The Consequences of Surgical Trauma on Intra-Peritoneal Tumour Recurrence and/or Adhesion Formation

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The Consequences of Surgical Trauma on Intra-Peritoneal Tumour Recurrence and/or Adhesion Formation

De consequenties van chirurgisch trauma op intra-peritoneale tumor ontwikkeling en/of de vorming van adhesies

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Chapter 1

Aim and Outline of the Thesis

S. ten Raa
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BACKGROUND

Potentially curative resection of colorectal or pancreatic carcinoma is often compromised due to locoregional or peritoneal recurrences. Furthermore in an average of 85% of patients that have received intra-abdominal surgery (oncological or other), post-operative adhesions are formed, which are a major source of post-operative morbidity.

The pathogenesis of the processes responsible for postoperative adhesion formation or intra-abdominal tumour recurrence is only partly clarified. Previous studies at our laboratory have suggested that the peritoneal defence mechanism triggered by surgical trauma to the peritoneum not only promotes adhesion formation but also stimulates tumour recurrence. Implicating a common denominator. The amount of peritoneal trauma has shown to be correlated with the amount of intra-abdominal adhesion formation or tumour recurrence. Furthermore in a rat model the effects of a surgical peritoneal trauma could be captivated in lavage fluids and passively transferred to naïve recipients. These observations have made the inflammatory reaction induced by surgical trauma our main subject of investigation.

AIM AND OUTLINE OF THE THESIS

To be able to prevent or reduce surgical complications like postoperative intra-abdominal adhesion formation and postoperative intra-abdominal tumour recurrence, the mechanism by which the complications occur need to be clarified.

Part one focus on the consequences of surgical peritoneal trauma on peritoneal carcinomatosis. The presence of tumour cell clusters at the time of surgery as well as seeding of tumour cells due to surgical handling or clamping may result in macrometastatic disease. The post-operative wound microenvironment provides advantageous conditions for tumour recurrence as tumour development was influenced by locally produced growth factors, inflammatory cytokines, and reactive oxygen species produced by inflammatory cells. One of the aims of this thesis is to analyse the effects of surgery on tumour development in more detail. For instance aimed to pinpoint the effect of local and remote surgical trauma on outgrowth of seeded tumour cells as well as on established tumour cell clusters (*chapter 3*). Analysing the different components of the inflammatory reaction (*chapter 4-6*). This may lead to the rationale design of new therapeutic strategies targeting early (post-operative) tumour development.

Part two focuses on the consequences of surgical peritoneal trauma on intra-abdominal adhesion formation. The main denominator of the inflammatory reaction for postoperative peritoneal recurrence is the neutrophil and its main products, reactive oxygen species. As suggested in previous studies the mechanism by which postoperative tumour recurrence is promoted also promotes post-operative adhesion formation. The role of neutrophils and oxygen free radicals in postoperative adhesions is analysed in *chapter 8*. Finally the need for

treatment strategies to reduce postoperative adhesion formation and peritoneal recurrence is high. Icodextrin, a glucose polymer, has immerged as a save and promising anti-adhesive agent. We further analysed the mechanisms by which icodextrin might be able to reduce adhesion formation and whether this influences peritoneal carcinomatosis (*chapter 9*).

Chapter 2

Introduction:

The Consequences of Surgical Trauma on Intra-Peritoneal Tumour Recurrence

S. ten Raa
J. Jeekel
C.H.J. van Eijck

CLINICAL IMPACT

The course of colorectal and pancreatic carcinomas after potentially curative resection is often compromised due to local or regional recurrences, mostly presenting within the first two years after operation.[1,2]

