humidified, 5% CO_2 incubator in polystyrene culture flasks (75 cm^2; Corning BV, Schiphol-Rijk, The Netherlands) precoated with collagen type I (15 $\mu g/cm^2$ collagen S (type I), cell biology Roche Diagnostics).

Tumour cell line

CC531 is a moderately differentiated, weakly immunogenic colon adeno-carcinoma induced in a WAG/Rij rat by 1,2-dimethylhydrazine. A cell line was established and maintained by serial passage after trypsinization in culture medium.[13] CC531 tumour cells were cultured in RPMI 1640 medium supplemented with 5% foetal calf serum, glutamin (2 mM) and penicillin (10^5 U/L), and passaged once weekly using trypsin (0.05%) and EDTA (0.02%).

Cytokine and growth factor pre-incubation

Recombinant rat interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) were obtained from R&D Systems, Uithoorn, The Netherlands. Epidermal growth factor (EGF), insulin like growth factor-I (IGF-I) and transforming growth factor-β (TGF-β) were obtained from Bachem, Bern, Switserland. Interferon-γ (IFN-γ) was obtained from Sanvertech, Heerhugowaard, The Netherlands. Pre-incubation of the mesothelial monolayer was performed using increasing concentrations of these mediators.

To demonstrate time-dependency, IL-1β pre-incubation of the mesothelial monolayer was performed at different time intervals (0 to 24 hours pre-incubation).

Anti-rat IL-1β was obtained from R&D Systems. To inactivate IL-1β, pre-incubation was performed with a hundred-fold excess of anti-IL-1β during 1 hour at 37°C according to instructions of the manufacturer. After this, the formed IL-1β/anti-IL-1β complex was added to the mesothelial monolayer for pre-incubation.

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 medium supplemented with 0.5% bovine serum albumin (RPMI/0.5%BSA, BSA obtained from Sigma-Aldrich). Trypsinized CC531 cells ($1x10^6$ cells/ml) were incubated in RPMI/0.5%BSA at 37°C for 45 minutes with occasional mixing.

Adhesion assay

To quantify tumour cell adhesion to 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 Packard, Groningen, The Netherlands), precoated 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 1.5×10^4 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 2 days as determined by light microscopy. In order to determine the effect of the inflammatory cytokines and growth factors 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 RPMI/0.5%BSA to remove free dye.

Medium from the experimental wells was removed and 200 µl RPMI/0.5%BSA containing 30.000 calcein labelled tumour cells was added. Plates were centrifuged for 1 minute at 80 g on a Heraeus centrifuge and incubated at 37°C for 60 minutes. After this, the medium of each well was removed and washed twice with 200 µl RPMI/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 establish whether overnight incubation of the mesothelial monolayer with the different cytokines was of influence on cell growth, a DNA measurement was performed. In this assay, the DNA content of the mesothelial cells was measured using the bisbenzimide fluorescent dye (Roche Diagnostics) as previously described by Hofland *et al.*[15]

Expression of cell adhesion molecules

CC531 and mesothelial cells were prepared for staining by cytospin preparation, fixed in acetone for one 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 mouse anti rat monoclonal antibodies to ICAM-1 (kindly provided by E. de Heer, University Hospital Leiden, Leiden, The Netherlands), E-cadherin (provided by L. Litvinov, University Hospital Leiden, Leiden, The Netherlands), CD44 (kind gift from J. Ate, Academic Medical Centre, Amsterdam, The Netherlands) and VCAM-1 (A. Duijvestijn, University of Maastricht, Maastricht, The Netherlands). Negative controls were incubated with PBS only. The cytospins were subsequently incubated with biotinylated goat-anti-mouse secondary antibodies (Dako, Glostrup, Denmark), rinsed and finally incubated with a Streptavidin-biotinylated horseradish peroxidase complex (Dako, Glostrup, Denmark). Cytospins were developed in 3.3' diaminobenzidine tetrachloride. To detect CD49a, CD49b and CD29 integrin chains, hamster anti-rat primary antibodies were used followed by a FITC conjugated secondary antibody (Pharmingen, Leiden, The Netherlands). The expression of cell adhesion molecules was quantified by two separate observers using a 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 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 within the cells (data not shown).

A dilution series was made by using labelled CC531 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 20-30% of the total amount of tumour cells added.

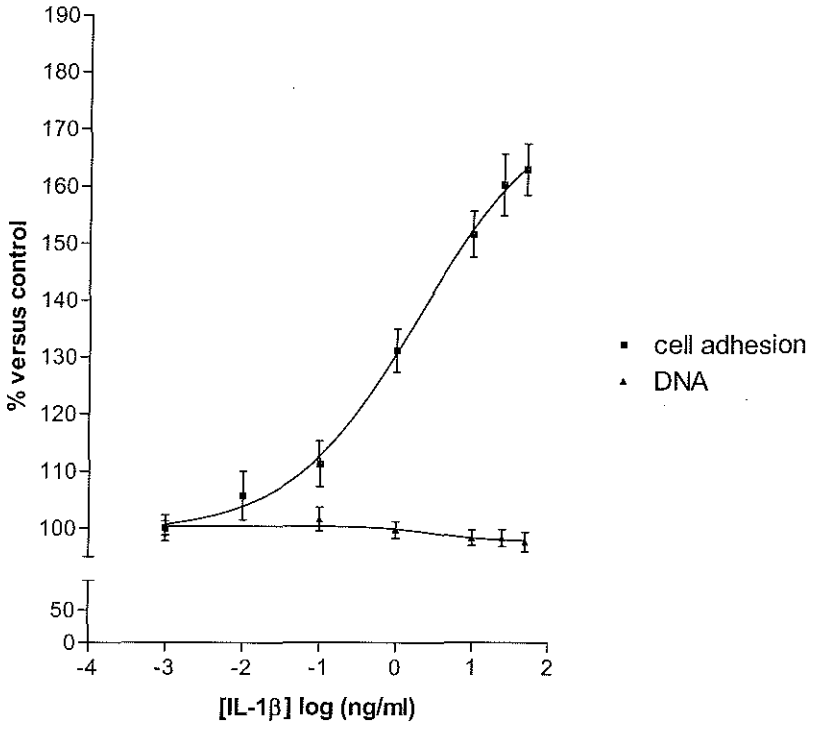


Figure 7.1

Tumour cell adhesion to, and growth of a mesothelial monolayer after pre-incubation with varying concentrations IL-1β. Data are expressed as the mean (n=6) +SEM. EC_{50} for tumour cell adhesion is 2 ng/ml IL-1β. Pre-incubation did not significantly modify total DNA of the monolayer.

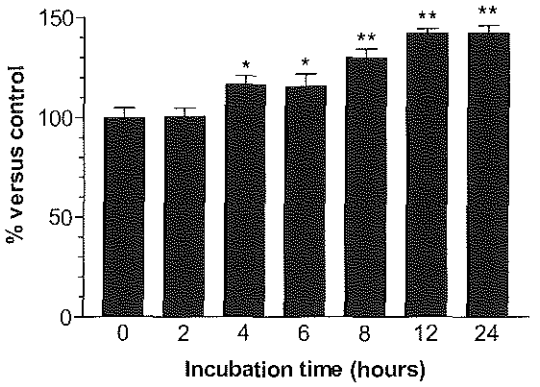


Figure 7.2

Tumour cell adhesion to a mesothelial monolayer after pre-incubation with 10 ng/ml IL-1β at varying time intervals. Data are expressed as the mean (n=6) +SEM. * $p<0.01$ and ** $p<0.001$

Effect of cytokine pre-incubation

Pre-incubation of the mesothelial monolayer with IL-1β resulted in enhanced tumour cell adhesion. This effect was dose dependent. Significant stimulation was already achieved with 0.1 ng/ml IL-1β (11% more cell adhesion *versus* non pre-incubated mesothelium, $p \leq 0.05$). Maximum stimulation was achieved with 50 ng/ml IL-1β ($p \leq 0.001$). The augmented adhesion at this concentration was at least 60% more than adhesion to non pre-incubated mesothelium. The EC_{50} of the IL-1β effect is 2 ng/ml (figure 7.1 and table 7.1).

At least 4 hours of pre-incubation were needed to induce enhanced cell adhesion (10 ng/ml IL-1β, $p \leq 0.01$). Maximal cell adhesion was achieved after pre-incubation for 12 hours with this concentration IL-1β (42% stimulation). The observed data suggest that IL-1β stimulation is time dependent and therefore involves secondary protein synthesis (figure 7.2).

After blocking with anti-IL-1β, stimulation with 1 ng/ml IL-1β (0,1 μg/ml anti-IL-1β) was completely inhibited and stimulation with 10 ng/ml IL-1β (1 μg/ml anti-IL-1β) partially inhibited (50%). Pre-incubation of mesothelium with anti-IL-1β alone did not affect tumour cell adhesion. These results underline the specificity of IL-1β stimulation and

demonstrates the involvement of a specific receptor related pathway at which enhanced tumour cell adhesion to the mesothelium is induced (figure 7.3).

Interestingly, TNF-α (1-100 ng/ml) and IFN-γ (1-100 ng/ml), known to upregulate several cell adhesion molecules (eg ICAM-I and VCAM-I), did not affect adhesion of CC531 cells to the mesothelial monolayer. Pre-incubation with IL-6 (1-100 ng/ml) did not result in altered tumour cell adhesion either (table 7.1).

Of the studied growth factors, EGF proved a potent stimulator of tumour cell adhesion. With light microscopy, morphologic changes were seen in cell appearance of the monolayer. There was a dose dependent stimulation of cell adhesion to overnight pre-incubated mesothelium. Maximal stimulation was achieved at 10^{-9} M. The EC_{50} is 2.6 x 10^{-11} M EGF (figure 7.4 and table 7.2).

Pre-treatment with TGF-β (0.1-10 ng/ml) lead to a minor (maximal 16%) though significant stimulation in cell adhesion ($p<0.05$). IGF-I (10^{-8}–10^{-7} M) did not affect tumour cell adhesion (table 7.2).

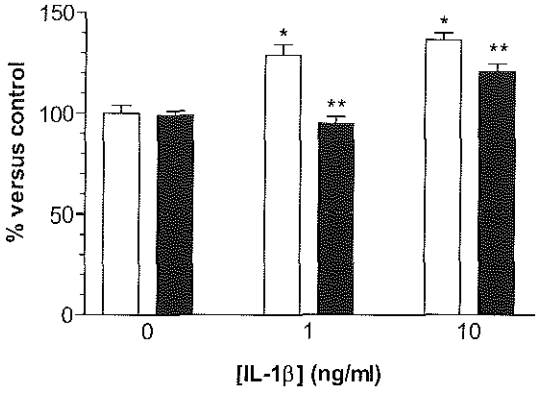


Figure 7.3

Tumour cell adhesion after pre-incubation of a mesothelial monolayer with 1 and 10 ng/ml IL-1β, with additional blocking using anti IL-1β (0.1 and 1 μg/ml respectively). Open bars represent IL-1β pre-treated monolayers and closed bars represent blocking of IL-1β stimulation with anti IL-1β. Values are the mean of n=4+SEM. * $p<0.01$ (IL-1β *vs* control) and ** $p<0.01$ (IL-1β *vs* anti IL-1β)

DNA-assay

Pre-incubation with IL-1β (≥12 hours) 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 is not caused by an increase in the number of mesothelial cells (figure 7.1).

EGF induced a significant growth of the mesothelial cells, consistent with the increased cell adhesion to mesothelium. At 10^{-10} M EGF stimulation, cell growth was stimulated by 10%, coinciding with 31% more cell adhesion. At 10^{-9} M EGF and higher concentrations this stimulation was 17%, coinciding with 39% stimulation. The EC_{50} for stimulation of mesothelial cell growth is 9.5×10^{-11} M EGF (figure 7.4).

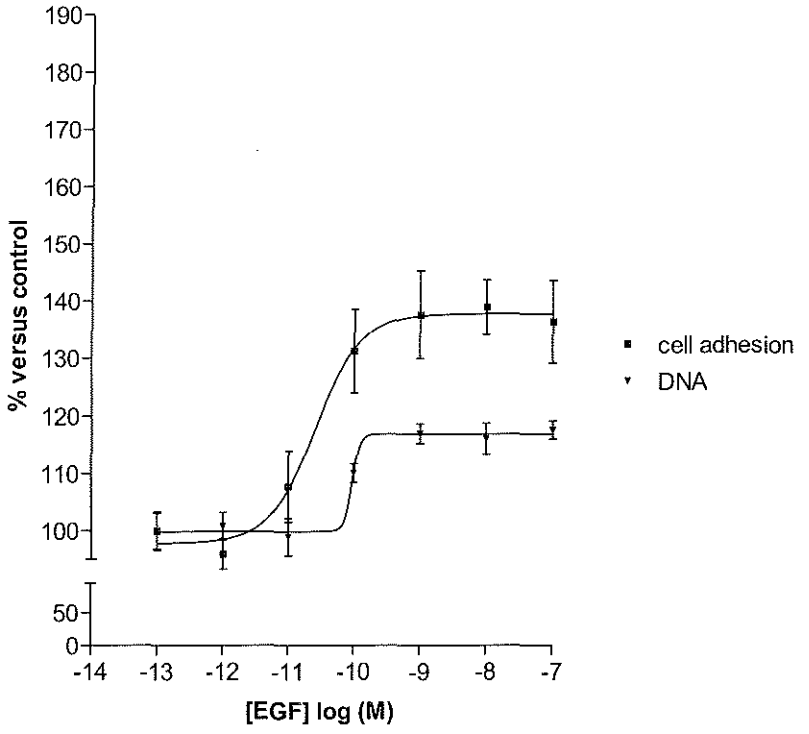


Figure 7.4

Tumour cell adhesion to, and growth of a mesothelial monolayer after pre-incubation with increasing concentrations EGF. Data are expressed as the mean (n=6) +SEM. The EC_{50} for tumour cell adhesion is 2.6×10^{-11} M EGF. Pre-incubation significantly enhanced total DNA of the monolayer indicating stimulated mesothelial cell growth (EC_{50} 9.5×10^{-11} M).

Concentration (ng/ml)	IL-1β	TNF-α	IL-6	TGF-β	IFN-γ
0.1	111 ± 14*			111 ± 11*	
1	131 ± 15**	110 ± 13	106 ± 7	116 ± 6*	94 ± 14
10	152 ± 14**	112 ± 13	100 ± 10	111 ± 9*	97 ± 14
50	163 ± 14**	102 ± 12	102 ± 7		98 ± 12
100		97 ± 9	102 ± 6		108 ± 14

Table 7.1

Tumour cell adhesion after pre-incubation of a mesothelial monolayer with cytokines or growth factors. Means (n=6, % *vs* control) +SD are shown. * $p<0.05$, ** $p<0.01$

Concentration (M)	EGF	IGF-I
10^{-11}	108 ± 14	
10^{-10}	131 ± 18*	
10^{-9}	138 ± 19**	
10^{-8}	139 ± 12**	101 ± 8
10^{-7}	132 ± 18**	100 ± 6

Table 7.2

Tumour cell adhesion after pre-incubation of a mesothelial monolayer with growth factors. Means (n=6, % *vs* control) + SD are shown. * $p<0.05$, ** $p<0.01$

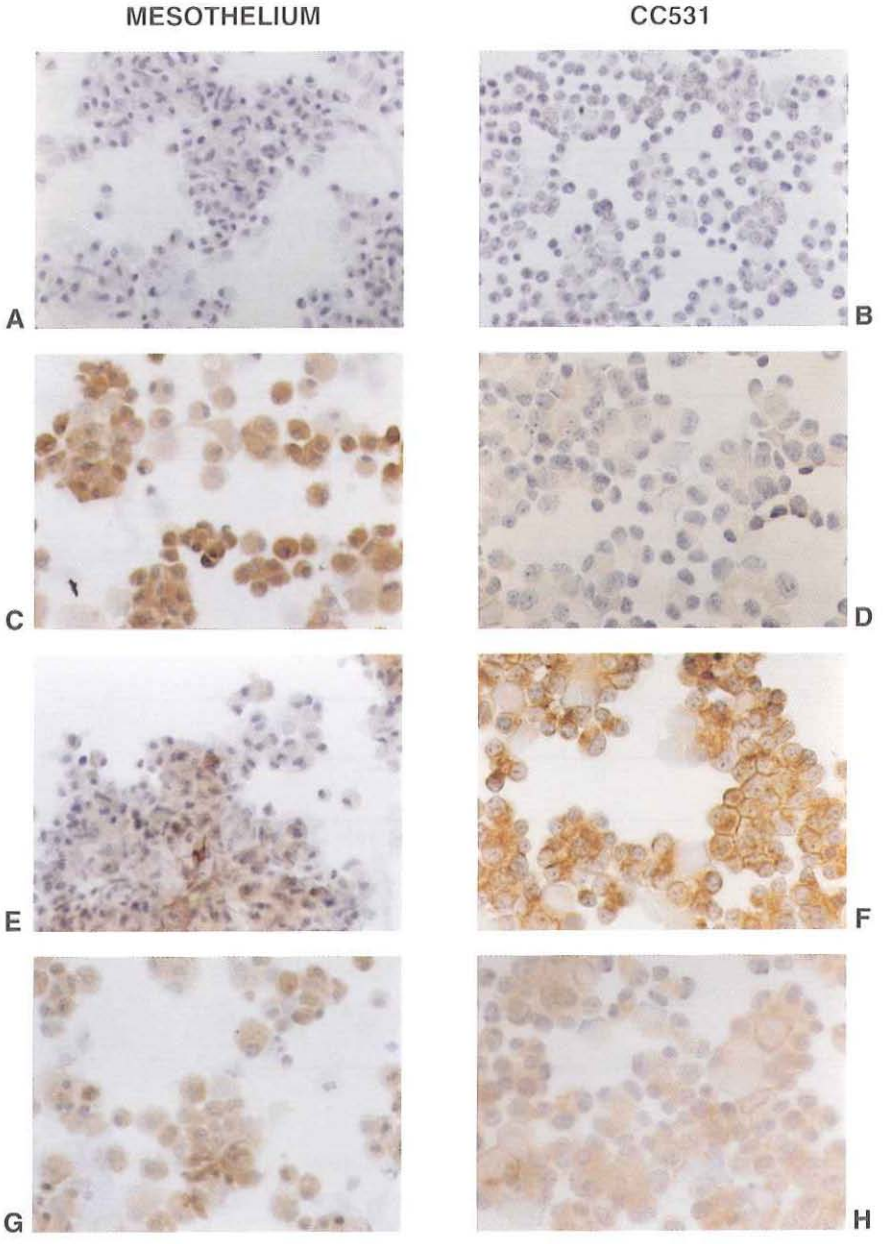


Figure 7.5

Expression of cell adhesion molecules by mesothelial and CC531 cells. Figures A and B: negative control; C and D: ICAM-I; E and F: E-cadherin; G and H: CD44.

Expression of cell adhesion molecules

A variety of cell adhesion molecules are expressed on both cell types offering sites for specific cell-cell and cell-matrix adhesion. Results are shown in figure 7.5 and table 7.3.

Cell adhesion molecule	**Mesothelial cells**	**CC531 cells**
ICAM-I	+	±
VCAM-I	+	−
E-cadherin	+	+
CD44	+	+
CD49a	+	±
CD49b	+	+
CD29	+	+

Table 7.3

Cell adhesion molecules expressed by mesothelial and CC531 cells.

DISCUSSION

For manifest tumour recurrence to develop, adhesion and growth of per-operatively spilled tumour cells is necessary. Cytokines and other inflammatory mediators, produced in great amount after surgical trauma, are known to influence tumour recurrence *in vivo*.[5;6;16] The source of these inflammatory mediators is ample. Following surgical trauma, the reactive inflammatory process will cause migration of macrophages and leukocytes to the injured site in order to promote the wound healing process.[17] In case of peritoneal trauma, the mesothelial cells lining the peritoneum take active part in the inflammatory process by producing a variety of neutrophil and monocyte chemokines such as IL-8 and IL-1β.[18;19] In order to re-establish the traumatised monolayer lining, additional stimulating factors are produced with an autocrine function.[18] Therefore locally, at traumatised sites, a rich microenvironment with high concentrations of inflammatory cytokines and growth factors are expected.

Enhanced reciprocal mesothelial-tumour cell interactions can be established in this way in a selected area.

The results of this study indicate that tumour cells adhere to a monolayer mesothelium. However, pre-treatment of mesothelium with inflammatory cytokines and growth factors can lead to more than 60% enhanced tumour cell adhesion. A significant increase in the adherence of tumour cells was seen after stimulation with IL-1β. The demonstrated time dependency of this effect, points in the direction of secondary RNA and protein synthesis required for enhanced cell adhesion. IL-1β is known to regulate the cell adhesion molecules VCAM-1 and ICAM-1 on human mesothelial cells in a time and dose dependent manner.[8;9] In the presented studies pre-treatment with IL-1β did not lead to enhanced mesothelial cell growth. The stimulus after IL-1β pre-treatment therefore, seems to be based on enhanced adhesion per cell. An upregulation of cell adhesion molecules expressed on the mesothelial cell surface is therefore likely to play a role in IL-1β enhanced tumour cell adhesion. These effects on cell adhesion are similar to those reported by others in a different model system.[20-22] In those studies, IL-1 promoted the adherence of human colorectal carcinoma cell lines and melanoma cell lines to endothelial cells in a time and dose dependent manner. Differential stimuli were seen with the melanoma cell lines occurring from highly significant enhancement to no significant change in tumour cell adhesion.[21;22]

In the presented study, EGF stimulated cell adhesion coincided with an increase in the number of mesothelial cells. EGF has been shown to be a potent growth factor for mesothelium in addition to augmenting cell adhesion and migration.[23;24] Typical reversible morphologic changes to a fibroblastic phenotype, as seen in our study, accompanied by increased expression of CD29 integrins have been demonstrated.[24]

The exact mechanism by which the increase of tumour cell adhesion to traumatised mesothelium comes about is yet to be unravelled. In several studies it has been demonstrated that TNF-α, TGF-β, IL-1β and IFN-γ have the potential to upregulate the expression of cell adhesion molecules, hereby increasing the prospect of cell adhesion.[8-10;21;25;26] However, upregulation of adhesion molecules does not always correspond with enhanced cell adhesion.[21;25] As shown in this study, enhanced tumour cell adhesion also coincides with mesothelial cell growth. Therefore, two mechanisms seem to play a role in this process. Firstly, specific enhanced adhesion per cell through

upregulation of adhesion molecules and secondly, enhanced adhesion by increase of cell number to adhere to.