The reported incidence of peritoneal seeding varies considerably. Peritoneal lavage fluid and serosal stamp cytology obtained peri-operatively, during intentionally curative surgery for colorectal cancer, proved tumour positive in 0% to 42%.[3,4] The conversion rate from negative pre-resection to positive post-resection in lavage fluid, apparently as a result of the surgery has shown to be up to 10%.[5,6] Tumour positive peritoneal washings are reported in up to 29% of patients receiving potentially curative resection for pancreatic carcinoma.[7] The prognostic significance of peritoneal seeding has proven to be difficult to determine. Several authors have made an attempt to correlate the finding of positive cytology with various clinicopathologic parameters, including pathologic TNM stage, malignancy grade, locoregional recurrence rate, and/or survival. Yamamoto et al.[8] and Kanellos et al.[9] found positive cytology to be significantly correlated with the risk of intraperitoneal recurrence. The relative risk of intraperitoneal recurrence in those patients with positive cytology as opposed to patients with negative cytology was 16.5 and 2.9, respectively. Moreover studies have shown that positive cytology correlated with impaired overall or disease-free survival.[5,7,10] The incidence of intraperitoneal recurrence in colorectal carcinoma defined as recurrence in the bowel anastomosis or in the resection bed, was reported in 5% to 18% after curative resection of colon cancer. Peritoneal carcinomatosis was reported in 4% to 12% after curative resection of colon cancer and in 2% to 19% after curative resection of rectal cancer.[3] For pancreatic carcinoma peritoneal dissemination is reported in 6% to 56% after potentially curative resection. Peritoneal carcinomatosis as the sole pattern of recurrence or as a component of failure was found in 4% and 21%, respectively.[7,11]

Surgery remains the only curative option for colorectal and pancreatic carcinoma, but intra-abdominal surgical trauma might favour loco-regional and peritoneal recurrence. The relation between surgical trauma, wound healing and tumour recurrence has been acknowledged since early observations of recurrent malignancies in operative wounds.[12,13] The reported increased risk of tumour recurrence that correlates with peri-operative blood transfusions, may be caused by the circumstances that necessitate them, such as extensive tissue trauma.[14] An association between surgical trauma and tumour development was furthermore supported by a number of experimental studies.[15-18] The amount of surgical trauma was found to correlate with the extensiveness of tumour recurrence.[18] Minimizing surgical trauma through the use of laparoscopy has shown to reduce tumour recurrence in experimental models.[19,20]

These data indicate that, in patients with gastrointestinal and pancreatic carcinoma, intraperitoneal recurrence is a rather common phenomenon with important clinical consequences.

PATHOGENESIS

Early preoperative tumour cell seeding and peroperative intra-abdominal shedding of tumour cells, due to handling of the tumour and leakage from dissected lymphatic channels, are inevitable causes of peritoneal metastasis.[1,21] Several studies have shown the presence of free malignant cells within the peritoneal cavity prior to and during surgery.[3,7,11] Schott et al.[4] demonstrated the presence of malignant cells in 22% of peritoneal lavage fluids of patients operated for colorectal cancer. Even in early stages of colorectal cancer free malignant cells were found in the peritoneal cavity.[4] Previous experimental data have demonstrated that the proliferative and metastatic potentials of these spilled tumour cells are very well preserved.[4,22] Peroperative occurrence of tumour cells in the peritoneal cavity has been shown to negatively correlate with the postoperative survival rate.[4,23] Previously described clinical and experimental studies showed that surgical trauma promotes intra-abdominal tumour recurrence.[14,18,20] The number of spilled tumour cells and their possibilities to give rise to implantation of metastases can be reduced by employing gentle operating techniques and materials. The dynamic cascade of peritoneal healing, induced by peritoneal damage, seems to be important in the process of intra-peritoneal adhesion and growth of tumour cells.[18,24,25]

Several theories speculate on the mechanisms of intra-peritoneal tumour recurrence as a consequence of peroperatively spilled intra-peritoneal tumour cells. Presumed implantation strategies of tumour cells include the theory of metastatic efficiency and the tumour cell entrapment hypothesis.[21] According to the theory of metastatic efficiency the implantation of spilled tumour cells on raw tissue surfaces is very efficient as opposed to the inefficient implantation on intact surfaces. Van den Tol et al.[18] showed a significant correlation between the amount of peritoneal trauma and the degree of tumour load at damaged but also at not directly traumatised peritoneal surfaces. The tumour cell entrapment hypothesis proposes that the fibrinous exudate, formed as an initial response to surgical trauma of the peritoneum, facilitates implantation of cancer cells onto raw tissue due to entrapment of the spilled tumour cells in the fibrin.