Studies on cell adhesion have generally been performed to investigate the adhesive pattern of leukocytes during inflammation on activated and non-activated endothelial cells. Most studies harmoniously conclude that inflammatory cytokines lead to an upregulation of ICAM-I, VCAM-I, E-selectin and P-selectin expression, hereby inducing leukocyte rolling and extravasation.[10] Studies on mesothelial cells show a similar upregulation of cell adhesion molecules (ICAM-I and VCAM-I) after pre-treatment with cytokines.[8;9;19] This is attributed to the fact that the mesothelium plays an active role in trafficking and attachment of leukocytes to the peritoneal membrane.[8;9;19] Tumour cells may utilise this altered expression of cell adhesion molecules during inflammation by facilitated lodging to mesothelial cells.

The expression of adhesion molecules determined on the cells used in this study provides ample possibility for cell-cell and cell-matrix interaction under non-stimulated conditions. From the known interactions of adhesion molecules, the results suggest that CD44, E-cadherin and CD29 integrins may be important in tumour and mesothelial cell interactions. *In vivo* kinetics of cell adhesion molecule expression following surgical trauma are currently being investigated. Preliminary results show an upregulation of VCAM-I mRNA on mesothelial cells of the small bowel mesentery. However, it is likely that for manifest tumour recurrence more than one subset of cell adhesion molecules play a role. Ovarian carcinoma cells have been shown to use several adhesion pathways for cell binding to mesothelium *in vitro*, engaging a combined role for CD29 integrin chain and CD44.[27;28] Nakashio *et al* demonstrated CD44H and integrin CD29 mediated adhesion of gastric cancer cells to mesothelium *in vivo* and *in vitro*.[26] To fully understand the underlying mechanisms of enhanced cell adhesion after pre-treatment with cytokines and growth factors, it is therefore important to determine the variety of adhesion molecules involved and their relation to complementary sites.

In conclusion, our data suggest that tumour cells benefit from the inflammatory response by utilising cytokine induced adhesion stimulation. IL-1β appears to be an important mediator of this process. Therefore, in addition to stimulating tumour growth, inflammatory metabolites contribute actively to manifest tumour recurrence in two ways.

The apparent relation between the inflammatory process after surgical trauma and tumour recurrence necessitates further unravelling of the specific mechanisms

involved. This may lead to potential new therapeutic possibilities aimed at abating tumour cell adhesion and growth.

REFERENCES

1. Abulafi AM, Williams NS. Local recurrence of colorectal cancer: the problem, mechanisms, management and adjuvant therapy [see comments]. **Br J Surg** 1994; **81**: 7-19.
2. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. **World J Surg** 1991; **15**: 609-616.
3. Cunliffe WJ, Sugarbaker PH. Gastrointestinal malignancy: rationale for adjuvant therapy using early postoperative intraperitoneal chemotherapy. **Br J Surg** 1989; **76**: 1082-1090.
4. Bouvy ND, Marquet RL, Jeekel J, Bonjer HJ. Laparoscopic surgery is associated with less tumour growth stimulation than conventional surgery: an experimental study [see comments]. **Br J Surg** 1997; **84**: 358-361.
5. van den Tol PM, van Rossen EE, van Eijck CH, Bonthuis F, Marquet RL, Jeekel H. Reduction of peritoneal trauma by using nonsurgical gauze leads to less implantation metastasis of spilled tumor cells. **Ann Surg** 1998; **227**: 242-248.
6. Hofer SO, Shrayer D, Reichner JS, Hoekstra HJ, Wanebo HJ. Wound-induced tumor progression: a probable role in recurrence after tumor resection. **Arch Surg** 1998; **133**: 383-389.
7. Bittinger F, Brochhausen C, Kohler H, et al. Differential expression of cell adhesion molecules in inflamed appendix: correlation with clinical stage. **J Pathol** 1998; **186** : 422-428.
8. Jonjic N, Peri G, Bernasconi S, et al. Expression of adhesion molecules and chemotactic cytokines in cultured human mesothelial cells. **J Exp Med** 1992; **176**: 1165-1174.
9. Klein CL, Bittinger F, Skarke CC, et al. Effects of cytokines on the expression of cell adhesion molecules by cultured human omental mesothelial cells. **Pathobiology** 1995; **63**: 204-212.
10. Meager A. Cytokine regulation of cellular adhesion molecule expression in inflammation. **Cytokine Growth Factor Rev** 1999; **10**: 27-39.
11. Akedo H, Shinkai K, Mukai M, et al. Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: a model for tumor invasion. **Cancer Res** 1986; **46**: 2416-2422.
12. van der Wal BC, Hofland LJ, Marquet RL, van Koetsveld PM, van Rossen ME, van Eijck CH. Paracrine interactions between mesothelial and colon-carcinoma cells in a rat model. **Int J Cancer** 1997; **73**: 885-890.
13. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. **Int J Cancer** 1984; **33**: 689-692.
14. Catterall JB, Gardner MJ, Jones LM, Thompson GA, Turner GA. A precise, rapid and sensitive in vitro assay to measure the adhesion of ovarian tumour cells to peritoneal mesothelial cells. **Cancer Lett** 1994; **87**: 199-203.

15. Hofland LJ, van Koetsveld PM, Lamberts SW. Percoll density gradient centrifugation of rat pituitary tumor cells: a study of functional heterogeneity within and between tumors with respect to growth rates, prolactin production and responsiveness to the somatostatin analog SMS 201-995. **Eur J Cancer** 1990; **26**: 37-44.
16. Hofer SO, Molema G, Hermens RA, Wanebo HJ, Reichner JS, Hoekstra HJ. The effect of surgical wounding on tumour development. **Eur J Surg Oncol** 1999; **25**: 231-243.
17. Slavin J. The role of cytokines in wound healing. **J Pathol** 1996; **178**: 5-10.
18. Holmdahl L, Ivarsson ML. The role of cytokines, coagulation, and fibrinolysis in peritoneal tissue repair. **Eur J Surg** 1999; **165**: 1012-1019.
19. Nagy JA. Peritoneal membrane morphology and function. **Kidney Int Suppl** 1996; **56**: S2-11.
20. Dejana E, Bertocchi F, Bortolami MC, et al. Interleukin 1 promotes tumor cell adhesion to cultured human endothelial cells. **J Clin Invest** 1988; **82**: 1466-1470.
21. Lee KH, Lawley TJ, Xu YL, Swerlick RA. VCAM-1-, ELAM-1-, and ICAM-1-independent adhesion of melanoma cells to cultured human dermal microvascular endothelial cells. **J Invest Dermatol** 1992; **98**: 79-85.
22. Rice GE, Gimbrone MAJ, Bevilacqua MP. Tumor cell-endothelial interactions. Increased adhesion of human melanoma cells to activated vascular endothelium. **Am J Pathol** 1988; **133**: 204-210.
23. Franzen L, Ghassemifar R, Schultz G. Specific binding of EGF in connective tissue repair. **Eur J Cell Biol** 1993; **60**: 346-350.
24. Leavesley DI, Stanley JM, Faull RJ. Epidermal growth factor modifies the expression and function of extracellular matrix adhesion receptors expressed by peritoneal mesothelial cells from patients on CAPD. **Nephrol Dial Transplant** 1999; **14**: 1208-1216.
25. Kim SJ, Kim NS, Lee JL. Effect of cytokines on the expression of cell adhesion molecule and on the adhesion of melanoma cells to endothelial cells. **J Korean Med Sci** 1993; **8**: 41-52.
26. Nakashio T, Narita T, Akiyama S, et al. Adhesion molecules and TGF-beta1 are involved in the peritoneal dissemination of NUGC-4 human gastric cancer cells. **Int J Cancer** 1997; **70**: 612-618.
27. Lessan K, Aguiar DJ, Oegema T, Siebenson L, Skubitz AP. CD44 and beta1 integrin mediate ovarian carcinoma cell adhesion to peritoneal mesothelial cells. **Am J Pathol** 1999; **154**: 1525-1537.
28. Strobel T, Cannistra SA. Beta1-integrins partly mediate binding of ovarian cancer cells to peritoneal mesothelium in vitro. **Gynecol Oncol** 1999; **73**: 362-367.

Chapter VIII

THE ROLE OF INTEGRINS IN PERITONEAL TUMOUR RECURRENCE OF COLON CARCINOMA

Submitted for publication

In prior in vivo experiments described in chapter 3 we demonstrated a preferential adhesion of colon carcinoma cells to areas of the peritoneum where mesothelial cells had been exfoliated. In this study we consider the interaction of tumour cells with surgically traumatised peritoneum in depth.

In vitro studies demonstrated a preferential adhesion of tumour cells to collagen type I (50% versus <30% adhesion to other submesothelial matrix (ECM) components and mesothelial cells). Tumour cells contained mRNA encoding for ICAM-I, α_1, α_2 and β_1 integrins and expressed these adhesion molecules on the cell surface. Mesothelial cells expressed mRNA encoding for the ICAM-I receptor ($\alpha_L\beta_2$). Unlike α integrin chains, β_1 mediated tumour cell adhesion to all ECM proteins. Adhesion to collagen I was fully mediated by $\alpha_2\beta_1$ integrin. Blocking integrin or ICAM-I mediated adhesion had no effect on tumour-mesothelial cell interaction.

We conclude that for tumour cell-ECM interaction, β_1 is used as a common integrin subunit. An intact mesothelial monolayer forms a natural barrier against tumour cell adhesion. Interruption of this protective surface, as happens in surgical trauma, enhances tumour cell adhesion to submesothelial surfaces. In the event of tumour cell adhesion to intact peritoneum, this occurs through an integrin and ICAM-I independent mechanism.

INTRODUCTION

Tumour recurrence remains a frequent complication after intentionally curative resection of colorectal malignancies. Apart from distant metastases, isolated loco-regional recurrence rates vary between 3 and 35%.[1-4] Patterns of first recurrence show the resection site is the most common anatomic location and is often combined with recurrence on peritoneal surfaces.[1;2;4;5] Per-operative shedding of tumour cells due to handling the tumour and leakage from dissected lymphatic channels are the most likely causes of tumour recurrence. Adhesion of these spilled tumour cells and subsequent growth results in manifest peritoneal tumour recurrence.[5]

In an experimental rat model we demonstrated earlier that surgical trauma of the peritoneum correlates with tumour recurrence *in vivo*.[5] At the site of inflicted trauma, the mesothelial cells lining the peritoneum were exfoliated, hereby exposing the underlying extra cellular matrix (ECM). Recurrence of CC531 colon carcinoma cells was primarily seen at the traumatised sites, in addition to other non-traumatised peritoneal surfaces.[6;7] Tumour cell adhesion to either exposed underlying matrix components or mesothelial cells of the intact peritoneum may be the key component in the process of tumour recurrence.

To realise adhesion, tumour cells make use of specific receptors expressed on the cell surface. Integrins are cell surface receptors mediating cell adhesion to ECM components and also, to a lesser extent, other cells. ICAM-I, the counterpart of $\alpha_L\beta_2$ integrin, is uniformly expressed by mesothelial cells and used by leukocytes to enter the abdominal cavity. The aim of this study was to explore the role of integrins in the adhesive pattern of CC531 colon carcinoma cells to different ECM components and mesothelium. Furthermore, the role of ICAM-I in tumour cell adhesion was assessed to explore a malign use of normally expressed adhesion molecules. With these experiments an attempt was made to clarify the use of integrins in the modalities of interaction between CC531 cells and the peritoneum following surgical trauma.

MATERIALS AND METHODS

Animals

Inbred WAG/Rij rats, obtained from Harlan-CPB, Zeist, The Netherlands were used. The rats were bred under specific pathogen-free conditions, kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 hours light/ 12 hours dark), fed with laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee in Animal Research of the Erasmus University Rotterdam.

Cell culture

CC531 is a moderately differentiated, weakly immunogenic colon adeno-carcinoma induced in the WAG/Rij rat by 1,2-dimethylhydrazine. A cell line was established from this carcinoma and maintained by serial passage after trypsinization in culture medium.[8] For continuous culture RPMI 1640 medium supplemented with 5% foetal calf serum, L-glutamin (2 mM) and penicillin (10^5 U/L) was used.

Mesothelial cells were isolated from the small bowel mesentery of WAG/Rij rats and brought into culture as described before.[9] Briefly, resected mesenterial sheaths were incubated in collagenase (1g/L) and dispase (2.4 10^3 U/L) (Boehringer, Mannheim, Germany). The detached mesothelial cells were pelleted and resuspended in RPMI 1640 medium supplemented with 10% foetal calf serum, L-glutamin (2 mM), penicillin (10^5 U/L) and fungizone (1.25 mg/L). Mesothelial monolayers were established in 96 well plates, precoated with collagen type I (17.5 $\mu g/cm^2$). For all experiments, primary cell cultures were used.

Medium, supplements and foetal calf were purchased at Life Technologies, Breda, The Netherlands.

Calcein-AM solution and incubation

The dye solution, calcein-AM, used to quantify tumour cell adhesion was prepared by dissolving 50 µg calcein-AM (Molecular Probes, Leiden, The Netherlands) in 5 µl anhydrous dimethyl sulphoxide. This solution was added to 5 ml of the RPMI medium supplemented with 0.5% BSA. Trypsinized CC531 cells ($1x10^6$ cells/ml) were incubated in this solution at 37°C for 45 minutes with occasional mixing. Calcein incubation did not influence the viability of CC531 cells as determined by trypan blue exclusion.

Cell attachment assay

To quantify tumour cell adhesion, a standardised cell adhesion assay was developed according to methods from Catterall *et al.*[10] Flat-bottomed 96 well culture plates were coated according to prescription of the manufacturer with collagen I (17.5 μg/ml), laminin (33 μg/ml), fibronectin (20 μg/ml) (Boehringer Mannheim, Mannheim, Germany), collagen IV (20 μg/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands) or with a mesothelial monolayer grown to confluence. To evaluate the contribution of integrins in the adhesion of tumour cells to extra cellular matrix components, calcein-labelled CC531 cells were pre-incubated with the function blocking antibodies anti-α_1, anti-α_2 and anti-β_1 (concentration 0.005 μg/ml to 1 μg/ml) for 30 minutes at room temperature. After incubation, the coated plates were washed with medium and tumour cells were added at a density of 30,000/200μl. The effect of anti-α_1, α_2, β_1 and ICAM-1 antibodies on tumour cell adhesion to a monolayer mesothelium was assessed by either pre-incubating the tumour cells or the mesothelial cells with antibodies before adding tumour cells (density of 30,000/200μl) to the mesothelium. Cell adhesion was measured after 60 minutes.

Total cell adhesion was assessed after washing away unbound cells and by measuring total fluorescence on a Perkin Elmer plate reader using 485 excitation and 530 emission filters. On each plate a standard curve was prepared by adding different numbers of calcein-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.

Adhesion assays were performed with RPMI medium supplemented with 0.5% bovine serum albumin (BSA, Sigma-Aldrich, Zwijndrecht, The Netherlands). All function-blocking antibodies were purchased at Pharmingen (Leiden, The Netherlands) and also used for flow cytometry analysis.

Cellular integrin expression profile using RT-PCR and Flow cytometry

Cellular integrin expression profiles were analysed by RT-PCR and flow cytometry. For the RT-PCR study, oligonucleotides were devised to specifically amplify rat sequences. The primers were based on rat cDNA sequences present in the database or were chosen in regions of high similarity between mouse and human cDNA sequences (see table 8.1). The specificity of the oligonucleotides was checked by RT-PCR procedure using RNA from various rat tissues such as spleen, liver, lung, kidney and lymph nodes. Total RNA was isolated with RNAzol™ B (Campro Scientific B.V., Veenendaal, The Netherlands) according to recommendations by the manufacturer. The procedure is essentially an improved version of the RNA isolation method described by Chomczynski and Sacchi.[11] One μg of total RNA was used as a template in a reverse transcriptase reaction containing 400 U M-MLV-Reverse Transcriptase, 40 U RNasin, 0.5 mM dNTP's (Promega, Madison, WI) and 20 ng random primers (Gibco-BRL, Gaitherburg, MD) using the M-MLV reaction buffer supplied by Promega. Part of the RT reaction mixture was subjected to PCR amplification using the primers sets described above. RT-negative and water controls were included in each experiment and never gave rise to a detectable amplified DNA fragment. The amplified DNA fragments were cloned into the pCR2.1™ cloning vector (Invitrogen, Carlsbad, CA) and sequenced to verify the nature of the amplified product. The presence of mRNAs encoding various integrins and ICAM-I was determined in total RNA preparations of CC531 colon carcinoma cells and mesothelial cells using an identical RT-PCR protocol.

Expression of α_1, α_2 and β_1 on tumour cells and mesothelial cells was measured using FACS analysis and analysed using CellQuest software. (Becton Dickinson, San Jose, CA, USA). The indirect immunofluorescence method was used to stain the cells. Cells ($5x10^5$ in 50μl) were incubated with 50μl PBS/0,2%BSA containing the specific primary antibody or isotype control antibody (dilution 1:10 to 1:10,000) at room temperature during 10 minutes and subsequently rinsed twice with 2ml PBS/0,2%BSA solution. Then the cells were incubated with 50μl PBS/0,2%BSA containing FITC-conjugated secondary antibody (dilution 1:50) for another 10 minutes. The cells were washed twice again with 2ml PBS/0,2%BSA solution. IgG and IgM isotype controls served as negative controls. All monoclonal antibodies (purified hamster anti-rat α_1, α_2 and β_1; purified mouse anti-rat ICAM-1), FITC labelled immunoglobulins (FITC anti-hamster IgG and –IgM, FITC anti-mouse IgG) and isotype controls (hamster IgG and IgM) were purchased at Pharmingen (Leiden, The Netherlands).

Statistical analysis

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.

All experiments were carried out at least twice with comparable results.

	Sequence	Size of PCR product
ICAM-1	5'-GTTCTTGTGTTCCCTGGAAGGCC (forward) 5'-GCACTGTCAGGTGCACGTCC (reverse)	757 bp
α_1	5'-CTACAAGATGGAGGATGGG (forward) 5'-GCCTCAGTGAATCAAGGG (reverse)	790 bp
α_2	5'-CATGGATGGCTTTAATGATGTG (forward) 5'-GTTGACAACATCAGAGGGC (reverse)	561 bp
α_4	5'-CTGACGTGATTACAGGAAGC (forward) 5'-CCATACACAAATGAAGTTGGG (reverse)	747 bp
α_L	5'-GCTGTGTACATCTTCAATGGG (forward) 5'-CCACAGTTCTTCTCAAAAGGG (reverse)	667 bp
β_1	5'-CCCAATGATGGCCAGTGTCACC (forward) 5'-GCACCACATTCACAGATGCCCC (reverse)	967 bp
β_2	5'-GGCCCTCAACGAGATCACCG (forward) 5'-CCAGCTCCTGGCTGCTCCG (reverse)	1015 bp

Table 8.1

Cell adhesion molecule primers used for RT-PCR.

RESULTS

Adhesion of CC531 cells to ECM proteins and mesothelial cells

Because adhesion is thought to represent the initial step in tumour recurrence, the adhesive properties of CC531 cells to matrix proteins and a mesothelial monolayer were investigated. Adhesion of CC531 cells reached a plateau at 60 minutes as demonstrated in preliminary analysis of binding kinetics (data not shown). CC531 tumour cells exhibited a preferential adhesion to type I collagen (53% of total cell number added *versus* <30% to other adhesive surfaces; $p \leq 0.01$). Data from representative experiments is shown in figure 8.1.

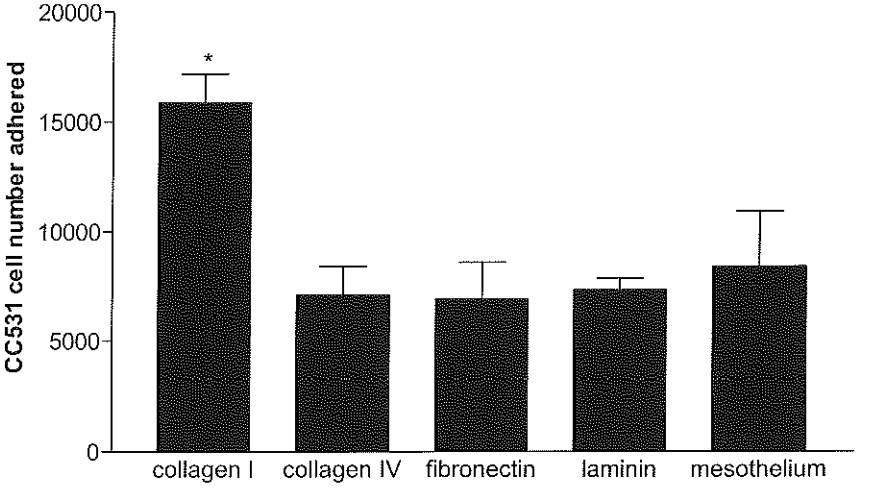


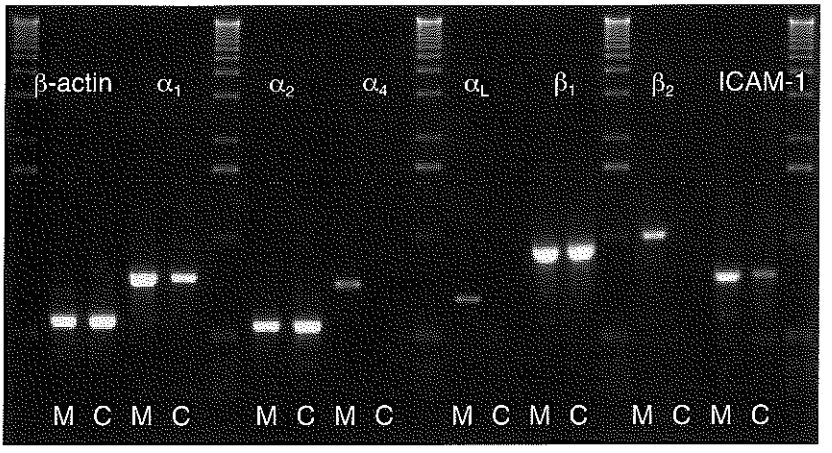
Figure 8.1

Tumour cell adhesion after 60 minutes to different ECM components and a monolayer mesothelium. Values are n=4 +SD, the results of a representative experiment are shown. *$p \leq 0.01$ *versus* collagen IV, fibronectin, laminin and monolayer mesothelium.

Analysis of integrin expression

RT-PCR was performed to investigate the ability of the tumour cells to express cell adhesion molecules. The integrity of total RNA isolated from CC531 and mesothelium was checked on an agarose gel. Amplified fragments of the expected size were found. The results show that CC531 cells express a mRNA pattern of adhesion molecules that allow interaction with ECM components. Mesothelial cells express ICAM-I and a distinct set of integrins, differing from the expression by tumour cells. The results are shown in figure 8.2.

A preferential adhesion to collagen I shown in the adhesion study, suggested that CC531 cells express the integrins mediating this interaction ($\alpha_1\beta_1$ and $\alpha_2\beta_1$) on the cell surface. FACS analysis demonstrated both cell types expressed α_2 and β_1 integrin chains. The α_1 integrin chain was detected on mesothelial cells, expression on CC531 cells however, was extremely low. As a positive cellular control rat lymphocytes were used and showed significant expression of the three integrins (data not shown). A representative result is shown in figure 8.3. With immuno histochemistry, ICAM-1 and VCAM-1 expression were demonstrated on mesothelial cells (chapter 7).



Cell type	α_1	α_2	α_4	α_L	β_1	β_2	ICAM-1
Mesothelium	+	+	+	+	+	+	+
CC531	+	+	–	–	+	–	+

Figure 8.2

Indicated is the presence (+) or absence (-) of a correctly sized amplified DNA fragment.

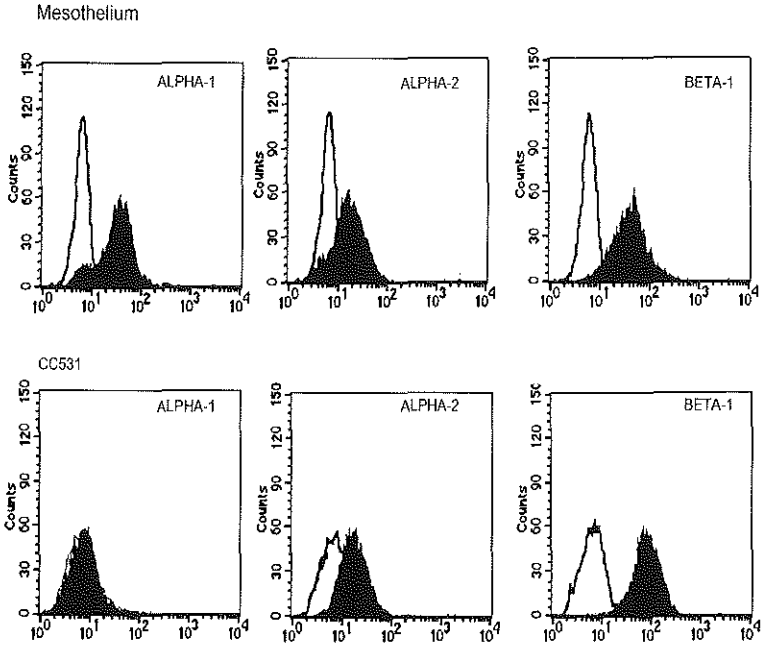


Figure 8.3

Detection of surface membrane expression of α_1, α_2 and β_1 (all at 1:100 dilution) on CC531 colon carcinoma and mesothelial cells by flow cytometry.

Effect of function blocking antibodies on tumour cell adhesion

Adhesion to type I collagen was completely inhibited by adding 0.05 μg/ml α_2 antibody. A clear dose-dependency of this statistically significant inhibitory effect is shown in figure 4. A similar pattern of inhibition was seen with type IV collagen and laminin. Cell adhesion to fibronectin was not affected significantly (figure 8.4). When adding α_1 antibody (concentrations ranging from 0.1 - 1 μg/ml) a slight inhibition of tumour cell adhesion was seen to type IV collagen and laminin (20-40% inhibition of the total number of cells added, inhibition at 0,5 μg/ml, data not shown). The inhibitory effect of β_1 antibody (concentrations ranging from 0.01 - 1 μg/ml) was most evident on laminin, followed by collagen I and IV and fibronectin (figure 8.5). Combining α and β chain antibodies ($\alpha_1\beta_1$ and $\alpha_2\beta_1$, concentrations of either integrin chain ranging from 0.1 - 1 μg/ml) did not cause a synergistic inhibition on tumour cell adhesion.

Pre-incubation of the tumour cells or the mesothelium with integrin antibodies did not affect tumour cell adhesion to a monolayer mesothelium indicating that α_1, α_2 nor β_1 play a role in this interaction. ICAM-1, expressed by CC531 and mesothelium, was also inhibited using a function blocking antibody (concentrations ranging from 0.01 - 10 μg/ml). Surprisingly, there was no effect on tumour cell adhesion indicating that ICAM-1 does not play an effective role in this interaction. The same ICAM-1 antibody was used in binding studies with FACS scan demonstrating the presence of ICAM-I on mesothelial cells and the validity of the antibody.

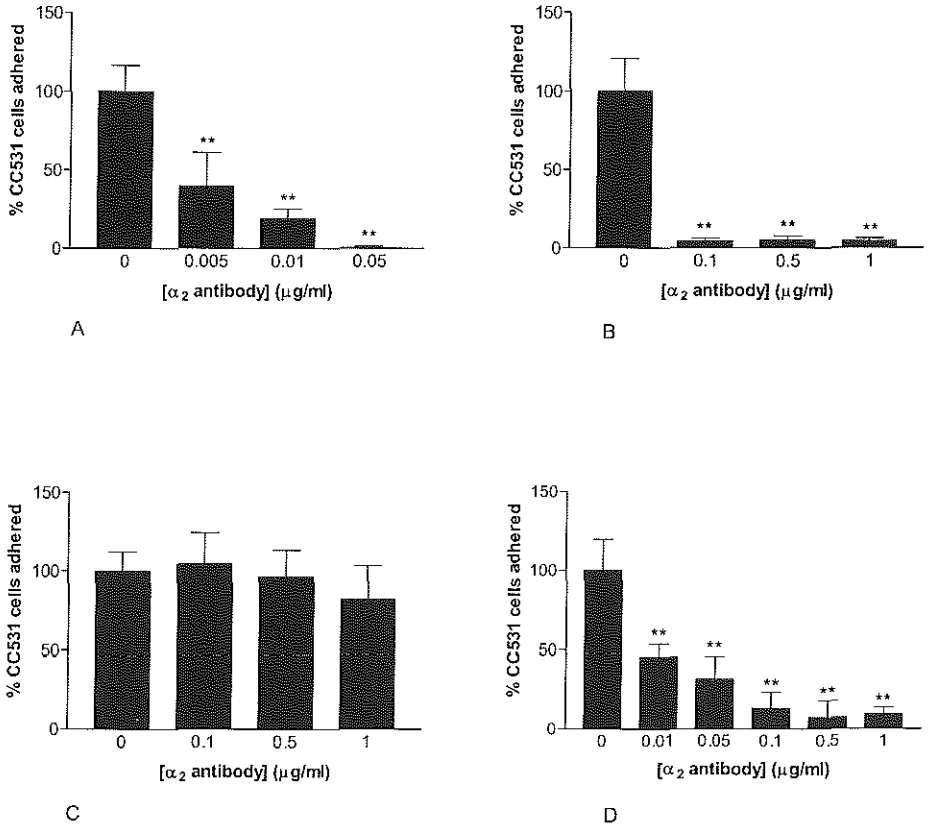


Figure 8.4

Dose dependent inhibition of tumour cell adhesion to collagen I (A), collagen IV (B), fibronectin (C) and laminin (D) after pre-incubation with varying concentrations of α_2 antibody ranging from 1 to 0.005 μg/ml. Values are n=6 +SD. *p≤0.01 and **p≤0.001 *versus* control.

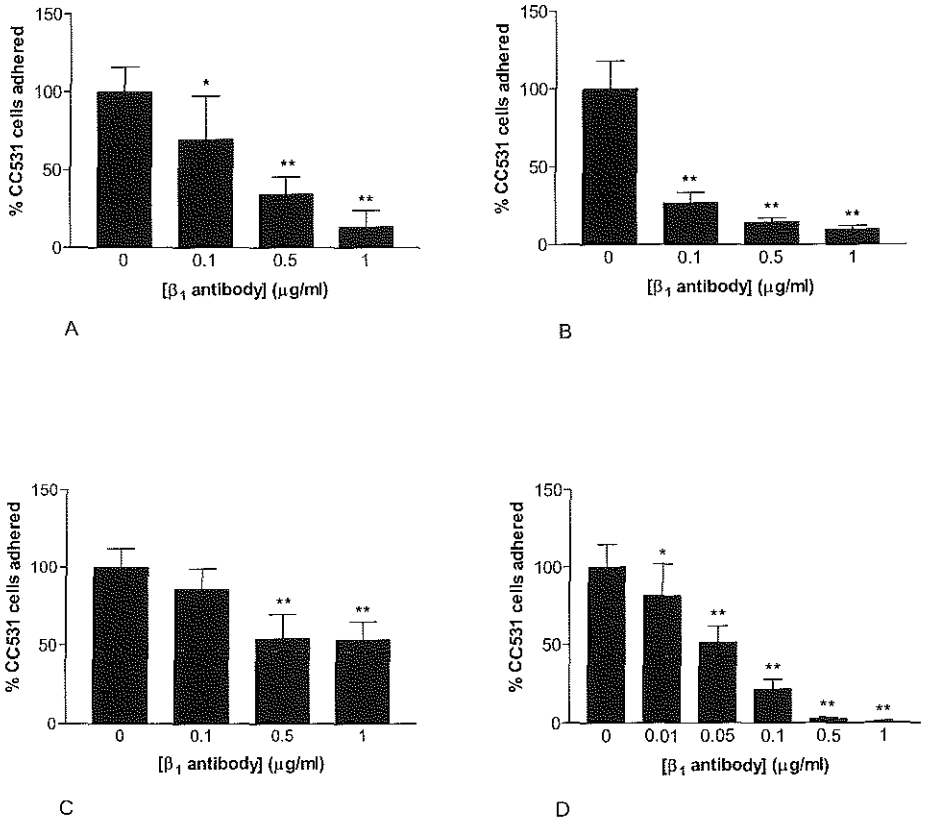


Figure 8.5

Dose dependent inhibition of tumour cell adhesion to collagen I (A), collagen IV (B), fibronectin (C) and laminin (D) after pre-incubation with varying concentrations of β_1 antibody ranging from 1 to 0.01 μg/ml. Values are n=6 +SD. *p≤0.01 and **p≤0.001 *versus* control.

DISCUSSION

The primary purpose of this study was to analyse the functional role of integrins in the adhesion of CC531 colon carcinoma cells to mesothelium and ECM components. In an experimental model in rats we demonstrated earlier (chapter 3) that surgical trauma of the peritoneum resulted in more adhesion and growth of CC531 cells *in vivo*.[6] This was most evident at the traumatised sites. Paraffin embedded sections of this area revealed that the mesothelial lining of the peritoneum had been damaged, hereby uncovering the underlying submesothelial matrix proteins (see figure 1, chapter 4).[6;7] These results indicate that tumour cells adhere more efficiently to submesothelial matrix components. This study evaluated the interaction of adhesion of CC531 cells to mesothelium and ECM components *in vitro*. Our data demonstrate that CC531 cells adhere preferentially to type I collagen. Collagen I is the primary structural component of most matrices together with fibronectin. Type IV collagen and laminin comprise the basal lamina on which most cell linings rest.[12] Adhesive patterns of human colon carcinoma cells also show differential adhesion to fibronectin, collagen and laminin.[13;14] The favoured adhesion to collagen in our study suggests that tumour cells express integrins known to mediate this interaction ($\alpha_1\beta_1$ and $\alpha_2\beta_1$).[15-17]

In the presented study the role of α_1, α_2 and β_1 subunits in mediating the adhesion of CC531 cells to different ECM components and mesothelium was studied using a variety of methods. With RT-PCR the expression of mRNA for α_1, α_2, α_4, α_L, β_1 and β_2 integrin chains and ICAM-1 was demonstrated. Flow cytometry demonstrated α_2 and β_1 subunits were prominently expressed on CC531 and mesothelial cells. Human colon carcinoma cells display a similar pattern of integrin expression. In similar studies using human colorectal cancer cell lines, α_2 and β_1 integrins were abundantly expressed, while ICAM-I and other α and β integrin chains were differently expressed.[13;14]

The activity of integrins expressed at the cell surface however, is versatile. Numerous studies have reported the pathways of inside-out and outside-in signalling of integrins as means of activation. The $\alpha_2\beta_1$ integrin has been shown to be induced from being totally inert to an active collagen receptor.[15;18] To correlate between integrin expression and function we therefore additionally investigated the functional capacity of expressed integrin chains in cell adhesion kinetics.

In these adhesion experiments, function blocking antibodies directed against integrin α_2 and β_1 subunits significantly inhibited adhesion to laminin, type I and type IV collagen,

providing direct evidence for the functional role of $\alpha_2\beta_1$ in tumour cell adhesion to these matrix proteins. The β_1 subunit antibody also inhibited adhesion to fibronectin showing the wide use of a common β_1 subunit. In contrast, although very moderately expressed on the cell surface, the α_1 subunit significantly inhibited tumour cell adhesion to type IV collagen and laminin. Similar results were reported of ovarian epithelial carcinoma cells binding collagen and laminin, although antibodies directed against the β_1 subunit inhibited adhesion more effectively than antibodies against α_2.[19] Adhesion studies of the human colon carcinoma SW480 demonstrated inhibited cell adhesion to laminin and fibronectin by anti-β_1, anti-α_2 had no effect (collagen not investigated).[14] Binding of HT29 to collagen and laminin was also effectively inhibited by anti-β_1. Moreover, anti-β_1 inhibited the invasive capacity of HT29 through a reconstituted basement membrane (Matrigel).[13;14] The association of the intracellular domain of β1 with cytoskeletal components[15] probably plays a vital role in the function of this integrin.

In our study, none of the integrin antibodies affected tumour cell adhesion to a mesothelial monolayer. Blocking of the ICAM-1 receptor, which is commonly expressed on mesothelial cells, did not affect adhesion either. Although the counterpart $\alpha_L\beta_2$ is not expressed by CC531 (as demonstrated by RT-PCR), a reversed interaction is possible with $\alpha_L\beta_2$ on mesothelium and ICAM-I on the tumour cells. These results indicate that neither ICAM-1 nor its counterpart $\alpha_L\beta_2$, nor $\alpha_1\beta_1$ or $\alpha_2\beta_1$ integrins, contribute to the tumour cell – mesothelial cell interaction. Studies on the adhesive properties of human colon cancer cell lines also demonstrate that β_1 integrin does not mediate the adhesion of human colon carcinoma cell lines to mesothelium.[13;14]

Through integrin mediated signal transduction, cell-matrix interactions may contribute to regulation of adhesion, growth, migration and differentiation of cells.[15;16;18] The role of $\alpha_2\beta_1$ has been demonstrated in several types of cancer including colon-, ovarian- and lungcancer, osteosarcoma and melanoma. In melanoma it plays a role in cell adhesion, progression and matrix reorganisation.[15] Additionally, adhesion to an ECM composed primarily of collagen through $\alpha_2\beta_1$ integrin has been shown to increase production of metalloproteinases enabling dissemination of cancer cells.[19;20] Disruption of cell adhesion by antibodies against integrin receptors could therefore prove useful as a means of inhibiting these pathways of tumour recurrence. The inhibition of cell adhesion obtained in the present study with antibodies against α_2 and β_1 subunits might indicate a possible approach for new therapy. However, non-selective peptides

(ie RGD-directed integrins) may be a more feasible tool for *in vivo* blocking of integrin mediated adhesion. Because most cells express surplus β1 subunits[21] and the demonstrated role of this integrin chain in tumour cell adhesion[13-15;18;19;21], aiming therapy at this integrin seems plausible.

In summary, we have shown that tumour cells show preferential adhesion to sub-mesothelial collagen. An intact mesothelial monolayer serves as a natural barrier against tumour cell adhesion. In the event of tumour cell adhesion to intact peritoneum, this occurs through an integrin and ICAM-I independent mechanism. As in similar studies using human colon carcinoma cell lines, we demonstrated that for tumour cell - ECM interaction, β_1 is used as a common integrin subunit. Variability in cell adhesion to ECM is primarily mediated through α integrin chains. Preventing tumour cell adhesion through blocking the β_1 integrin receptor, may prove a powerful tool in the prevention of post surgical tumour recurrence.

REFERENCES

1. Brodsky JT, Cohen AM. Peritoneal seeding following potentially curative resection of colonic carcinoma: implications for adjuvant therapy. **Dis Colon Rectum** 1991; **34**: 723-727.
2. Galandiuk S, Wieand HS, Moertel CG, et al. Patterns of recurrence after curative resection of carcinoma of the colon and rectum. **Surg Gynecol Obstet** 1992; **174**: 27-32.
3. Michelassi F, Vannucci L, Ayala JJ, Chappel R, Goldberg R, Block GE. Local recurrence after curative resection of colorectal adenocarcinoma. **Surgery** 1990; **108**: 787-792.
4. Obrand DI, Gordon PH. Incidence and patterns of recurrence following curative resection for colorectal carcinoma. **Dis Colon Rectum** 1997; **40**: 15-24.
5. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. **World J Surg** 1991; **15**: 609-616.
6. van den Tol PM, van Rossen EE, van Eijck CH, Bonthuis F, Marquet RL, Jeekel H. Reduction of peritoneal trauma by using nonsurgical gauze leads to less implantation metastasis of spilled tumor cells. **Ann Surg** 1998; **227**: 242-248.
7. van den Tol MP, van S, I, Bonthuis F, Marquet RL, Jeekel J. Reduction of intraperitoneal adhesion formation by use of non-abrasive gauze. **Br J Surg** 1997; **84**: 1410-1415.
8. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. **Int J Cancer** 1984; **33**: 689-692.
9. van der Wal BC, Hofland LJ, Marquet RL, van Koetsveld PM, van Rossen ME, van Eijck CH. Paracrine interactions between mesothelial and colon-carcinoma cells in a rat model. **Int J Cancer** 1997; **73**: 885-890.
10. Catterall JB, Gardner MJ, Jones LM, Thompson GA, Turner GA. A precise, rapid and sensitive in vitro assay to measure the adhesion of ovarian tumour cells to peritoneal mesothelial cells. **Cancer Lett** 1994; **87**: 199-203.
11. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. **Anal Biochem** 1987; **162**: 156-159.
12. Nagy JA. Peritoneal membrane morphology and function. **Kidney Int Suppl** 1996; **56**: S2-11.
13. Ebert EC. Mechanisms of colon cancer binding to substratum and cells. **Dig Dis Sci** 1996; **41**: 1551-1556.
14. Schlaeppi M, Ruegg C, Tran-Thang C, et al. Role of integrins and evidence for two distinct mechanisms mediating human colorectal carcinoma cell interaction with peritoneal mesothelial cells and extracellular matrix. **Cell Adhes Commun** 1997; **4**: 439-455.
15. Heino J. Integrin-type extracellular matrix receptors in cancer and inflammation. **Ann Med** 1993; **25**: 335-342.

16. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. **Cell** 1992; **69**: 11-25.
17. Menger MD, Vollmar B. Adhesion molecules as determinants of disease: from molecular biology to surgical research. **Br J Surg** 1996; **83**: 588-601.
18. Ruoslahti E. Integrins as signaling molecules and targets for tumor therapy. **Kidney Int** 1997; **51**: 1413-1417.
19. Moser TL, Pizzo SV, Bafetti LM, Fishman DA, Stack MS. Evidence for preferential adhesion of ovarian epithelial carcinoma cells to type I collagen mediated by the alpha2beta1 integrin. **Int J Cancer** 1996; **67**: 695-701.
20. Riikonen T, Westermarck J, Koivisto L, Broberg A, Kahari VM, Heino J. Integrin alpha 2 beta 1 is a positive regulator of collagenase (MMP-1) and collagen alpha 1(I) gene expression. **J Biol Chem** 1995; **270**: 13548-13552.
21. Ruoslahti E. Integrin signaling and matrix assembly. **Tumour Biol** 1996; **17**: 117-124.

Part V

Mesothelial and Tumour Cell Growth *in vitro*

Chapter IX

PARACRINE INTERACTIONS BETWEEN MESOTHELIAL AND COLON CARCINOMA CELLS IN A RAT MODEL

Adapted from the original publication in the **International Journal of Cancer** 1997; **73**: 885-890

This study used a co-culture system with Transwell tissue-culture inserts to investigate the role of primary cultures of rat peritoneal mesothelial cells on the proliferation of rat colon carcinoma cells (CC531). Mesothelial cells grown to form a monolayer, significantly inhibited the growth of CC531 cells, while, conversely, CC531 cells stimulated growth of the mesothelial cells. Receptor-binding studies demonstrated the presence of high-affinity IGF-I receptors on the mesothelial and CC531 cells. Both cell types also produced IGF-I, as measured by radioimmunoassay. IGF-I stimulated DNA synthesis in mesothelial cells, but had no effect on the growth of CC531 cells. In co-culture, it was found that IGF-I potentiated the inhibitory effect of mesothelial cells on CC531 cells. The effect of IGF-I on mesothelial cell proliferation was additive to the stimulatory effect of CC531 cells. The study provides evidence for the existence of a paracrine loop between mesothelial and colon carcinoma cells, giving more insight into the basic cellular mechanisms that may modulate the growth of intra-peritoneal colon carcinoma. Inhibition of CC531 proliferation by an intact monolayer of rat mesothelial cells might explain the earlier finding that tumour cells grow poorly in a surgically undisturbed abdomen.