The role of the wound environment has only been partly elucidated. The presence of tumour cell clusters at the time of surgery as well as seeding of tumour cells due to surgical handling or clamping may result in peritoneal metastases. The post-operative wound microenvironment provides advantageous conditions for tumour recurrence as tumour development was influenced by locally produced growth factors, inflammatory cytokines, and reactive oxygen species produced by inflammatory cells.[26,27] The mesothelium plays an important role in this process. Mesothelial cells not only attend the peritoneal lining, but also

play a key role in the peritoneal inflammatory reaction. Upon activation mesothelial cells also produce a great variety of cytokines, chemoattractants and growth factors, both with pro-inflammatory as well as anti-inflammatory actions. Moreover the activated mesothelial cells will recruit the inflammatory cells like polymorphonuclear cells and macrophages. In an experimental rat model lavage fluid from peritoneal traumatized rats were administered intraperitoneal together with tumour cells to naïve recipients, which resulted in significantly more peritoneal tumourload compared to rats receiving just tumour cells.[18]

Successful development of metastases depends on several successive processes, such as adhesion, implantation and proliferation of seeded tumour cells. Experimental data suggest that wound healing mediators affect both tumour cell adhesion and growth, though involved mechanisms are largely unknown.[25,26] Tumour cells are able to adhere to extracellular matrix products as well as to mesothelial cells. Specialized proteins termed cell adhesion molecules mediate this process. Several cell adhesion molecules have been investigated. Schlaeppi et al.[28] showed that integrins are related to the tumour cell to extracellular matrix adhesion. Whereas intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and CD44 are responsible for tumour cell adhesion to mesothelial cells.[28-30]

As shown development of peritoneal metastasis is a complex cascade of events stimulated by the postoperative environment. The exact mechanisms are still to be explored.

PREVENTION

The amount of surgical trauma is directly related to tumour recurrence. Clearly, the surgeon must reduce operative trauma to the peritoneum, by avoiding unnecessary handling, manipulation of the tumour and by minimizing the use of abrasive material. Less invasive surgical approaches like laparoscopy should be performed whenever possible.[20]

Unravelling the mechanisms, which stimulate postoperative tumour development, may lead to the rationale design of new therapeutic strategies. Remodelling the postoperative inflammatory response could be a promising venue. Intraperitoneal administration of granulocyte/ macrophage-colony-stimulating factor has shown to inhibit tumour growth even in the post-operative period.[31] Van Rossen et al.[27] showed a significant reduction of peritoneal metastases in a rat model by scavenging reactive oxygen species that are produced by recruited inflammatory cells. Other promising results were seen using cyclo-oxygenases and prostaglandins in a mouse model.[32] Blocking the ability of tumour cells to adhere to the mesothelial cell or to the extracellular matrix components could be another level at which peritoneal metastasis can be prevented. Mainly in vitro studies using anti-integrin antibodies have shown that these antibodies were able to block adhesion and invasion of different human coloncarcinoma cell-lines to the extracellular matrix.[28]

Still, more research has to be performed to fully understand the mechanisms involved in loco-regional recurrence and peritoneal metastasis. Herewith finding a key mechanism by which peritoneal metastasis can be reduced/prevented.