INTRODUCTION

Loco-regional recurrence after surgically treated large bowel cancer is seen in a majority of patients. Tumour cells that gain access to the abdominal cavity may adhere to the mesothelium. The resection site is at greatest risk, but all traumatised peritoneal and intestinal surfaces are capable of trapping tumour cells.[1] Studies have been performed on tumour cell adhesion ad outgrowth, but the mechanisms by which this occurs are not completely understood.[2] It has been shown that lymphocytes and platelets release growth factors that may cause proliferation of tumour cells.[1] The alleged importance of the surrounding tissue on the growth of epithelial tumour cells led us to investigate the growth of colon carcinoma cells in the absence and in the presence of a monolayer of mesothelial cells in a rat model. We co-cultured both cell populations using Transwell tissue culture inserts with microporous membranes. Insulin like growth factors are known to have major autocrine and paracrine effects on many cellular functions. A role of IGFs in the transformation and proliferation of cancer cells has become increasingly evident in the past few years. Several studies indicate that IGFs may influence the aetiology of colon cancer by acting as potent autocrine growth factors.[3] Human mesothelial cells and colon carcinoma cells have been found to express the IGF-I receptor, and both produce IGF-I.[4;5] We therefore studied the effects of IGF-I in this co-culture system, to gain more insight into the basic cellular mechanisms that modulate intra-peritoneal tumour growth.

MATERIALS AND METHODS

Animals

To isolate mesothelial cells, inbred WAG/Rij rats, obtained from Harlan-CPB, Zeist, The Netherlands were used. The rats were bred under specific pathogen-free conditions, kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 hours light/ 12 hours dark), fed with laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee in Animal Research of the Erasmus University Rotterdam.

Isolation of mesothelial cells

The rats were decapitated and the peritoneal cavities were opened. Window like transparent triangular sheets of mesentery were seen, bordered by 2 adjacent jejunal arteries, located within streaks of adipose tissue. The centre of each sheet was picked up with small forceps and

the transparent part of mesentery was cut along the border of the sheet with scissors.[2] Immediately after removal, the sheets were placed in pre-warmed isolation medium (Hank's balanced slat solution, HBSS) and washed twice. The isolation medium was HBSS supplemented with 5% human serum albumin (CLB, Amsterdam, The Netherlands) penicillin (10^5 U/L), glutamin (2 mM) and fungizone (1.25 mg/L). After washing the sheets twice in this medium, they were incubated in a mixture of collagenase (1 g/L) and dispase (2.4×10^3 U/L) (Roche Diagnostics, Almere, The Netherlands). Following incubation during 15 minutes at 37°C and continuous gentle shaking, the detached mesothelial cells were pelleted by centrifugation at 300 g for 5 minutes. Cell viability was determined by trypan blue and always exceeded 95%. The pelleted mesothelial cells were resuspended in culture medium. The pelleted mesothelial cells were re-suspended in culture medium and were counted and cultured as described below. Culture medium consisted of RPMI 1640 supplemented with 5% foetal calf serum (FCS), glutamin (2 mM), penicillin (10^5 U/L) and fungizone (1.25 mg/L). Medium and supplements were obtained from Life Technologicals BV (Breda, The Netherlands).

Tumour cell line

CC531 is a moderately differentiated, weakly immunogenic colon adeno-carcinoma induced in a WAG/Rij rat by 1,2-dimethylhydrazine. A cell line was established and maintained by serial passage after trypsinization in culture medium.[6] CC531 tumour cells were cultured in RPMI 1640 medium supplemented with 5% FCS, glutamin (2 mM) and penicillin (10^5 U/L), and passaged once weekly using trypsin (0.05%) and EDTA (0.02%).

Co-culture

CC531 tumour cells were trypsinized, counted and seeded in multiwell plates in a concentration of 40,000 cells per well. Mesothelial cells were seeded at 100,000 cells per Transwell in Transwell microporous membranes (0.4 μm; Costar, Badhoevedorp, The Netherlands). The Transwells were transferred into wells containing 1 ml of culture medium. After one day, the co-culture experiment started by transferring the Transwells into wells containing 1 ml of culture medium with of without CC531 tumour cells. Thereafter, the CC531 tumour cells (with or without mesothelial cells) were incubated with or without 10 nM IGF-I (Bissendorf, Biochemical, Hannover, Germany) for 5 days. At the end of the incubation the DNA content of the cells was measured using bisbenzimide fluorescent dye (Boehring Diagnostics, La Jolla, CA) as described before.[7]

Measurement of IGF-I production by CC531 tumour cells and mesothelial cells

The radioimmunoassay kit for IGF-I (code 3015840, Biosource Europe, Fleurus, Belgium) was used to measure IGF-I production by CC531 and mesothelial cells. CC531 (40,000) and

mesothelial cells (200,000) were cultured for 3 days with 1 ml of culture medium. After 3 days the media were collected and stored at –20°C until further analysis. The control medium was culture medium, which was also incubated for 3 days at 37°C.

IGF-I receptor binding studies

Reaction conditions were the same as those describe by Zhong et al.[8] The radioligand used in the binding studies was 3-[^{125}I]iodotyrosyl-IGF-I (Bachem, Heidelberg, Germany). Briefly, membrane preparations (corresponding to 15-30 μg protein) of CC531 tumour cells and mesothelial cells cultured in RPMI with 5% FCS were incubated in a total volume of 100 μl at room temperature for 90 minutes with 30,000 to 50,000 cpm radioligand and increasing concentrations of unlabelled IGF-I in HEPES buffer (10 mM HEPES, 5 mM $MgCl_2$ and 0.2 g/l bacitracin, pH 7.6) containing 0.2% BSA (Sigma, St Louis, MO). After incubation, 1 ml ice-cold HEPES buffer was added to the assay mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 minutes at 500 g in an Eppendorf micro centrifuge.[9] The remaining pellet was washed twice with ice-cold HEPES buffer, and the final pellet was counted in a gamma counter. Specific binding was taken to be the total binding minus binding in the presence of 10 μM unlabelled IGF-I.

Immunocytochemical determination of keratin, vimentin and von Willebrand factor

Mesothelial cells can be characterised by positive keratin and vimentin staining and negative von Willebrand factor (vWf) staining. In order to exclude the presence of fibroblasts and endothelial cells, staining for vimentin and vWf was done. Mesothelial cells were cultured on type I collagen 15 mg/cm2 (collagen S (type I), cell biology Boehringer) coated 75 cm^2 flasks. After the cells had formed a confluent monolayer of polygonal cells with a typical cobblestone appearance, the cells were trypsinized, transferred to glass coverslips and dried in air.

Staining for keratin was done using a DAKO PAP kit system (anti-serum Z622; DAKO, Glostrup, Denmark). Staining for vimentin was done using a DAKO PAP kit system (anti-serum L1843; DAKO). The F8/86 antibody (DAKO) was used to detect the possible presence of vWf.

Statistical analysis of data

CC531 and mesothelial cells were cultured for 24, 48 or 72 hr, after which DNA content was measured and the doubling time was calculated. IGF-I receptor binding data were analysed by the method of Scatchard.[10]

All values are expressed as mean +SD, n=3 wells per treatment group. All data were analysed using analysis of variance (ANOVA) to determine overall differences between treatment groups.

Subsequently a comparison between treatment groups was made using the Newman-Keuls test. $P<0.05$ was considered to be statistically significant. All experiments were carried out at least twice with comparable results.

RESULTS

Staining of mesothelial cells

Figure 9.1 shows that all rat mesothelial cells stained positive for keratin and vimentin, while no vWF staining was observed. This confirms the mesothelial origin of the cells.

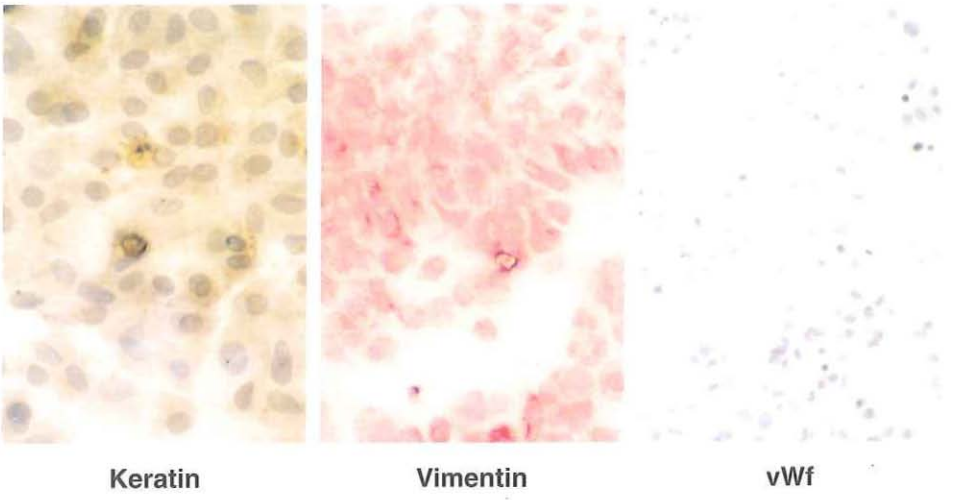


Figure 9.1
Immunocytochemical staining of rat mesothelial cells for keratin, vimentin and vWf.

Growth curves of CC531 and mesothelial cells

Figure 9.2 shows that CC531 cells have a mean doubling time of 45 hours. After 48 hours in serum-free conditions, the growth decreased by 29%, suggesting loss of cell viability. Rat mesothelial cells have a mean doubling time of 43 hours (figure 9.2). In contrast to the CC531 cells, these cells grew in serum-free conditions, with a mean doubling time of 58 hours.

In order to study the interaction between rat mesothelial and CC531 cells in conditions of proliferating CC531 cells; experiments were done in serum-containing medium.

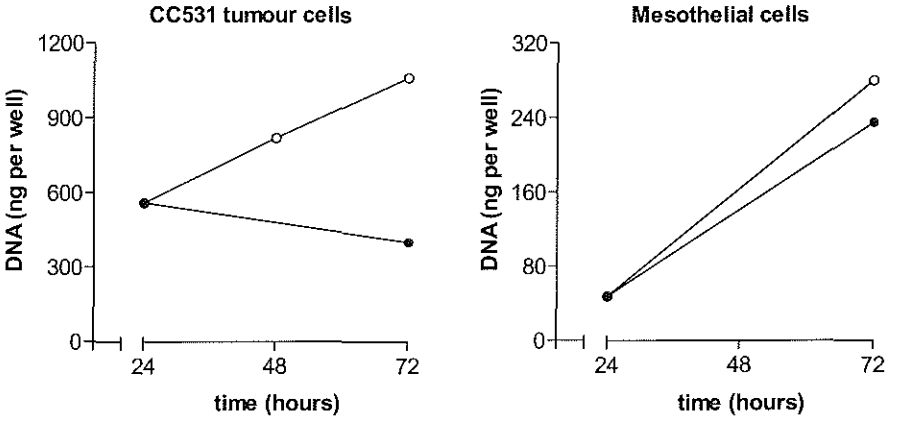


Figure 9.2

Growth rate of 40,000 CC531 tumour cells and 20,000 mesothelial cells. The cells were cultured during 72 hours. • serum free; ○ 5% FCS.

IGF-I production by CC531 tumour cells and mesothelial cells

Table 9.1 shows the IGF-I concentration in the conditioned media of the CC531 and rat mesothelial cells. Both cell types produced IGF-I. However, IGF-I production by rat mesothelial cells expressed per μg DNA was significantly higher than that by CC531 cells.

	IGF-I production	**IGF-I receptors**	
	(pmoles/μg DNA)	K_D	Number (fmoles/mg)
CC531 cells	427 ± 32	1.2	135
Mesothelial cells	3062 ± 464	2.7	2451

Table 9.1

IGF-I production and IGF-I receptor expression in CC531 colon carcinoma and rat mesothelial cells. Values of IGF-I production represent the mean of 4 individual wells ± SD. IGF-I value in medium alone: 0.1 nmol/L.

IGF-I receptor binding studies

Using the radioligand ^{125}I-labelled IGF-I, specific binding was demonstrated on membrane preparations of CC531 tumour and rat mesothelial cells. Binding of ^{125}I-labelled IGF-I on membranes of cultured CC531 and rat mesothelial cells was specific and could be displaced in a dose dependent manner with increasing concentrations of unlabelled IGF-I (figure 9.3). Scatchard analysis (not shown) of these data revealed a single class of high-affinity binding sites with an apparent dissociation constant (K_D) of 1.2 nM and 2.72 nM, and a maximum binding capacity (B_{max}) estimated to be 135 fmoles/mg and 2451 fmoles/mg membrane protein for CC531 and rat mesothelial cells respectively. Rat mesothelial cells showed significantly higher specific ^{125}I-labelled IGF-I binding than CC531 cells. See table 9.1.

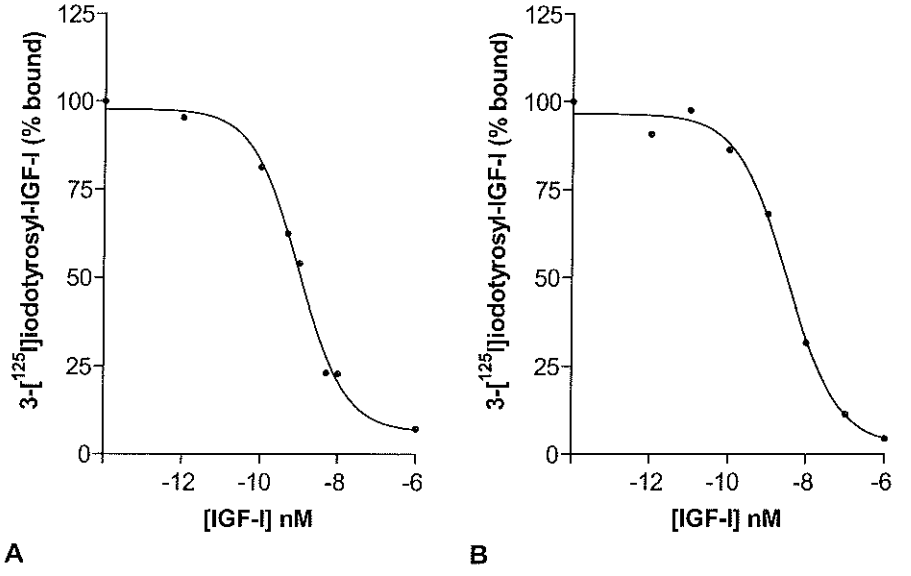


Figure 9.3

A: Dose-dependent displacement by unlabeled IGF-I of ^{125}I-labeled IGF-I binding on a membrane preparation of cultured CC531 tumour cells. The incubation time was 90 min. B: Dose dependent displacement by unlabeled IGF-I of ^{125}I-labeled IGF-I binding on a membrane preparation of cultured mesothelial cells. The incubation time was 90 min.

Mesothelial-CC531 tumour cell interactions and the role of IGF-I

In a co-culture system using Transwell tissue-culture inserts, we established the effects of rat mesothelial cells on the growth of CC531 tumour cells and vice versa. Figure 9.4A shows that rat mesothelial cells significantly inhibited the proliferation of CC531 tumour cells by 25% after 5 days of culture ($p<0.01$ *vs* control CC531 cells). IGF-I alone had no effect on the proliferation of CC531 tumour cells. However, IGF-I treatment potentiated the inhibitory effect of rat mesothelial cells on CC531 tumour cells (-35%, $p<0.01$ *vs* –25% with rat mesothelial cells). On the other hand, CC531 cells significantly stimulated rat mesothelial cell proliferation by 54% ($p<0.01$ *vs* rat mesothelial cells alone, figure 9.4B). IGF-I (10^{-8}M) significantly stimulated rat mesothelial cell proliferation by 27% *vs* control cells. The effect of IGF-I was additive to the stimulatory effect of CC531 cells on rat mesothelial cell proliferation (+75%, $p<0.01$ *vs* with CC531 cells).

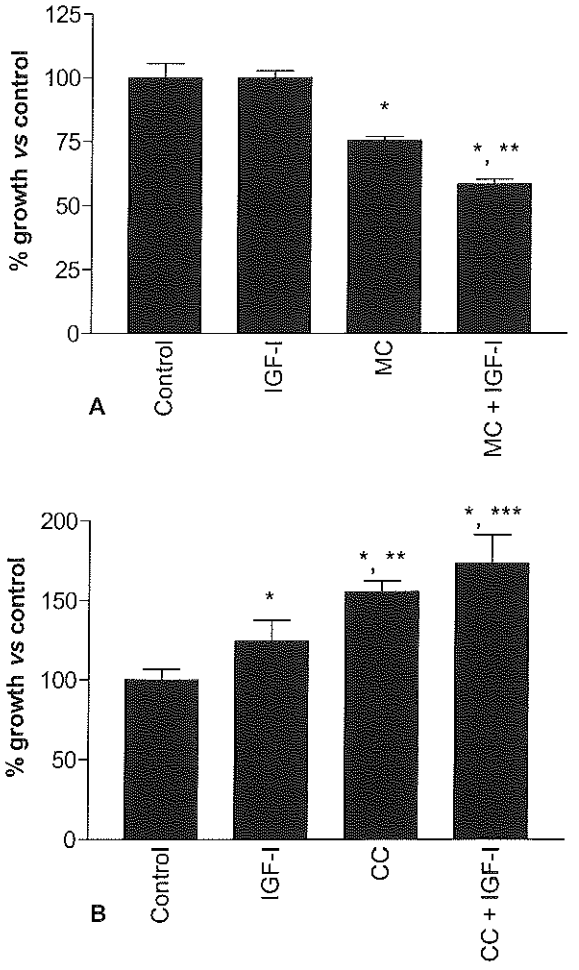


Figure 9.4

Mesothelial and CC531 cells were cultured separately as well as co-cultured during 5 days. The data are the mean ± (SD) of triplicate wells expressed as percentage of growth *vs* control.

Figure 4A shows the effect of mesothelial cells (MC) on the growth of CC531 tumour cells with or without IGF-I (10 nM). *p<0.01 *vs* CC531 cells without MC, ** p<0.01 *vs* CC531 cells with MC alone. Figure 4B shows the effect of CC531 tumour cells on the growth of mesothelial cells with or without IGF-I (10 nM). *p<0.01 *vs* MC without IGF-I, ** p<0.01 *vs* MC without CC531 cells, *** p<0.01 *vs* CC531 cells alone.

DISCUSSION

Several investigators have demonstrated that traumatised mesothelial surfaces are privileged sites for tumour cell recurrence. [1;11] It is conceivable that the process of enhanced tumour growth on traumatised tissue is biphasic. First, trauma of the peritoneum and the ensuing inflammatory response leads to up-regulation of mesothelial adhesion molecules, thus promoting the anchoring of tumour cells. Second, the subsequent healing of the peritoneum will lead to growth promotion of the adhered tumour cells through the action of locally produced growth factors. Using the *in vivo* counterparts of the cell types employed in the current study, namely seeding of CC531 cells in the peritoneal cavity we have demonstrated that tumour growth was greatly enhanced after surgical trauma. It was found that laparoscopic removal of a bowel segment, representing a minor surgical intervention, led to less adherence of intra-peritoneal tumour cells than when conventional surgery was performed, indicating that the degree of surgical trauma was proportional to the extent of tumour growth.[12] In addition, we observed that growth of a regenerating liver following partial hepatectomy led to a marked propagation of intra-hepatic CC531 tumour growth.[13] It is clear that these *in vivo* models, when coupled to a relevant *in vitro* system, provide unique possibilities to unravel surgery-induced variables such as the kinetics of adhesion molecule expression and the role of cytokines and growth factors. Although mesothelial cells can secrete many factors, including IGF-I[5], which can influence the proliferation of carcinoma cells, the exact role of these factors on tumour cell growth is still unknown. We therefore developed an *in vitro* model in which the role of a monolayer mesothelial cells on growth regulation of colon carcinoma cells was investigated in a co-culture system.

This study provides preliminary evidence that paracrine growth factors produced by mesothelial cells appear to inhibit proliferation of the colon carcinoma cells. On the other hand, paracrine factors produced by the colon carcinoma cells stimulate the proliferation of mesothelial cells.

Colon carcinoma cells have been shown to secrete many growth factors like IGF-II[4], IL-4 and TNF-α[14], which can have stimulatory effects on mesothelial cell proliferation. Less is known about the stimulating properties of IGF-I. Locally produced IGF-I has been shown to be an important regulatory factor of breast cancer cell growth.[15] In the presented paper, we demonstrate the presence of high affinity IGF-I receptors on CC531 cells and production of IGF-I by these cells. However, IGF-I has no effect on

the proliferation rate of CC531 cells. It has been shown that, although almost all colon cancers express a significant concentration of high affinity IGF-I receptors, growth effect of IGF-I on colon cancer cell lines were not always observed.[16] It appears likely that endogenously secreted IGF-II desensitises the cells to exogenously added IGFs.[3] High affinity IGF-I receptors are also present on mesothelial cells, which produce IGF-I and are stimulated by it. Lee et al. have demonstrated this for human mesothelial cells.[5] Rat mesothelial cells possess a higher number of IGF-I receptors and produce more IGF-I than CC531 cells, as demonstrated in this paper. Since CC531 cells and rat mesothelial cells possess IGF-I receptors and produce IGF-I, this may be an important factor in the paracrine loop between these cells.

IGF-I treatment potentiated the inhibitory effect of rat mesothelial cells on CC531 cells. This could be explained by an increase of IGF-binding protein (IGF-BP) by mesothelial cells, since IGF-BPs are known to regulate the action of IGF-I. The stimulation of the mesothelial cells by IGF-I, thereby enhancing the production of inhibitory factors, may explain a stronger inhibition of the proliferation of CC531 cells. The effect of IGF-I was additive to the stimulatory effect of the CC531 cells on mesothelial cell proliferation. Increased secretion of IGF-I by CC531 cells may (partly) explain the stimulation in growth of the mesothelial cells. Since rat mesothelial cells themselves secrete IGF-I and have a high number of IGF-I receptors, an autocrine loop of rat mesothelial cell growth by IGF-I is likely. This is also evident from our observation that rat mesothelial cells grow well in serum free conditions. However additional experiments using neutralising anti-sera to IGF-I or IGF-I receptors are needed to demonstrate a paracrine and/or autocrine loop between mesothelial and colon carcinoma cells with relation to IGF-I and its receptor.

In conclusion, our study provides evidence for the existence of a paracrine loop between mesothelial cells and colon carcinoma cells, providing more insight into the basic cellular mechanisms that may modulate the growth op intra peritoneal colon carcinoma. Inhibition of CC531 cell proliferation by mesothelial cells in an intact monolayer might explain the finding that tumour cells grow poorly in a surgically uncompromised abdomen.[12]

REFERENCES

1. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. **World J Surg** 1991; **15**: 609-616.
2. Akedo H, Shinkai K, Mukai M, et al. Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: a model for tumor invasion. **Cancer Res** 1986; **46**: 2416-2422.
3. Singh P, Rubin N. Insulinlike growth factors and binding proteins in colon cancer. **Gastroenterology** 1993; **105**: 1218-1237.
4. Lamonerie T, Lavialle C, de Galle B, Binoux M, Brison O. Constitutive or inducible overexpression of the IGF-2 gene in cells of a human colon carcinoma cell line. **Exp Cell Res** 1995; **216**: 342-351.
5. Lee TC, Zhang Y, Aston C, et al. Normal human mesothelial cells and mesothelioma cell lines express insulin-like growth factor I and associated molecules. **Cancer Res** 1993; **53**: 2858-2864.
6. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. **Int J Cancer** 1984; **33**: 689-692.
7. Hofland LJ, van Koetsveld PM, Lamberts SW. Percoll density gradient centrifugation of rat pituitary tumor cells: a study of functional heterogeneity within and between tumors with respect to growth rates, prolactin production and responsiveness to the somatostatin analog SMS 201-995. **Eur J Cancer** 1990; **26**: 37-44.
8. Zhong P, Cara JF, Tager HS. Importance of receptor occupancy, concentration differences, and ligand exchange in the insulin-like growth factor I receptor system. **Proc Natl Acad Sci U S A** 1993; **90**: 11451-11455.
9. Viguerie N, Tahiri-Jouti N, Ayral AM, et al. Direct inhibitory effects of a somatostatin analog, SMS 201-995, on AR4- 2J cell proliferation via pertussis toxin-sensitive guanosine triphosphate-binding protein-independent mechanism. **Endocrinology** 1989; **124**: 1017-1025.
10. Scatchard G. The attractions of proteins for small molecules and ions. **Ann NY Acad Sci** 1949; **51**: 660-672.
11. Cunliffe WJ, Sugarbaker PH. Gastrointestinal malignancy: rationale for adjuvant therapy using early postoperative intraperitoneal chemotherapy. **Br J Surg** 1989; **76**: 1082-1090.
12. Bouvy ND, Marquet RL, Jeekel H, Bonjer HJ. Impact of gas(less) laparoscopy and laparotomy on peritoneal tumor growth and abdominal wall metastases [see comments]. **Ann Surg** 1996; **224**: 694-700.
13. Slooter GD, Marquet RL, Jeekel J, Ijzermans JN. Tumour growth stimulation after partial hepatectomy can be reduced by treatment with tumour necrosis factor alpha. **Br J Surg** 1995; **82**: 129-132.

14. Barth RJJ, Camp BJ, Martuscello TA, Dain BJ, Memoli VA. The cytokine microenvironment of human colon carcinoma. Lymphocyte expression of tumor necrosis factor-alpha and interleukin-4 predicts improved survival. **Cancer** 1996; **78**: 1168-1178.
15. van der Burg B, Isbrucker L, van Selm-Miltenburg AJ, de Laat SW, van Zoelen EJ. Role of estrogen-induced insulin-like growth factors in the proliferation of human breast cancer cells. **Cancer Res** 1990; **50**: 7770-7774.
16. Guo YS, Narayan S, Yallampalli C, Singh P. Characterization of insulinlike growth factor I receptors in human colon cancer. **Gastroenterology** 1992; **102**: 1101-1108.

Chapter X

PARACRINE MODULATORS OF PERITONEAL TUMOUR RECURRENCE IN A RAT MODEL

Submitted for publication

Peritoneal dissemination is a common cause of post-surgical tumour recurrence. Surgically traumatised surfaces are predestined sites for successful implantation of tumour cells. In this process paracrine interactions between regenerating mesothelium and tumour cells may play an important role. In this study we investigated the effect of actively proliferating mesothelial cells, as seen during peritoneal wound healing, on the growth of colon carcinoma cells, using a co-culture system with Transwell tissue-culture inserts. The effect of acute phase cytokines like IL-1β, IL-6, IFN-γ and TNF-α, in addition to the peptide growth factors IGF-I, EGF, TGF-α, TGF-β and bFGF, known to play a role in wound healing, are investigated on their direct as well as paracrine growth modulating potential.

We demonstrate that among the different factors tested, IGF-I was the only one exerting statistically significant growth stimulatory effects on the tumour cells in the autocrine setting. On the other hand, mesothelial cell proliferation was significantly stimulated by IGF-I, TGF-α, EGF and bFGF. During co-culture, tumour cells stimulate the growth of mesothelial cells. In contrast to our previous findings with a monolayer mesothelial cells, actively proliferating mesothelial cells stimulate the growth of tumour cells. Combining IGF-I and tumour cells resulted in mesothelial cell growth which consisted of more than the sum of separate stimulating properties, demonstrating the existence of a synergistic paracrine loop. Furthermore, we provide evidence that direct proliferative responses and upregulation of paracrine growth stimulatory factors may be mediated via different pathways.

This study illustrates that different paracrine loops are present between tumour cells and activated mesothelial cells. Stimulation of tumour growth by proliferating mesothelial cells might explain the preferential tumour growth to surgically traumatised peritoneal membrane.

INTRODUCTION

The success of surgical treatment for gastro-intestinal cancer is often limited due to local or regional tumour recurrence by cells that have been seeded at the time of operation. The resection site is the most common location for disease spread. Several theories have been put forward to explain preferential recurrence to surgically traumatised tissue. Experimental studies suggest the prosperous wound environment stimulates residual tumour cell growth.[1] The tumour cell entrapment hypothesis proposes that per-operatively spilled tumour cells or emboli nestle in the fibrinous matrix of the wound where they are stimulated to grow by growth factors released to initiate the healing process at traumatised surfaces.[2] Furthermore, implantation of cancer cells on intact basement membrane surfaces is an inefficient process compared to implantation onto raw tissue surfaces that result from surgical dissection.[2] The common factor in these theories is encouraged tumour growth in the wound microenvironment.

The apparent correlation between wound healing and tumour recurrence is of relevance to all surgeons with an interest in cancer management. Experimental studies unravelling the specific interactions between wound healing and tumour recurrence have demonstrated that mediators in wound fluid significantly enhance tumour growth.[1] In various *in vivo* studies we previously demonstrated a correlation between the extent of surgical peritoneal injury and local tumour recurrence.[3;4] Moreover, using a sub-renal capsule assay, in which growth of tumour lumps can be determined objectively and where promotion of tumour cell adherence is irrelevant, a similar correlation between peritoneal injury and tumour growth was found (see also chapter 3).[3;4] Within hours after injury, wound-healing mediators can be captivated in a lavage fluid and enhance tumour recurrence in naive recipients (see also chapters 3 and 4).[1;4;5] These results suggest that during the acute phase of wound healing, tumour-enhancing factors are produced.

In the *in vitro* experiments described in the previous chapter, we demonstrated that mesothelial cells grown to form a monolayer, exert inhibitory effects on tumour cell growth. IGF-I potentiated this inhibitory effect by mesothelial cells, indicating a paracrine loop between mesothelial and colon carcinoma cells.[6] These results may explain the inferior growth of tumour cells during sustained integrity of the mesothelial monolayer in surgically undisturbed peritoneal cavities.

The aim of this study was to investigate the effect of growing, activated mesothelial cells, as seen during peritoneal wound healing, on the growth of colon carcinoma cells. Acute phase cytokines like IL-1β, IL-6, IFN-γ and TNF-α, in addition to peptide growth factors IGF-I, EGF, TGF-α, TGF-β, and bFGF, known to play a role in wound healing, are investigated on their cell growth modulating potential. Furthermore, the effect of these factors is investigated in a paracrine setting using a Transwell co-culture system, in which the interaction between mesothelial and tumour cells can be investigated. The results of these experiments allow us to determine which factors play a direct and/or indirect role in growth of colon carcinoma.

MATERIALS AND METHODS

Animals

To isolate mesothelial cells, inbred WAG/Rij rats, obtained from Harlan-CPB, Zeist, The Netherlands were used. The rats were bred under specific pathogen-free conditions, kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 hours light/ 12 hours dark), fed with laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee in Animal Research of the Erasmus University Rotterdam.

Isolation of mesothelial cells

Mesothelial cells (MC) were isolated from the small bowel mesentery, according to techniques modified from Akedo et al[7] as described previously.[6] In brief, under ether anaesthesia the abdomen of a rat was shaved, cleaned with alcohol 70% and subsequently opened. Window like transparent triangular sheets of mesentery were isolated and collected in Hank's Balanced Salt Solution (HBSS) containing 5% human serum albumin (CLB, Amsterdam, The Netherlands) penicillin (10^5 U/L), glutamin (2 mM) and fungizone (1.25 mg/L). After washing the sheets twice in this medium, they were incubated in a mixture of collagenase (1 g/L) and dispase (2.4×10^3 U/L) (Roche Diagnostics, Almere, The Netherlands). Following incubation during 15 minutes at 37°C and continuous gentle shaking, the detached mesothelial cells were pelleted by centrifugation at 300 g for 5 minutes. Cell viability was determined by trypan blue and always exceeded 95%. The pelleted mesothelial cells were resuspended in culture medium. Culture medium consisted of RPMI 1640 supplemented with 10% foetal calf serum (FCS), glutamin (2 mM), penicillin (10^5 U/L) and fungizone (1.25 mg/L). Medium and supplements were obtained from Life Technologicals BV (Breda, The Netherlands).

The mesentery derived cells grew forming a monolayer in a 37°C, fully humidified, 5% CO_2 incubator in polystyrene culture flasks (75 cm^2; Corning BV, Schiphol-Rijk, The Netherlands) precoated with collagen type I (15 $\mu g/cm^2$ collagen S (type I), cell biology Roche Diagnostics). For all experiments, mesothelial cells in their second passage were used. Isolated mesenteries from at least three different rats were used.

Colon carcinoma

CC531 is a moderately differentiated, weakly immunogenic colon adeno-carcinoma induced in a WAG/Rij rat by 1,2-dimethylhydrazine. A cell line was established and maintained by serial passage after trypsinization in culture medium.[8] CC531 tumour cells were cultured in RPMI 1640 medium supplemented with 5% FCS, glutamin (2 mM) and penicillin (10^5 U/L), and passaged once weekly using trypsin (0.05%) and EDTA (0.02%).

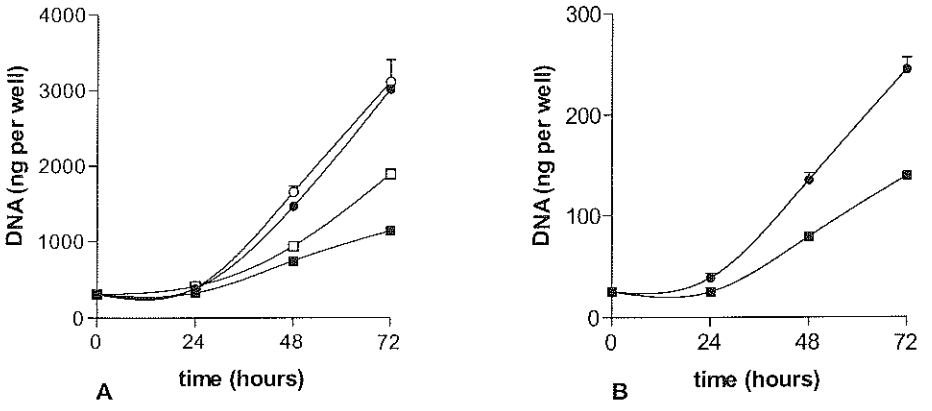


Figure 10.1

(A) Growth rate of 20,000 CC531 tumour cells • 5% FCS; ■ 1% FCS; ○ 1% FCS + 10^{-8}M IGF-I; □ 1% FCS + 10^{-8}M IGF-I. (B) Growth rate of 10,000 mesothelial cells • 5% FCS; ■ 1% FCS. The values are expressed as the mean of four replicates with SEM.

Growth curves

The culture medium used in all experiments was RPMI supplemented with FCS, glutamin (2 mM) and penicillin (10^5 U/L). Growth curves were made using different concentrations FCS and cell densities to establish the ideal culture conditions for studying growth stimulation and inhibition. Tumour and mesothelial cell doubling time was calculated as the number of doublings in the exponential growth phase ('b-a' in which a = $\log_2$ (DNA t=24 hours) and b = $\log_2$ (DNA t = 72 hours).

To investigate the effects of growth factors and cytokines on cell growth, dose response curves were made. Pre-incubation was performed with different factors and initiated immediately after seeding of tumour or mesothelial cells in 24 well plates. Growth response to interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) (purchased from R&D Systems, Uithoorn, The Netherlands), insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β) (all from Bachem, Bern, Switserland) and interferon-γ (Sanvertech, Heerhugowaard, The Netherlands) was studied.

Co-culture

Tumour cells were trypsinized, counted and seeded in 24 well plates in a concentration of 20,000 cells per well. The plates were then centrifuged at 80 g to force floating cells to the well bottom. Mesothelial cells were seeded at 10,000 cells per Transwell (Transwell microporous membranes 0.4 μm, Costar, Badhoevedorp, The Netherlands). After moistening of the membrane, the Transwells were transferred into wells containing the CC531 cells and 1 ml of culture medium.

The effect of growth factors and cytokines on the paracrine milieu was investigated by adding optimal concentrations, as determined in the obtained growth curves, to the culture medium immediately after seeding the tumour cells. After three days, plates and Transwells were collected for determination of the DNA content.

DNA-assay

To establish an effect on cell growth, analysis of the DNA content was performed. The DNA content of the tumour and mesothelial cells was determined using the bisbenzimide fluorescent dye (Roche Diagnostics) as previously described.[9] In short, at the end of the incubation period the plates and Transwells 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 of data

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. All data are expressed as mean ±SEM.

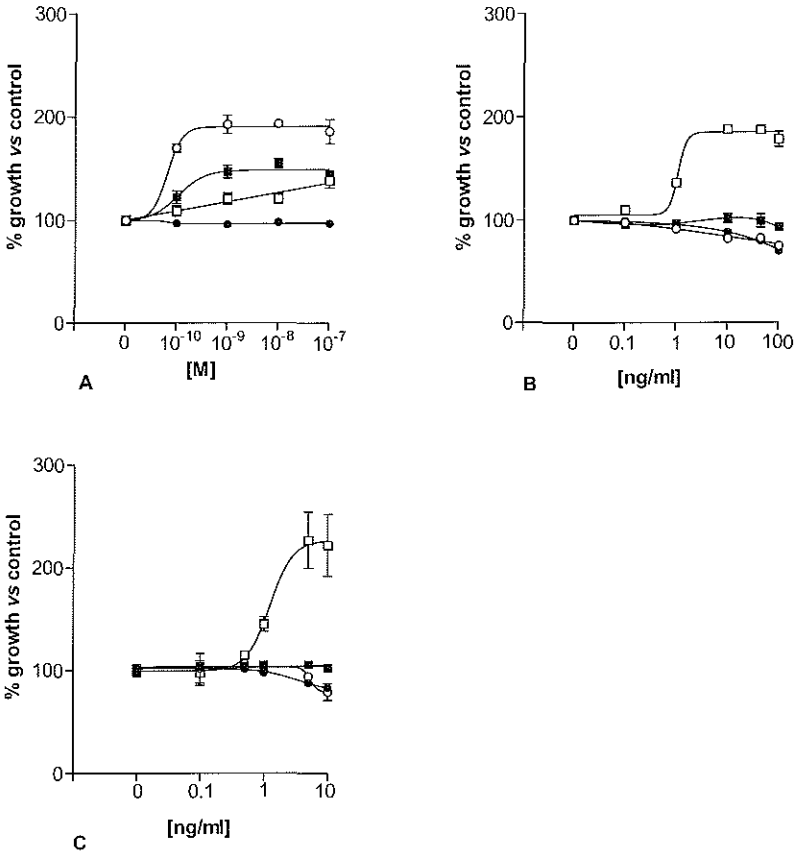


Figure 10.2

Growth response of CC531 tumour and mesothelial cells to cytokines and growth factors. The data are the mean ± (SEM) of quadruplicate wells expressed as percentage of growth *vs* the control during 72 hours incubation. Figure A shows CC531 tumour cells • +EGF (no effect); ■ +IGF-I (≥ 10^{-10}M, $p<0.01$;EC_{50} $1.1x10^{-10}$M). and mesothelial cells ○ +EGF (≥ 10^{-10}M, $p<0.01$; EC_{50} $6.7x10^{-11}$M); □ +IGF-I (≥ 10^{-8}M, $p<0.05$; EC_{50} $3.8x10^{-9}$M). Figure B shows CC531 tumour cells • +IL-1β (≥10 ng/ml, $p<0.05$); ■ +bFGF (no effect) and mesothelial cells ○ +IL-1β (≥1 ng/ml, $p<0.05$); □ +bFGF (≥1 ng/ml, $p<0.01$; EC_{50} 1.1 ng/ml). Figure C shows CC531 tumour cells • +TGF-β (≥5ng/ml, $p<0.01$); ■ +TGF-α (no effect). Mesothelial cells ○ +TGF-β (no effect); □ +TGF-α (≥1 ng/ml, $p<0.05$; EC_{50} 1.2 ng/ml).

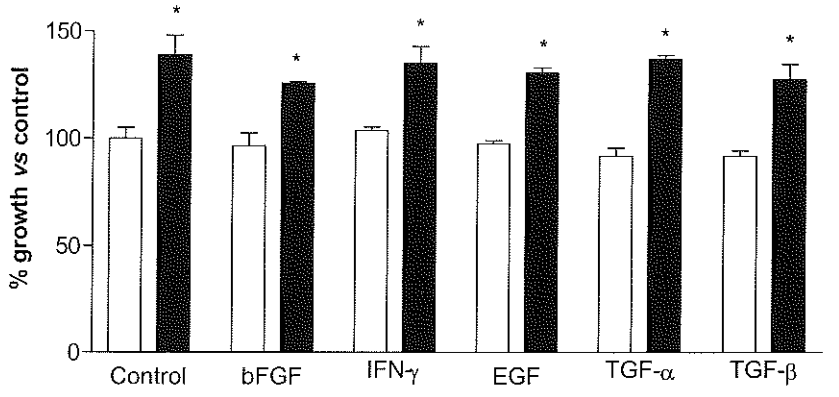
Results

Growth curves

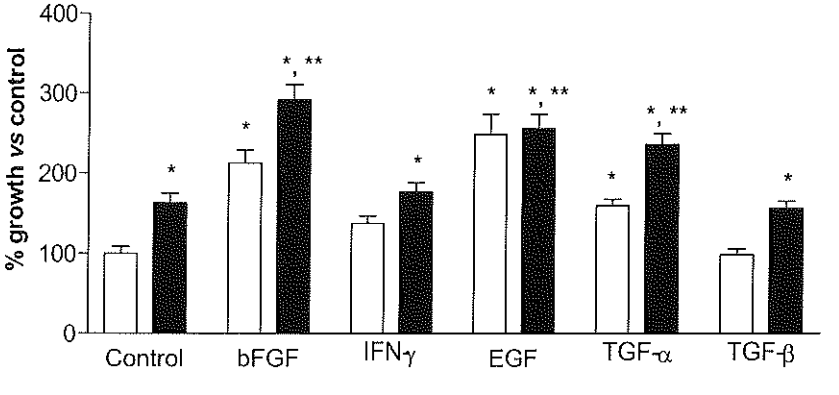
Based on the results (data not shown) using different seeding densities, for all growth curves tumour cells were seeded at a density of 20,000 cells per well and mesothelial cells were seeded at a density of 10,000 cells per well. The amount of DNA significantly increased from t=24 to t=72 hours of culture (in all instances $p<0.01$) indicating that the cells are actively growing during this period. In order to compare the 'growth rates' of the CC531 tumour and mesothelial cells, the number of doublings which took place between 24 and 72 h culture period was calculated. There were major differences in number of tumour cell doublings between 1 and 5% FCS and when IGF-I was added to 1% serum. After 72 hours, the wells containing tumour cells in 5% FCS were full-grown with a mean doubling time (MDT) of 3.0. In 1% FCS, the tumour cells grew with a MDT of 1.8, leaving ample growing opportunity in the wells. In 0.5 % FCS, tumour cells grew slowly with a MDT of 1.2 (not shown). Mesothelial cell growth appeared more autonomous and was less affected by the concentration of FCS, with a MDT of 2.7 in 5% FCS, 2.5 in 1% FCS and 2.1 in 0.5% FCS (figure 10.1A and B).

In chapter 9 we demonstrated the presence of IGF-I receptors on CC531 cells. Therefore, this growth factor was used to determine which time point is preferential for assessing maximal effect on cell growth. In 5% FCS, no statistically significant IGF-I (10^{-8}M) effect was established at all (figure 10.1A) as demonstrated in the previous chapter.[6] In 1% FCS however, statistically significant enhanced cell growth (60% stimulation) was evident after 72 hours (MDT 2.9, $p<0.01$), while not at earlier time points. Based on these experiments we concluded the optimal concentration for studies on the proliferation of tumour and mesothelial cells was 1% FCS enriched growth medium with a cut off point after 72 hours.

In this setting IGF-I was the only growth factor to stimulate CC531 cell proliferation. However, mesothelial cells were growth stimulated by IGF-I, TGF-α, EGF and bFGF. IL-1β and TGF-β slightly inhibited mesothelial and tumour cell growth when used at higher concentrations (Figure 10.2). IFN-γ, IL-6 and TNF-α had no effect on either cell type (data not shown).



A



B

Figure 10.3

Response of CC531 tumour and mesothelial cells to growth factors and inflammatory mediators (bFGF 10 ng/ml; IFN-γ 10 ng/ml; EGF 10^{-8}M; TGF-α 1 ng/ml; TGF-β 1 ng/ml). The data are the mean ± (SEM) of quadruplicate wells expressed as percentage of growth *vs* the non-stimulated control after 72 hours incubation. In figure A the growth of CC531 tumour cells is shown in the autocrine (open bars) and the paracrine milieu (filled bars). * $p<0.01$ *vs* control cells without co-culture. Figure B shows the growth of mesothelial cells in the autocrine (open bars) and the paracrine milieu (filled bars). * $p<0.05$ *vs* control cells without co-culture, ** $p<0.01$ *vs* control cells with co-culture.

Co-culture

In a Transwell® co-culture system the paracrine interactions between CC531 tumour cells and actively proliferating mesothelial cells were investigated. Both cell types stimulated each other in growth. Tumour cells stimulated the proliferation of mesothelial cells by 40 to 60% ($p<0.01$ *vs* control mesothelial cells). Mesothelial cells stimulated tumour cells to grow with 40 to 60% as well ($p<0.01$ *vs* control tumour cells). The different growth factors and inflammatory mediators that were tested and shown in figure 10.3 did not influence the autocrine or paracrine growth milieu of CC531 tumour cells. Mesothelial cells were highly responsive to growth factors, resulting in stimulated cell growth in an autocrine and paracrine setting. The effect of bFGF and TGF-α in the paracrine milieu was additive to the effect of CC531 tumour cells, the effect of EGF was not additive (figure 10.3).

In chapter 9 we demonstrated a role of IGF-I in the paracrine loop between a monolayer of mesothelial cells and proliferating tumour cells. An inhibitory effect of mesothelial cells on tumour cells was shown, which was potentiated by IGF-I.[6] In the present study we wanted to elucidate the role of IGF-I in the paracrine loop between active, proliferating mesothelial cells and tumour cells. Contrary to mesothelial cells forming a monolayer, proliferating mesothelial cells stimulated tumour growth. Adding IGF-I to the autocrine milieu of mesothelial and tumour cells lead to proliferation of both cell types. In the paracrine setting, a synergistic effect was seen regarding mesothelial cell growth after adding tumour cells with IGF-I. The effect of IGF-I in the paracrine milieu of tumour cells was partly additive to the stimulation of mesothelial cells (figure 10.4).

Apart from the slightly inhibited tumour cell growth by IL-1β (dose response curve shown in figure 10.2) none of the acute phase cytokines significantly affected the growth of mesothelial or tumour cells (figure 10.5). However, in the paracrine setting a significant proliferation of mesothelial cell growth was seen after adding tumour cells and IL-1β ($p<0.01$ *vs* paracrine control) indicating this factor plays a role in the paracrine loop between growing tumour and mesothelial cells (figure 10.5).

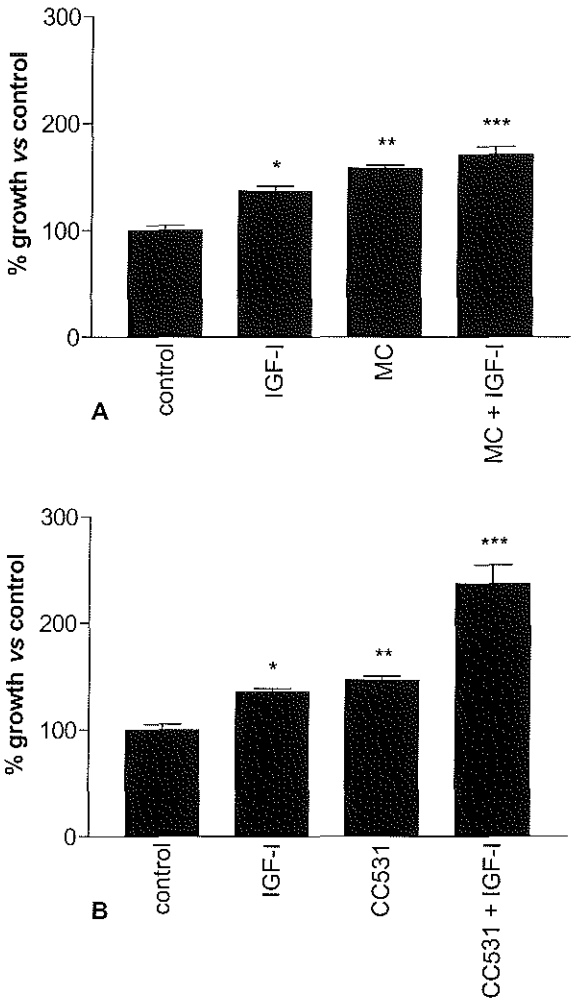


Figure 10.4

Mesothelial and CC531 cells were cultured separately as well as co-cultured during 72 hours. The data are the mean ± (SEM) of quadruplicate wells expressed as percentage of growth *vs* control.

Figure A shows the effect of mesothelial cells (MC) on the growth of CC531 tumour cells with or without IGF-I (10^{-9}M). *p<0.01 *vs* CC531 cells without IGF-I, ** p<0.01 *vs* CC531 cells without MC, *** p<0.01 *vs* CC531 cells with or without IGF-I. Figure B shows the effect of CC531 tumour cells on the growth of mesothelial cells with or without IGF-I (10^{-8}M). *p<0.05 *vs* MC without IGF-I, ** p<0.05 *vs* MC without CC531 cells, *** p<0.01 *vs* MC with or without IGF-I and CC531 cells.

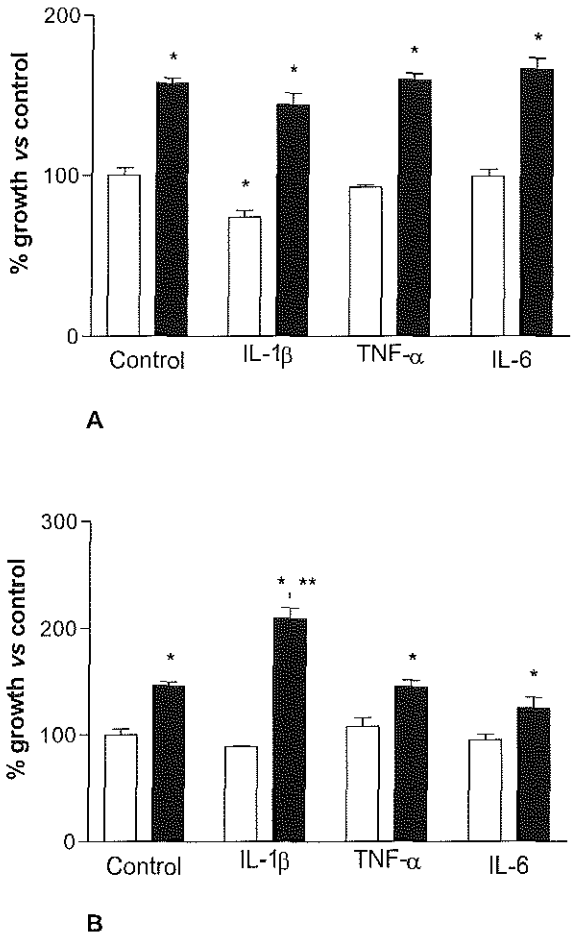


Figure 10.5

Response of CC531 tumour and mesothelial cells to the acute phase cytokines IL-1β (10 ng/ml), TNF-α (10 ng/ml) and IL-6 (10 ng/ml). The data are the mean ± (SEM) of quadruplicate wells expressed as percentage of growth *vs* the non-stimulated control after 72 hours incubation. In figure A the growth of CC531 tumour cells is shown in the autocrine (open bars) and the paracrine milieu (filled bars). * $p<0.01$ *vs* control cells without co-culture. Figure B shows the growth of mesothelial cells in the autocrine (open bars) and the paracrine milieu (filled bars). * $p<0.05$ *vs* control cells without co-culture , ** $p<0.01$ *vs* control cells with co-culture.

DISCUSSION

Tumour recurrence at wound sites is a well known phenomenon and has been demonstrated in clinical and experimental settings by several investigators.[1;2;4] Exact mechanisms of the role of wound healing in tumour growth are not clear. Wound healing is based upon the synthesis and release of several growth factors and cytokines at the site of injury which act through autocrine and paracrine pathways to regulate healing. These wound site derived growth factors are thought to take part in enhancing local tumour growth.[1;2;10]

Peritoneal tissue repair exhibits a more diverse process. Regardless of the type of injury, a common pathway of inflammation and restoration follows. The peritoneal mesothelium plays an active role in this process by producing chemoattractants, prostaglandins and growth factors.[11-14] Most studies investigating tumour growth during wound healing, study the autocrine events of wound related growth factors and cytokines. Pivotal interactions through cross-talking of cells are neglected in this way. Lack of insight in these facets of peritoneal tissue repair and tumour recurrence impedes the development of effective tumour modulating agents.

In this study we evaluated both direct as well as paracrine-mediated effects of different growth factors and acute phase cytokines using a co-culture system of colon carcinoma cells and proliferating mesothelial cells. We demonstrate that the growth stimulating effects of the tumour cells in the autocrine setting are preferentially mediated through IGF-I. Mesothelial cells appeared more susceptible to the growth stimulating effects of different wound healing factors. In our study, significant enhancement of mesothelial cell proliferation is seen after stimulation with IGF-I, TGF-α, EGF and bFGF. These findings are supported by other studies, which use human or rat mesothelial cells, investigating peritoneal repair after wounding.[6;15-17] The presence of receptors for different growth factors has been demonstrated on mesothelial cells.[6;18;19] The EGF receptor, which binds EGF as well as TGF-α, is expressed by mesothelial cells and plays an active role in peritoneal tissue repair[16-18] as has been shown for bFGF[16] and IGF-I as well.[6] Mesothelial cells also express the TGF-β and IL-1β receptor.[14;19] The presence of an IGF-I receptor and absence of EGF receptor on CC531 tumour cells[6;20] explains the response to IGF-I and the lacking response to EGF and TGF-α by these cells.

In the previous chapter we demonstrated an inhibitory effect of a quiescent, non-proliferating mesothelial monolayer, on CC531 tumour cell growth. This inhibitory effect was potentiated by IGF-I.[6] The aim of the present study was to elucidate the role of IGF-I in the paracrine loop between active, proliferating mesothelial cells and tumour cells. The presented results show that, contrary to quiescent cells, proliferating mesothelial cells stimulate tumour growth. Addition of IGF-I to either cell type stimulates cell growth and implies a role for IGF-I in an autocrine loop. Mesothelial and CC531 tumour cells produce IGF-I although the production by mesothelial cells is six-fold higher (chapter 9).[6] At paracrine level the stimulation of tumour cells by mesothelial cells and IGF-I is higher than by mesothelial cells or IGF-I alone, however it is not fully additive. This indicates that IGF-I production by mesothelial cells may be the stimulative factor enhancing tumour growth. In return the combination of IGF-I and tumour cells causes more stimulation of mesothelial cell growth than either factor alone. Moreover, the stimulating effect is more than the sum of the isolated effect of tumour cells and IGF-I. This allows the speculation of a synergistic paracrine loop involving IGF-I. Based on these results we may speculate that IGF-I stimulation of CC531 tumour cells leads to secondary production of a growth factor, which has proliferative activity for mesothelial cells.

The *in vivo* experiments described in chapter 4 demonstrated a potential pivotal role of IGF-I in post-surgical tumour recurrence. Others and we demonstrated that after abdominal surgery, serum IGF-I levels decrease.[21;22] Minimal invasive surgery, and therefore minimal peritoneal trauma, coincides with diminished tumour recurrence and affects IGF-I levels less than conventional abdominal surgery.[21] Furthermore, we detected elevated concentrations IGF-I in peritoneal lavage fluid after peritoneal surgical trauma (chapter 4). Combining these *in vivo* data and the presented *in vitro* results inevitably demonstrates a pivotal role of IGF-I in tumour recurrence in our model. Although the presented data reflect interactions in a rat model, the distance to a human model may only be a short step.

Of the cytokines, IL-1β slightly inhibited tumour cell growth but augmented mesothelial cell proliferation during co-culture with tumour cells. Mesothelial cell growth is highly sensitive to bFGF, TGF-α, and EGF. Increased levels of these growth factors in the supernatant might explain mesothelial cell proliferation after co-culture with tumour cells and IL-1β. This suggests that the direct proliferative responses and upregulation of paracrine growth stimulatory factors are mediated via different pathways. Further

studies are required however, to elucidate the nature of these paracrine growth stimulating factors.

In conclusion, the results of this study show that actively proliferating mesothelial cells stimulate the growth of CC531 tumour cells. In contrast to the inhibition of tumour growth by quiescent mesothelial cells, this may explain the preferential tumour growth to surgically traumatised peritoneum. It seems different paracrine loops are present during activated mesothelial responses than during peritoneal equilibrium when mesothelial cells form a monolayer. IL-1β and IGF-I, released during peritoneal wound healing stimulate, directly or indirectly, growth of mesothelial and tumour cells. Further unravelling of the effect of cytokines and growth factors in the paracrine loop between tumour and mesothelial cells may provide new insights in manipulating tumour recurrence.

REFERENCES

1. Hofer SO, Shrayer D, Reichner JS, Hoekstra HJ, Wanebo HJ. Wound-induced tumor progression: a probable role in recurrence after tumor resection. **Arch Surg** 1998; **133**: 383-389.
2. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. **World J Surg** 1991; **15**: 609-616.
3. Bouvy ND, Marquet RL, Jeekel J, Bonjer HJ. Laparoscopic surgery is associated with less tumour growth stimulation than conventional surgery: an experimental study [see comments]. **Br J Surg** 1997; **84**: 358-361.
4. van den Tol PM, van Rossen EE, van Eijck CH, Bonthuis F, Marquet RL, Jeekel H. Reduction of peritoneal trauma by using nonsurgical gauze leads to less implantation metastasis of spilled tumor cells. **Ann Surg** 1998; **227**: 242-248.
5. van Rossen M, van den Tol MP, Aalbers AGJ, et al. 34th Congress of the European Society for Surgical Research (ESSR). Bern, Switzerland, April 22-24, 1999. Abstracts. **Eur Surg Res** 1999; **31 Suppl 1**: 1-244.
6. van der Wal BC, Hofland LJ, Marquet RL, van Koetsveld PM, van Rossen ME, van Eijck CH. Paracrine interactions between mesothelial and colon-carcinoma cells in a rat model. **Int J Cancer** 1997; **73**: 885-890.
7. Akedo H, Shinkai K, Mukai M, et al. Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: a model for tumor invasion. **Cancer Res** 1986; **46**: 2416-2422.
8. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. **Int J Cancer** 1984; **33**: 689-692.
9. Hofland LJ, van Koetsveld PM, Lamberts SW. Percoll density gradient centrifugation of rat pituitary tumor cells: a study of functional heterogeneity within and between tumors with respect to growth rates, prolactin production and responsiveness to the somatostatin analog SMS 201-995. **Eur J Cancer** 1990; **26**: 37-44.
10. Hofer SO, Molema G, Hermens RA, Wanebo HJ, Reichner JS, Hoekstra HJ. The effect of surgical wounding on tumour development. **Eur J Surg Oncol** 1999; **25**: 231-243.
11. Holmdahl L, Ivarsson ML. The role of cytokines, coagulation, and fibrinolysis in peritoneal tissue repair. **Eur J Surg** 1999; **165**: 1012-1019.
12. Nagy JA, Jackman R. Response of the peritoneal membrane to injury. **Semin Dialysis** 1998; **11**: 242-248.
13. Topley N, Williams JD. Effect of peritoneal dialysis on cytokine production by peritoneal cells. **Blood Purif** 1996; **14**: 188-197.
14. Yung S, Davies M. Response of the human peritoneal mesothelial cell to injury: an in vitro model of peritoneal wound healing. **Kidney Int** 1998; **54**: 2160-2169.

15. Lee TC, Zhang Y, Aston C, et al. Normal human mesothelial cells and mesothelioma cell lines express insulin-like growth factor I and associated molecules. **Cancer Res** 1993; **53**: 2858-2864.
16. Mutsaers SE, McAnulty RJ, Laurent GJ, Versnel MA, Whitaker D, Papadimitriou JM. Cytokine regulation of mesothelial cell proliferation in vitro and in vivo. **Eur J Cell Biol** 1997; **72**: 24-29.
17. Wingren U, Franzen L, Larson GM, Malcherek P, Schultz GS. Epidermal growth factor accelerates connective tissue wound healing in the perforated rat mesentery. **J Surg Res** 1992; **53**: 48-54.
18. Bermudez E, Everitt J, Walker C. Expression of growth factor and growth factor receptor RNA in rat pleural mesothelial cells in culture. **Exp Cell Res** 1990; **190**: 91-98.
19. Chegini N. The role of growth factors in peritoneal healing: transforming growth factor beta (TGF-beta). **Eur J Surg Suppl** 1997; 17-23.
20. de Jong KP, Brouwers MA, van Veen ML, et al. Serum Obtained from Rats after Partial Hepatectomy Enhances Growth of Cultured Colon Carcinoma Cells. **Invasion Metastasis** 1999; **18**: 155-164.
21. Bouvy ND, Marquet RL, Tseng LN, et al. Laparoscopic vs conventional bowel resection in the rat. Earlier restoration of serum insulin-like growth factor 1 levels. **Surg Endosc** 1998; **12**: 412-415.
22. Skjaerbaek C, Frystyk J, Orskov H, et al. Differential changes in free and total insulin-like growth factor I after major, elective abdominal surgery: the possible role of insulin- like growth factor-binding protein-3 proteolysis. **J Clin Endocrinol Metab** 1998; **83**: 2445-2449.

Part VI

General Discussion and Summary

Chapter XI

GENERAL DISCUSSION and MODES FOR CLINICAL APPLICATION

Peritoneal dissemination is a common cause for post-surgical tumour recurrence and represents the terminal stage of disease.[1-4] Patterns of first recurrence show the resection site is preferential, and distant metastases combined with loco-regional intra-abdominal recurrence is common.[4]

For successful metastasis, tumour cells undergo a series of sequential, interrelated steps including invasion through basement membranes, embolism and transport through lymphatics or vasculature, arrest in organs, adherence and growth.[5] In case of peritoneal recurrence due to intra-abdominal seeding or spill of tumour cells, the process of tumour metastasis is essentially facilitated, only leaving tumour cell adhesion and growth to be completed.

Most attempts at limiting peritoneal tumour recurrence are aimed at annulling manifest tumour foci. Examples are neo-adjuvant chemotherapy[6], photodynamic therapy[7], lavage of the abdominal cavity with chemotherapeutic agents[8] and post-operative loco-regional (chemo)radiotherapy.[9] A different approach to diminishing post-surgical peritoneal tumour recurrence is to interfere with the primary implantation process of seeded tumour cells. This necessitates a profound understanding of the underlying pathophysiologic mechanisms leading to tumour implantation.

The studies described in this thesis were aimed at unravelling links between surgical peritoneal trauma and peritoneal tumour recurrence. Different facets of the peritoneal response to surgical trauma were highlighted with the intention to elucidate which pathways may be used for specific therapeutical alternatives.

Consideration of experimental models

To investigate patterns of tumour recurrence *in vivo*, different rat models were used. In a cell seeding model the clinical situation of free intra-peritoneal tumour cells was mimicked. This model associates the combination of cell adhesion and growth, ultimately leading to manifest tumour recurrence. In the sub-renal capsule assay, the effect of surgical trauma on the growth of extra-peritoneal tumours was reproduced. This model represents the systemic effects on tumour growth that may occur after surgical trauma. Based on the results from the *in vivo* experiments, the initial impetus to separate the generalised term "tumour recurrence" into the more elementary mechanisms of tumour cell adhesion and tumour growth was made.

To study tumour cell adhesion, an *in vitro* model was designed. The peritoneum was recalled by culturing a monolayer of mesothelial cells on a matrix coating. Tumour cell adhesion could be studied after pre-incubating the mesothelial monolayer with different cytokines and growth factors, known to be produced after surgical peritoneal trauma, without the interference of unknown *in vivo* factors.

Tumour growth was assessed in an autocrine and paracrine setting. For growth factors to establish any effect on proliferation, cells must express receptors on the cell surface. If not, no interaction is achieved. Studies at this 'autocrine' level do not represent the *in vivo* situation entirely. Paracrine related mechanisms may affect tumour recurrence as well. To achieve paracrine interaction, mesothelial cells and tumour cells were co-cultured without making contact. Factors produced by both cell types could thus pass freely in the culture medium, allowing cross-talking between these cells.

Surgical peritoneal trauma and tumour recurrence

The observed preference of tumours to recur at surgically traumatised sites[4] led us to investigate if a correlation existed between peritoneal surgical trauma and tumour recurrence.

In the experiments described in chapter 2, indeed a significant correlation between the intensity of surgical trauma and tumour recurrence was demonstrated *in vivo*. Tumour recurrence was greatest at sites where the mesothelial cells were abraded. This suggests that tumour cells preferentially recur at the sub-mesothelial extra cellular matrix (figure 11.1A). However, enhanced tumour recurrence was also seen at not directly traumatised peritoneal sites. Apparently tumour cells recur at both the mesothelial and sub-mesothelial surfaces. After placing a solid tumour in the extra-

peritoneal space, the intensity of surgical trauma also correlated with extra-peritoneal tumour growth, suggesting that systemic factors produced after surgical trauma play an important role in enhanced tumour growth.

The apparent generalised character of tumour recurrence led to the hypothesis that factors produced after peritoneal trauma, may influence loco-regional and distant aspects of tumour recurrence. When these factors were captured in a lavage fluid and passively transferred to naive recipients, this hypothesis was confirmed. Further analysis of the fragments in the captured lavage fluid brought forward the presence of a cellular and soluble fraction. When these were individually injected into pristine abdominal cavities, without any form of additional surgical trauma, both induced significantly more peritoneal tumour recurrence. However, the effect of the soluble factors was inferior to that of the cellular fraction. This difference was apparent in the amount of peritoneal tumour load. Significantly more and larger tumours were seen after injection with the cellular compared to the soluble component.

The results of these experiments demonstrate that cellular and soluble factors are the mediators of surgical trauma. Furthermore, direct peritoneal injury and the extent of it is an additional factor influencing local tumour recurrence.

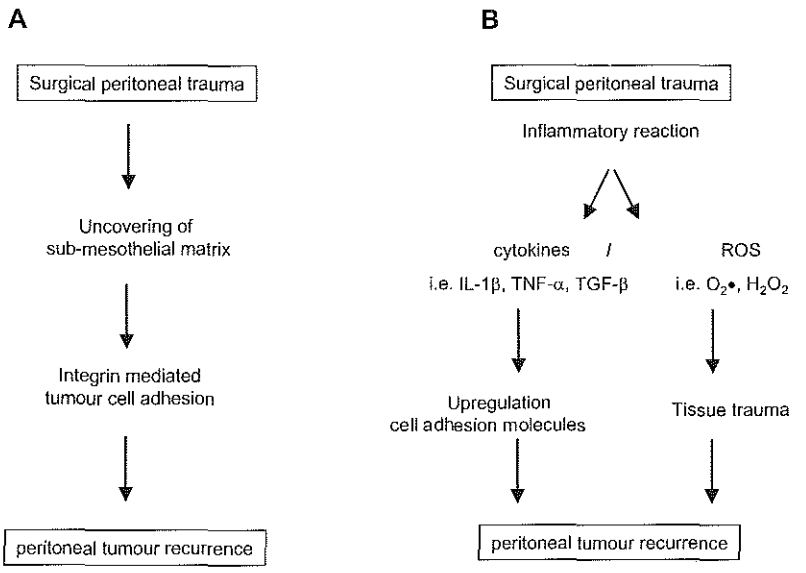


Figure 11.1

Peritoneal tumour recurrence after direct or indirect surgical trauma of the peritoneum.

A: Direct surgical trauma of the peritoneum; B: Indirect surgical trauma of the peritoneum.

The mesothelial monolayer as natural barrier for tumour recurrence

Normally, the mesothelium is a quiescent population that forms the first barrier free abdominal cells come across. Any form of peritoneal injury, including inflammation and surgical trauma, damages the monolayer of mesothelial cells lining the peritoneum (see figure 4.1, chapter 4).[10;11] During peritoneal inflammation, mesothelial cells round up, hereby increasing the intercellular space where patches of sub-mesothelial matrix components become denuded.[11] Tumour cells have been shown to produce factors that stimulate a similar reaction of mesothelial cell rounding after which adhesion to the matrix takes place.[12;13] This may be an unspecific reaction that can be elicited by intra-peritoneal injection of suspensions in general.[14] Drying, wetting or rubbing exfoliates mesothelial cells, also uncovering the layer underneath.[10;15;16] The *in vivo* studies described in this thesis demonstrated that at sites where the integrity of the mesothelial monolayer was lost, enhanced tumour recurrence could be found. This led to the conclusion that direct peritoneal injury augments local tumour recurrence.

The results described in chapter 8 clearly show a preferential adhesion of tumour cells to sub-mesothelial collagen compared to a monolayer mesothelium. Preferential adhesive patterns to matrix components have also been shown for human colon carcinoma and ovarian carcinoma cell lines.[17-19] Cell adhesion to matrix components is mediated by integrins. The integrin profile of tumour cells may explain preferential recurrence at sub-mesothelial matrices and enhanced tumour recurrence at surgically traumatised sites *in vivo* (figure 11.1A). The experiments in chapter 8 demonstrated that preferential tumour cell adhesion to collagen coincided with $\alpha_2\beta_1$ integrin expression. Variability in integrin expression by tumour cells will determine the affinity for different extra cellular matrix components.

The tumour–mesothelial cell interaction studies described in chapter 9 demonstrated that quiescent mesothelial cells inhibited the growth of tumour cells. Contrasting, actively proliferating mesothelial cells as seen during peritoneal healing[20;21], stimulated the growth of tumour cells due to the production of factors by these cells during proliferation (chapter 10). It seems different paracrine loops are present during activated mesothelial responses than during peritoneal equilibrium when mesothelial cells are quiescent. In addition to enhanced adhesion to matrix components, cross-talk between mesothelial and tumour cells may be an important mechanism for the preferential tumour growth at surgically traumatised peritoneal locations (figure 11.2).

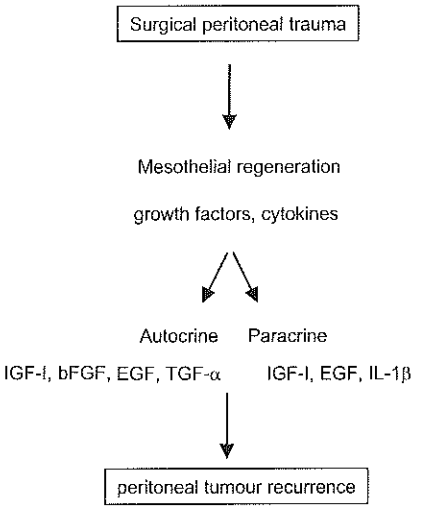


Figure 11.2

Mesothelial cell regeneration and peritoneal tumour recurrence.

Cellular mediators of surgical trauma

Peritoneal trauma induces an oscillation in abdominal cells. Apart from an augmented absolute cell amount, the ratio changes from primarily monocytes to granulocytes (PMN). This observation was made during the acute phase after surgery and most likely represents the PMN influx in response to chemoattractant factors. PMN generate reactive oxygen metabolites and discharge contents of granular organelles to ingest foreign particles or micro-organisms. Both oxygen dependent and oxygen independent processes participate in the killing of bacteria and also in damage to host tissue.[22-24] PMN also produce inflammatory mediators like IL-1β and TNF-α, however the capacity is low in comparison to peritoneal macrophages.[23;25]

Enhanced tumour recurrence after intra-peritoneal injection of primarily PMN, as accomplished in the experiments described in chapter 4, may therefore be based on several characteristics of these cells. First, through cytokine production an upregulation of cell adhesion molecules may be induced[26;27], hereby facilitating tumour adhesion (figure 1B). In addition, cytokine activated mesothelial cells produce chemoattractants such as IL-8, required for PMN recruitment.[28;29] 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. Secondly, mesothelial damage may be

induced in two ways. By the degranulation of organelles, but most likely through the production of reactive oxygen species (ROS).[24]

Excessive production of ROS and related tissue injury plays a fundamental role in a wide variety of disease processes.[24;30] In ischaemia reperfusion injury, ROS mediated injury has been recognised for a long time.[31] In a rather unusual way, the experiments described in this thesis also turned in the direction of ROS mediated enhanced tumour recurrence (figure 11.3). In chapter 5, red blood cell (RBC) related components were recognised as having a tumour impeding function. This effect mainly took place around the acute phase of surgery. The experiments in chapter 6 further unravel that anti-oxidant scavengers, contained by RBC, are responsible for inhibiting tumour recurrence.

Recent studies brought to light that ROS are potent inducers of the nuclear factor (NF) kappaB. Upon activation, NF-kappaB induces transcription of a number of genes involved in cell adhesion, immune and pro-inflammatory responses and growth.[32;33] Although the exact pathway by which ROS stimulate peritoneal recurrence was not unravelled, both peritoneal tissue damage and activation of NF-kappaB may play a role.

With the use of anti-oxidant scavengers, it was demonstrated that ROS stimulate tumour recurrence. This may be a pathway of the tumour promoting effect brought about by the cellular component of the lavage fluid since PMN are a major source of ROS. These experiments, although indirectly, stress the correlation between tissue trauma and tumour recurrence. Furthermore, as deduced from the experiments described in chapters 4 and 5, the timing of intervention methods is essential and should be aimed within the acute phase after surgical trauma.

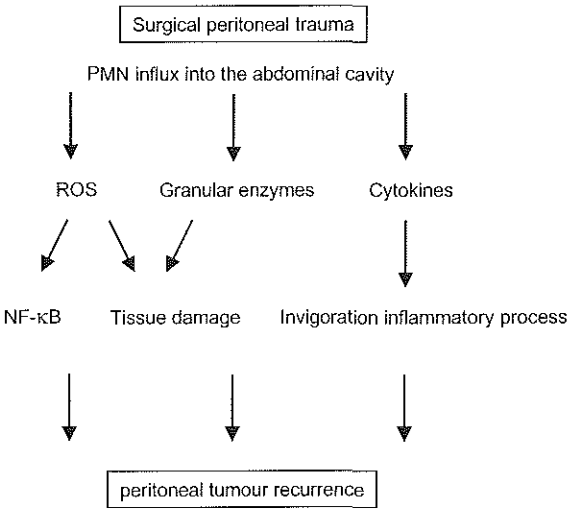


Figure 11.3

PMN mediated trauma of the peritoneum.

Soluble mediators of surgical trauma

The soluble fraction of the lavage fluid collected after surgical peritoneal trauma represents all substances, including growth factors and inflammatory cytokines, that are present in the abdominal cavity in the acute phase after surgical trauma. The dichotomy of cell adhesion and growth is now relevant to disclose exact mechanisms of tumour recurrence. Other than the demonstrated acute phase cytokines (IL-1β, IL-6, TNF-α) and IGF-I, different components of the wound healing process may also be expected in the soluble fraction.

Insulin-like Growth Factor-I (IGF-I) Serum IGF-I is a metabolic representation of trauma intensity.[34;35] Laparoscopic bowel resection affects circulating levels less than conventional surgery. In chapter 4, it is demonstrated that IGF-I in abdominal lavage fluid is also an indicator of trauma intensity. Enhanced concentrations IGF-I could be the result of efflux from the blood to the peripheral tissues[34] or of local production by mesothelial cells.[36]

The effect of IGF-I is mainly exerted during tumour growth. From the investigated growth factors, it was selected as the only factor that promoted tumour growth, which emphasises its vital role in tumour recurrence. The studies described in chapters 9 and 10 demonstrate IGF-I is used as an autocrine factor by tumour cells. During paracrine

interactions a synergistic loop appears to exist when IGF-I is added. These data demonstrate the tumour enhancing effect and the difference between autocrine and paracrine interactions mediated by IGF-I (figure 11.2).

Similar to the CC531 colon carcinoma cells used in the described studies, most human colon cancers express a significant concentration of IGF-I receptor-like binding sites.[37] Therefore, IGF-I related interactions might not be restrained to the tumour cell line used in this thesis.

Interleukin-1β (IL-1β) One of the first acute phase cytokines to appear after peritoneal trauma is IL-1β. Its whimsical appearance and volatility causes many futile attempts at demonstrating its presence.[38] The *in vitro* studies described in chapter 7 demonstrate a pivotal role of this cytokine in tumour cell adhesion. Already at low concentrations a significant increase in tumour cell adhesion to a mesothelial monolayer was seen. The time dependency suggested a secondary upregulation of cell adhesion molecules. IL-1β is known as an appropriate stimulus for NF-kappaB. Besides a direct IL-1β mediated upregulation of adhesion molecules, activation of this nuclear factor may be an indirect pathway ultimately leading to enhanced tumour recurrence.

Although IL-1β did not directly stimulate tumour and mesothelial cell growth, at paracrine level a significant augmentation of mesothelial cell proliferation was seen, illustrating that direct proliferative responses and upregulation of paracrine growth stimulatory factors are mediated via different pathways. These data place IL-1β as an important factor in different pathways leading to enhanced tumour recurrence (figures 11.1B and 11.2).

Other inflammatory cytokines and growth factors In the adhesion studies, TGF-β mildly enhanced tumour cell adhesion. EGF was a potent stimulator of tumour cell adhesion that coincided with mesothelial cell proliferation. In the growth studies EGF was indeed a potent stimulator of mesothelial cell growth. Apparently, enhanced adhesion and cell growth walk hand in hand.

Summarising, post surgically produced cytokines and growth factors secreted into the peritoneal fluid may cause a generalised state of mesothelial activation. Upregulation of cell adhesion molecules by these factors would clarify tumour recurrence at not directly traumatised peritoneal surfaces (figure 11.1B), whereas stimulated mesothelial cell proliferation would facilitate tumour adhesion to regenerating areas of traumatised peritoneum (figure 11.2).

Considerations for clinical application

Any form of peritoneal trauma is likely to enhance peritoneal tumour recurrence. Although it is the best treatment option for gastro-intestinal malignancies, surgery may not be the best intervention method to prevent recurrence. Adjuvant measures embraced with surgical intervention seem a logical step to eliminate post-operative loco-regional recurrent disease.

In this thesis several pathways of tumour recurrence have been brought into perspective. Figure 11.4 shows multiple pathways leading to enhanced tumour recurrence that are based on the experiments described in this thesis. *In vivo*, a combination of these pathways is likely to take place.

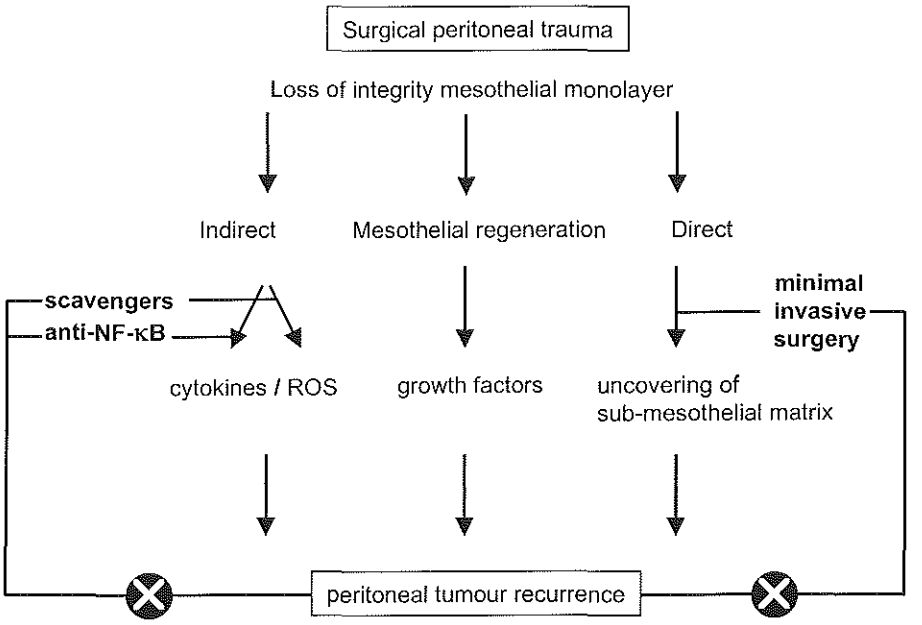


Figure 11.4

Pathways leading to tumour recurrence. Indirect peritoneal trauma: i.e. inflammatory mediators like PMN, cytokines and ROS; Growth stimulating factors during mesothelial regeneration: i.e. IGF-I and EGF; Direct peritoneal trauma: surgical handling of the peritoneum.

Adjuvant measures to prevent peritoneal tumour recurrence may be sought at different levels. Diminishing the extent of direct peritoneal tissue trauma may be achieved with the use of minimal invasive surgical techniques, like laporoscopy, and the use of less traumatic tools (i.e. gauzes). These surgical measures may be combined with the application of a protective layer to the resection site and peritoneum, as has been suggested with hyaluronic based gels and coatings (figure 11.4).

Preventing adhesion of per-operatively spilled tumour cells with the use of monoclonal antibodies might prove difficult, as the expression profile of adhesion molecules is likely to be highly variable between tumours. Integrin mediated adhesion to matrix components may be collectively blocked with the use of peptides containing integrin-binding sequences (i.e. RGD).[39;40] These peptides are capable of interfering with integrin function in general and have been shown to block experimental metastasis. Given the complexity of the cell adhesion apparatus, blocking one single receptor, however, cannot prohibit adhesion through another. Therefore, this pathway may prove difficult for clinical application.

Manipulation of the inflammatory reaction may be a more novel approach of diminishing tumour recurrence. Interference with inflammatory cytokines and growth factors with the use of antibodies may impede normal peritoneal wound healing and disturb the balance of the cytokine network inducing unknown side effects. The key role of NF-kappaB in regulating expression of a number of pro-inflammatory genes and adhesion molecules makes this protein an attractive target for selective therapeutic intervention (figure 11.4). Current studies are investigating the possibility of interfering with its regulatory system and form a promising new approach in preventing tumour recurrence.[33]

ROS mediated tissue injury can be restrained fairly easily with the use of anti-oxidant scavengers. In ischaemia-reperfusion injury, scavengers are widely used to protect organs from ROS mediated tissue injury. A wide variety of anti-oxidant scavengers, in addition to SOD and catalase, are being used in clinical practice (i.e. preservation fluids like UW). The use of a lavage fluid, containing these factors, may prove a manageable therapeutical measure to prevent recurrence by per-operatively seeded tumour cells without impeding peritoneal wound healing (figure 11.4).

References

1. Abulafi AM, Williams NS. Local recurrence of colorectal cancer: the problem, mechanisms, management and adjuvant therapy [see comments]. **Br J Surg** 1994; **81**: 7-19.
2. Delpero JR, Pol B, Le Treut P, et al. Surgical resection of locally recurrent colorectal adenocarcinoma. **Br J Surg** 1998; **85**: 372-376.
3. Schott A, Vogel I, Krueger U, et al. Isolated tumor cells are frequently detectable in the peritoneal cavity of gastric and colorectal cancer patients and serve as a new prognostic marker. **Ann Surg** 1998; **227**: 372-379.
4. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. **World J Surg** 1991; **15**: 609-616.
5. Gutman M, Fidler IJ. Biology of human colon cancer metastasis. **World J Surg** 1995; **19**: 226-234.
6. Kok TC, Van der Gaast A, Dees J, et al. Cisplatin and etoposide in oesophageal cancer: a phase II study. Rotterdam Oesophageal Tumour Study Group. **Br J Cancer** 1996; **74**: 980-984.
7. Pass HI, Donington JS. Use of photodynamic therapy for the management of pleural malignancies. **Semin Surg Oncol** 1995; **11**: 360-367.
8. Sugarbaker PH. Successful management of microscopic residual disease in large bowel cancer. **Cancer Chemother Pharmacol** 1999; **43 Suppl**: S15-S25
9. van Eijck CH, Link KH, van Rossen ME, Jeekel J. (Neo)adjuvant treatment in pancreatic cancer--the need for future trials. **Eur J Surg Oncol** 1999; **25**: 132-137.
10. van den Tol MP, van S, I, Bonthuis F, Marquet RL, Jeekel J. Reduction of intraperitoneal adhesion formation by use of non-abrasive gauze. **Br J Surg** 1997; **84**: 1410-1415.
11. Whitaker D, Papadimitriou JM, Walters MN. The mesothelium and its reactions: a review. **Crit Rev Toxicol** 1982; **10**: 81-144.
12. Akedo H, Shinkai K, Mukai M, et al. Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: a model for tumor invasion. **Cancer Res** 1986; **46**: 2416-2422.
13. Kimura A, Koga S, Kudoh H, Iitsuka Y. Peritoneal mesothelial cell injury factors in rat cancerous ascites. **Cancer Res** 1985; **45**: 4330-4333.
14. Strauli P, In-Albon A, Haemmerli G. Morphological studies on V2 carcinoma invasion and tumor-associated connective tissue changes in the rabbit mesentery. **Cancer Res** 1983; **43**: 5403-5410.
15. Ryan GB, Grobety J, Majno G. Mesothelial injury and recovery. **Am J Pathol** 1973; **71**: 93-112.
16. Watters WB, Buck RC. Scanning electron microscopy of mesothelial regeneration in the rat. **Lab Invest** 1972; **26**: 604-609.

17. Ebert EC. Mechanisms of colon cancer binding to substratum and cells. **Dig Dis Sci** 1996; **41**: 1551-1556.
18. Moser TL, Pizzo SV, Bafetti LM, Fishman DA, Stack MS. Evidence for preferential adhesion of ovarian epithelial carcinoma cells to type I collagen mediated by the alpha2beta1 integrin. **Int J Cancer** 1996; **67**: 695-701.
19. Strobel T, Cannistra SA. Beta1-integrins partly mediate binding of ovarian cancer cells to peritoneal mesothelium in vitro. **Gynecol Oncol** 1999; **73**: 362-367.
20. Nagy JA, Jackman R. Response of the peritoneal membrane to injury. **Semin Dialysis** 1998; **11**: 242-248.
21. Whitaker D, Papadimitriou J. Mesothelial healing: morphological and kinetic investigations. **J Pathol** 1985; **145**: 159-175.
22. Heinzelmann M, Simmen HP, Battaglia H, Friedl HP, Trentz O. Inflammatory response after abdominal trauma, infection, or intestinal obstruction measured by oxygen radical production in peritoneal fluid. **Am J Surg** 1997; **174**: 445-447.
23. Styrt B. Species variation in neutrophil biochemistry and function. **J Leukoc Biol** 1989; **46**: 63-74.
24. Weiss SJ. Tissue destruction by neutrophils [see comments]. **N Engl J Med** 1989; **320**: 365-376.
25. Pruimboom W, Verdoold A, Tak C, et al. Changes in eicosanoid and tumor necrosis factor a production by rat peritoneal macrophages during carrageenin-induced peritonitis. **Mediators Inflamm** 1994; **3**: 335-340.
26. Jonjic N, Peri G, Bernasconi S, et al. Expression of adhesion molecules and chemotactic cytokines in cultured human mesothelial cells. **J Exp Med** 1992; **176**: 1165-1174.
27. Klein CL, Bittinger F, Skarke CC, et al. Effects of cytokines on the expression of cell adhesion molecules by cultured human omental mesothelial cells. **Pathobiology** 1995; **63**: 204-212.
28. Meager A. Cytokine regulation of cellular adhesion molecule expression in inflammation. **Cytokine Growth Factor Rev** 1999; **10** : 27-39.
29. Topley N, Williams JD. Effect of peritoneal dialysis on cytokine production by peritoneal cells. **Blood Purif** 1996; **14**: 188-197.
30. Bulkley GB. The role of oxygen free radicals in human disease processes. **Surgery** 1983; **94**: 407-411.
31. Grace PA. Ischaemia-reperfusion injury. **Br J Surg** 1994; **81**: 637-647.
32. Chen F, Castranova V, Shi X, Demers LM. New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. **Clin Chem** 1999; **45**: 7-17.

33. Jobin C, Sartor RB. The IkappaB/NF-kappaB system: a key determinant of mucosal inflammation and protection. **Am J Physiol Cell Physiol** 2000; **278**: C451-C462
34. Bouvy ND, Marquet RL, Tseng LN, et al. Laparoscopic vs conventional bowel resection in the rat. Earlier restoration of serum insulin-like growth factor 1 levels. **Surg Endosc** 1998; **12**: 412-415.
35. Skjaerbaek C, Frystyk J, Orskov H, et al. Differential changes in free and total insulin-like growth factor I after major, elective abdominal surgery: the possible role of insulin- like growth factor-binding protein-3 proteolysis. **J Clin Endocrinol Metab** 1998; **83**: 2445-2449.
36. van der Wal BC, Hofland LJ, Marquet RL, van Koetsveld PM, van Rossen ME, van Eijck CH. Paracrine interactions between mesothelial and colon-carcinoma cells in a rat model. **Int J Cancer** 1997; **73**: 885-890.
37. Guo YS, Narayan S, Yallampalli C, Singh P. Characterization of insulinlike growth factor I receptors in human colon cancer. **Gastroenterology** 1992; **102**: 1101-1108.
38. Baigrie RJ, Lamont PM, Dallman M, Morris PJ. The release of interleukin-1 beta (IL-1) precedes that of interleukin 6 (IL-6) in patients undergoing major surgery. **Lymphokine Cytokine Res** 1991; **10**: 253-256.
39. Ruoslahti E. Integrin signaling and matrix assembly. **Tumour Biol** 1996; **17**: 117-124.
40. Ruoslahti E. Integrins as signaling molecules and targets for tumor therapy. **Kidney Int** 1997; **51**: 1413-1417.

Chapter XII

SUMMARY and CONCLUSIONS

Surgical treatment of gastro-intestinal malignancies is often complicated by loco-regional recurrence. Patterns of first recurrence show the resection site is preferential, and distant metastases combined with loco-regional intra-abdominal recurrence is common.

The studies described in this thesis were aimed at unravelling links between surgical peritoneal trauma and peritoneal tumour recurrence. In vivo and in vitro rat models were used to study which surgery related factors affected tumour cell adhesion or growth and consequently tumour recurrence. Different facets of the peritoneal response to surgical trauma were highlighted with the intention to elucidate which pathways may be used for specific therapeutical alternatives.

IN VIVO EXPERIMENTS

Surgical peritoneal trauma and tumour recurrence *in vivo*

Surgical trauma to the peritoneum causes the formation of fibrous adhesions. A correlation appears to exist between the intensity of surgical trauma and the amount of adhesion formation. The first experimental studies presented in this thesis investigated whether mechanisms leading to post-operative adhesion formation also applied to post-operative tumour recurrence.

The demonstrated results clearly point out that a similar correlation exists between the intensity of surgical peritoneal trauma and the amount of local tumour recurrence. However, after infliction of severe peritoneal trauma significantly more tumour recurrence was also seen at not directly traumatised sites in addition to enhanced tumour growth. It seems that the surgery related tumour enhancing effect is not a local phenomenon but has a generalised character.

The inflammatory response to surgical peritoneal trauma appeared to play a pivotal role in the process of tumour recurrence. Inflammatory mediators like cells and cytokines could be captivated in a lavage fluid and transferred to naive, non-operated recipients. Surgery related factors produced in the early hours after surgical peritoneal trauma, were potent stimulators of tumour recurrence. The cellular component of the post-surgical inflammatory process was the main stimulator of *in vivo* tumour recurrence. Cytokines also enhanced *in vivo* recurrence, but played an inferior role. No differences in cytokine concentration were detected in mildly or severely traumatised rats. However, elevated concentrations of IGF-I were detected in the abdomens of severely traumatised rats.

Intra-abdominal blood loss and peritoneal tumour recurrence *in vivo*

Operative skills seem to be a relevant factor in tumour prognosis. Excessive per-operative blood loss followed by transfusion has been associated with increased tumour recurrence. The *in vivo* studies described in this part of the thesis analysed the effect of intra-abdominal blood on peritoneal tumour cell adhesion and growth.

Simultaneous intra-abdominal injection of blood and tumour cells after surgical peritoneal trauma resulted in reduced adhesion of per-operatively spilled tumour cells, but did not affect tumour growth. The tumour inhibitive effect by blood only took place within 24 hours after surgical trauma and was mediated by intra-RBC substances.

The RBC related anti-oxidant scavengers catalase and superoxide dismutase (SOD) were among the effectors of tumour inhibition by RBC. Haemoglobin enhanced tumour recurrence significantly. The total inhibition of tumour recurrence that was achieved by combining catalase and SOD was not additional to their separate effect and consisted of 40%. From these results we concluded that neutralising the oxidative burst after surgical peritoneal trauma with the use of anti-oxidant scavengers may prove a successful approach for diminishing peritoneal tumour recurrence.

Based on these *in vivo* results, the initial impetus to separating the generalised term "tumour recurrence" into the more elementary mechanisms tumour cell adhesion and tumour growth was made. The following *in vitro* experiments were aimed at unravelling specific pathways of enhanced tumour cell adhesion and growth.

IN VITRO EXPERIMENTS

Mesothelial and tumour cell adhesive interactions *in vitro*

An *in vitro* cell adhesion model was designed to study the adhesive interactions between primary cultures of mesothelial cells and tumour cells. The effect of inflammatory cytokines and growth factors, produced after surgical trauma, on tumour cell adhesion could be investigated without the interference of unknown *in vivo* factors. Using the same model, adhesion to different proteins of the sub-mesothelial matrix mimicked tumour cell adhesion to damaged peritoneum.

Tumour cell adhesion to mesothelium pre-incubated with IL-1β and EGF resulted in a dose dependent stimulation of tumour cell adhesion of at least 60%. TGF-β pre-incubation resulted in a minor though significant stimulation of cell adhesion (maximal 16%). Enhanced tumour cell adhesion after IL-1β pre-incubation appeared to depend on an upregulation of cell adhesion molecules, whereas after EGF pre-incubation an increase in the number of mesothelial cells probably played a role.

When studying tumour cell adhesion to different sub-mesothelial matrix components, a preferential adhesion of tumour cells to collagen type I (50% *versus* <30% adhesion to other sub-mesothelial matrix (ECM) components and mesothelial cells) was seen. Tumour cells contained mRNA encoding for ICAM-I, α_1, α_2 and β_1 integrins and expressed these adhesion molecules on the cell surface. Unlike α integrin chains, β_1 mediated tumour cell adhesion to all ECM proteins. Tumour cell adhesion to mesothelium occurred through an integrin and ICAM-I independent mechanism.

Analysis of cell adhesion molecules expressed by both cell types elucidated the presence of several adhesion molecules offering potential interaction between both cell types.

Mesothelial and tumour cell growth *in vitro*

The response of mesothelial cells to growth factors highlights the reparative properties of this tissue. During wound healing, tumour cells present at the injured site may profit from the same mediators to stimulate tumour growth. Indeed, clinical evidence exists that surgically traumatised surfaces are predestined sites for successful implantation of tumour cells. This part of the thesis investigated which factors play a role in mesothelial and tumour cell proliferation and interaction.

We demonstrated that growth stimulation of tumour cells in the autocrine setting was preferentially mediated through IGF-I. Mesothelial cells appeared more susceptible to the growth stimulating effects of different wound healing factors. In our study, significant enhancement of mesothelial cell proliferation was seen after stimulation with IGF-I, TGF-α, EGF and bFGF.

During co-culture, interactions at paracrine level between mesothelial and tumour cells could be investigated. Under these conditions, the cell types do not make contact however, cross-talking takes place by soluble factors in the culture medium produced by both cell types. These studies demonstrated that quiescent mesothelial cells, during non-proliferating conditions, produced factors that significantly inhibited the growth of tumour cells, while, conversely, tumour cells stimulated the growth of mesothelial cells. Adding IGF-I to the culture medium potentiated the inhibitory effect of mesothelial cells on tumour cells. Actively proliferating mesothelial cells, as seen during peritoneal wound healing, stimulated tumour growth significantly. Under these circumstances IGF-I, added to the medium, showed additional stimulation of tumour growth.

Of the cytokines, IL-1β slightly inhibited tumour cell and mesothelial cell growth but augmented mesothelial cell proliferation during co-culture with tumour cells. This suggests that the direct proliferative responses and upregulation of paracrine growth stimulatory factors are mediated via different pathways.

It seems different paracrine loops are present during activated mesothelial responses than during peritoneal equilibrium when mesothelial cells are quiescent.

Conclusions

- Surgical abdominal trauma stimulates peritoneal tumour adhesion and growth. *(chapter 3)*
- Surgical abdominal trauma stimulates tumour growth. *(chapter 3)*
- The extent of surgical trauma correlates with tumour adhesion and growth. *(chapter 3)*
- Inflammatory cells (mainly PMN), present in the abdominal cavity after surgical trauma, stimulate tumour adhesion and/or growth. *(chapter 4)*
- Cytokines and growth factors, present in the abdominal cavity after surgical trauma, stimulate tumour adhesion and/or growth. *(chapter 4)*
- Simultaneous injection of blood and tumour cells in the peritoneal cavity inhibits adhesion of these tumour cells. *(chapter 5)*

- Intra red blood cell anti-oxidants (catalase and SOD) prevent peritoneal tumour adhesion and/or growth. *(chapter 6)*
- IL-1β stimulated tumour cell adhesion is associated with an upregulation of cell adhesion molecules. *(chapter 7)*
- EGF stimulated tumour cell adhesion coincides with mesothelial cell proliferation. *(chapter 7)*
- Tumour cells adhere preferentially to sub-mesothelial collagen (type I). *(chapter 8)*
- Significantly higher concentrations of IGF-I are found in surgically traumatised abdomens. *(chapter 4)*
- IGF-I stimulates tumour growth. *(chapter 10)*
- A monolayer mesothelial cells inhibits tumour cell proliferation. *(chapter 9)*
- Actively proliferating mesothelial cells stimulate tumour cell proliferation. *(chapter 10)*

These conclusions may, however, be restricted to the used rat model and colon carcinoma cell line (CC531) in the described experiments. General conclusions that may be drawn are:

- Surgical abdominal trauma stimulates peritoneal tumour recurrence through stimulating tumour cell adhesion and tumour growth.
- The inflammatory process after surgical trauma plays a pivotal role in the promotion of peritoneal tumour recurrence.
- Neutralisation of the oxidative burst after surgical trauma prevents tumour recurrence.
- Tumour cell adhesion is stimulated through an upregulation of cell adhesion molecules.
- Mesothelial proliferation stimulates tumour cell growth and adhesion.
- An intact peritoneum is the best way of preventing peritoneal tumour recurrence.

APPENDICES

LIST OF PUBLICATIONS

van der Wal BCH, Hofland LJ, Marquet RL, van Koetsveld PM, van Rossen MEE, van Eijck CHJ. Paracrine interactions between mesothelial and colon carcinoma cells in a rat model. **Int J Cancer** 1997; **73**: 885-890.

van den Tol MP, van Rossen MEE, van Eijck CHJ, Bonthuis F, Marquet RL, Jeekel J. Reduction of peritoneal trauma by using non-surgical gauze leads to less implantation metastasis of spilled tumour cells. **Ann Surg** 1998; **227**: 242-248.

Marquet RL, van Rossen MEE, van den Tol MP, Bonthuis F, Haverlag R, Jeekel J. Can exposure to latex cause adhesion formation? **Allergy** 1998; **53**: 1229-30.

Haverlag R, van Rossen MEE, van den Tol MP, Bonthuis F, Marquet RL, Jeekel J. Hyaluronate-based coating solution for prevention of surgical adhesions has no major effect on adhesion and growth of intraperitoneal tumour cells. **Eur J Surg** 1999;**165**: 791-795.

van Rossen MEE, Stoop MPO, Hofland LJ, van Koetsveld PM, Marquet RL, Jeekel J, van Eijck CHJ. Red blood cells inhibit tumour cell adhesion to the peritoneum. **Br J Surg** 1999; **86**: 509-513.

van Eijck CHJ, Link KH, van Rossen MEE, Jeekel J. (Neo)adjuvant treatment in pancreatic cancer. The need for future trials. **Eur J Surg Oncol** 1999;**25**: 132-7.

van Rossen MEE, Hofland LJ, van den Tol MP, van Koetsveld PM, Jeekel J, Marquet RL, van Eijck CHJ. Effect of inflammatory cytokines and growth factors on tumour cell adhesion to the peritoneum. Accepted for publication, **J Pathol**.

van Rossen MEE, van den Tol MP, Aalbers AGJ, Hofland LJ, van Koetsveld PM, Bonthuis F, Jeekel J, Marquet RL, van Eijck CHJ. The inflammatory sequelae of surgery provoke enhanced tumour recurrence; a crucial role for neutrophils and cytokines. Submitted for publication.

van Rossen MEE, Sluiter W, Bonthuis F, Jeekel J, Marquet RL, van Eijck CHJ. Scavenging of reactive oxygen species leads to diminished peritoneal tumour recurrence. Submitted for publication.

van Rossen MEE, Hofland LJ, Aalbers AGJ, van den Beemd MWM, Wiemer EAC, van Koetsveld PM, Jeekel J, Marquet RL, van Eijck CHJ. Identification of receptors on colon carcinoma cells binding extra cellular matrix components. Submitted for publication.

van Rossen MEE, Hofland LJ, van Koetsveld PM, Jeekel J, Marquet RL, van Eijck CHJ. Paracrine modulators of peritoneal tumour recurrence in a rat model. Submitted for publication.

SAMENVATTING VOOR DE LEEK

Chirurgische behandeling van tumoren uitgaande van het maagdarmkanaal wordt vaak gecompliceerd door het terugkomen van kanker in de buik (loco-regionale tumorrecurrence). Veelal is de plaats waar de oorspronkelijke tumor is verwijderd de voorkeurslocatie voor het terugkomen van kanker. Metastasen buiten de buik gecombineerd met recurrence in de buik wordt ook veel gezien.
De studies beschreven in dit proefschrift hebben als doel een mogelijke correlatie aan te tonen tussen chirurgisch trauma van het peritoneum (buikvlies) en peritoneale tumorrecurrence. *In vivo* en *in vitro* ratmodellen zijn gebruikt om te onderzoeken of factoren die in de buik geproduceerd worden na chirurgie, de aanhechting of de groei van tumorcellen beïnvloeden en daarmee tumorrecurrence.

Chirurgisch peritoneaal trauma en tumorrecurrence *in vivo*
Chirurgische beschadiging, of trauma, van het peritoneum veroorzaakt fibreuze adhesies tussen beschadigde oppervlakken in de buik. Eerder onderzoek heeft aangetoond dat er een correlatie bestaat tussen de intensiteit van het aangerichte chirurgisch trauma en de mate van adhesieformatie. De eerste experimentele studies beschreven in dit proefschrift onderzoeken naar aanleiding van deze resultaten de invloed van vergelijkbare mechanismen, die leiden tot adhesieformatie, op postoperatieve tumorrecurrence.
De resultaten van deze studies laten zien dat een vergelijkbare correlatie tussen trauma-intensiteit en tumorrecurrence aanwezig is. Niet alleen werd er meer tumorrecurrence aangetroffen op de chirurgisch beschadigde locaties, maar ook op niet beschadigd peritoneum. Bovendien bleken reeds aanwezige tumoren, die niet in de buik gelokaliseerd waren, harder te groeien. Het lijkt dan ook dat de factoren die geproduceerd worden na een chirurgisch trauma niet alleen locaal (in de buik) maar ook op afstand een tumor stimulerend effect hebben.
De ontstekingsreactie na chirurgisch peritoneaal trauma bleek een centrale rol te spelen bij tumorrecurrence. Ontstekingsmediatoren, zoals cellen en cytokinen, werden gevangen in een spoelvloeistof en overgebracht naar buiken van niet-geopereerde ontvangers. Na deze passieve overdracht werd tumorrecurrence ook gestimuleerd in de ontvangers. De cellulaire component van de ontstekingsreactie bleek het meest

krachtige tumorstimulerende effect te hebben, echter de overdracht van cytokinen alleen leidde ook tot significant meer tumorrecurrence.

Intra-abdominaal bloedverlies en peritoneale tumorrecurrence *in vivo*

Operatieve vaardigheid lijkt een belangrijke prognostische factor te zijn voor patiënten met kanker. Overmatig bloedverlies tijdens de operatie gevolgd door bloedtransfusie is geassocieerd met een slechtere prognose. De *in vivo* studies beschreven in dit deel van het proefschrift hebben het effect van de aanwezigheid van bloed in de buik op peritoneale tumorcel-aanhechting en –groei onderzocht.

Het achterblijven van bloed en tumorcellen in de buik na het aanrichten van chirurgisch trauma ging gepaard met minder aanhechting van tumorcellen. Werd het bloed toegevoegd nadat tumorcellen reeds aangehecht waren (24 uur later), dan was het tumor-remmend effect verdwenen. Anti-oxidantia, aanwezig in rode bloedcellen, bleken verantwoordelijk voor het tumor-remmende effect. Het gebruik van anti-oxidantia lijkt dan ook een succesvolle benadering te zijn voor het verminderen van peritoneale tumrorecurrence.

De hierna beschreven studies zijn verricht in een weefselkweekmodel (*in vitro*) waarin de elementaire mechanismen van tumorrecurrence, zoals tumorcel-aanhechting en –groei, onderzocht kunnen worden.

De aanhechting van tumorcellen op mesotheel

Alle organen in de buik worden bedekt door het peritoneum dat bestaat uit mesotheelcellen van slechts één laag dik met daaronder een matrix van eiwitten. Door de mesotheelcellen *in vitro* te kweken, kon de aanhechting van tumorcellen bestudeerd worden zonder dat onbekende '*in vivo*' factoren hier invloed op hadden.

Door de gekweekte mesotheellaag voor te behandelen met cytokinen en groeifactoren werd aangetoond dat sommige van deze factoren de aanhechting van tumorcellen op het mesotheel stimuleren. Bovendien bleek er een preferentiële aanhechting te bestaan van tumorcellen op de eiwitten die zich in de matrix onder de mesotheellaag bevinden. Deze aanhechting werd gereguleerd door specifieke aanhechtingsmoleculen waarvan de functie volledig geblokkeerd kon worden met de hulp van antilichamen.

Mesotheel- en tumor-groei *in vitro*

De reactie van mesotheelcellen op groeifactoren en cytokinen stipt de unieke herstelmogelijkheden van dit weefsel aan. Tijdens wondheling kunnen tumorcellen die aanwezig zijn op de plaats van de wond profiteren van dezelfde mediatoren als de mesotheelcellen wat leidt tot gestimuleerde tumorgroei. In dit deel van het proefschrift werd onderzocht welke factoren een rol spelen bij mesotheel- en tumor-groei en of zij de interactie tussen deze cellen beïnvloeden.

Mesotheelcellen groeiden significant sneller nadat verschillende groeifactoren toegevoegd werden aan het kweekmedium. Tumorcellen respondeerden alleen na toevoegen van één groeifactor aan het kweekmedium.

Door mesotheel- en tumor-cellen samen te kweken zonder dat ze contact maken met elkaar, kon onderzocht worden of deze cellen een interactie met elkaar aangaan door te communiceren via het kweekmedium. Deze studies toonden aan dat mesotheelcellen van één cellaag dik, zoals in de buik voorkomt, factoren produceren en uitscheiden die de groei van tumorcellen remmen. Werden tumorcellen samen gekweekt met groeiende mesotheelcellen, zoals tijdens wondheling, dan werd tumorgroei gestimuleerd. Omgekeerd, bleken tumorcellen de groei van mesotheelcellen te stimuleren.

Samenvattend kan gesteld worden dat chirurgisch abdominaal trauma de groei en aanhechting van tumorcellen stimuleert en daarmee peritoneale tumorrecurrence. Beschadigd peritoneum, waarbij de mesotheellaag is verdwenen, toont een voorkeursplaats voor tumorrecurrence. Tijdens het ontstekingsproces en de wondheling na chirurgische beschadiging komen factoren vrij in de buik die locaal de aanhechting en groei van tumorcellen stimuleren, maar tevens de tumorgroei op aftstand bevorderen.

Algemeen mag geconcludeerd worden dat een intact peritoneum de beste manier is om peritoneale tumorrecurrence te voorkomen.

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CURRICULUM VITAE AUCTORIS

Maria Elma Elizabeth van Rossen was born on 1st September, 1970 in Utrecht, The Netherlands. After finishing high school at the Huygens Lyceum in Voorburg in 1988, she continued her studies at the Faculty of Medicine of the Erasmus University in Rotterdam. During her undergraduate days her interest in surgery was aroused when she worked at the surgical department of hospitals in Budapest, Seville and Barcelona.
In 1996 she obtained her medical degree and was offered her present position as a PhD student by dr. C.H.J. van Eijck (Department of General Surgery, University Hospital Rotterdam Dijkzigt). During her four years as a researcher at the laboratory of Experimental Surgery & Oncology (Erasmus University Rotterdam) her work was awarded by the European Society for Surgical Research (Walter Brendel nomination and Young Researchers Award), the European School of Oncology (best scientific presentation on gastro-intestinal malignancies) and by the Symposium Experimenteel Onderzoek Heelkundige Specialismen (best scientific presentation).
In July 2000 she will start her training in Surgery at the Surgical Departments of the Albert Schweitzer Hospital (location Dordwijk) in Dordrecht (dr. K.G. Tan) and at the Academic Medical Center in Amsterdam (Prof. dr. D.J. Gouma).

LIST OF ABBREVIATIONS

bFGF	basic fibroblast growth factor
CAPD	continuous ambulatory peritoneal dialysis
E-cad	epithelial cadherin
ECM	extra cellular matrix
EGF	epidermal growth factor
GM-CSF	granulocyte monocyte-colony stimulating factor
ICAM	inter cellular adhesion molecule
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
IL-1RA	interleukin-1 receptor antagonist
NF-κB	nuclear factor kappa B
PAI	plaminogen activator inhibitor
PDGF	platelet derived growth factor
PMN	polymorph nuclear leukocytes
PG	prostaglandins
ROS	reactive oxygen species
SOD	superoxide dismutase
TGF	transforming growth factor
TNF	tumour necrosis factor
VCAM	vascular cell adhesion molecule