REFERENCES

1. Abulafi AM, Williams NS. Local recurrence of colorectal cancer: the problem, mechanisms, management and adjuvant therapy. Br J Surg 1994;81:7-19.
2. Improved survival with preoperative radiotherapy in resectable rectal cancer. Swedish Rectal Cancer Trial. N Engl J Med 1997;336:980-7.
3. Koppe MJ, Boerman OC, Oyen WJ, Bleichrodt RP. Peritoneal carcinomatosis of colorectal origin: incidence and current treatment strategies. Ann Surg 2006;243:212-22.
4. Schott A, Vogel I, Krueger U, Kalthoff H, Schreiber HW, Schmiegel W, Henne-Bruns D, Kremer B, Juhl H. 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;227:372-9.
5. Guller U, Zajac P, Schnider A, Bosch B, Vorburger S, Zuber M, Spagnoli GC, Oertli D, Maurer R, Metzger U, et al. Disseminated single tumor cells as detected by real-time quantitative polymerase chain reaction represent a prognostic factor in patients undergoing surgery for colorectal cancer. Ann Surg 2002;236:768-75; discussion 75-6.
6. Horattas MC, Evasovich MR, Topham N. Colorectal carcinoma and the relationship of peritoneal cytology. Am J Surg 1997;174:334-7; discussion 37-8.
7. Vogel I, Kruger U, Marxsen J, Soeth E, Kalthoff H, Henne-Bruns D, Kremer B, Juhl H. Disseminated tumor cells in pancreatic cancer patients detected by immunocytology: a new prognostic factor. Clin Cancer Res 1999;5:593-9.
8. Yamamoto S, Akasu T, Fujita S, Moriya Y. Long-term prognostic value of conventional peritoneal cytology after curative resection for colorectal carcinoma. Jpn J Clin Oncol 2003;33:33-7.
9. Kanellos I, Demetriades H, Zintzaras E, Mandrali A, Mantzoros I, Betsis D. Incidence and prognostic value of positive peritoneal cytology in colorectal cancer. Dis Colon Rectum 2003;46:535-9.
10. Wind P, Norklinger B, Roger V, Kahlil A, Guin E, Parc R. Long-term prognostic value of positive peritoneal washing in colon cancer. Scand J Gastroenterol 1999;34:606-10.
11. Nakao A, Fujii T, Sugimoto H, Kanazumi N, Nomoto S, Kodera Y, Inoue S, Takeda S. Oncological problems in pancreatic cancer surgery. World J Gastroenterol 2006;12:4466-72.
12. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 1986;315:1650-9.
13. Jewell WR, Romsdahl MM. Recurrent malignant disease in operative wounds not due to surgical implantation from the resected tumor. Surgery 1965;58:806-9.
14. Busch OR, Hop WC, Hoynck van Papendrecht MA, Marquet RL, Jeekel J. Blood transfusions and prognosis in colorectal cancer. N Engl J Med 1993;328:1372-6.
15. Weese JL, Ottery FD, Emoto SE. Do operations facilitate tumor growth? An experimental model in rats. Surgery 1986;100:273-7.
16. Murthy SM, Goldschmidt RA, Rao LN, Ammirati M, Buchmann T, Scanlon EF. The influence of surgical trauma on experimental metastasis. Cancer 1989;64:2035-44.
17. Lee JY, Murphy SM, Scanlon EF. Effect of trauma on implantation of metastatic tumor in bone in mice. J Surg Oncol 1994;56:178-84.
18. 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;227:242-8.
19. Allendorf JD, Bessler M, Horvath KD, Marvin MR, Laird DA, Whelan RL. Increased tumor establishment and growth after open vs laparoscopic surgery in mice may be related to differences in postoperative T-cell function. Surg Endosc 1999;13:233-5.

20. 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;10:490-4.
21. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. World J Surg 1991;15:609-16.
22. Fermor B, Umpleby HC, Lever JV, Symes MO, Williamson RC. Proliferative and metastatic potential of exfoliated colorectal cancer cells. J Natl Cancer Inst 1986;76:347-9.
23. van Eijck CH, Muller SOL, Meyer DCM, van Toorenbergen AW, Hop WC, van Dam JH, Jeekel J. A perspective study on the prognostic value of cytology and tumour markers (CEA and CA 19-9) in peritoneal washings of patients with periampullary tumours. HPS 2000:75-81.
24. 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;12:421-9.
25. van Rossen ME, Hofland LJ, van den Tol MP, van Koetsveld PM, Jeekel J, Marquet RL, van Eijck CH. Effect of inflammatory cytokines and growth factors on tumour cell adhesion to the peritoneum. J Pathol 2001;193:530-7.
26. 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;133:383-9.
27. van Rossen ME, Sluiter W, Bonthuis F, Jeekel H, Marquet RL, van Eijck CH. Scavenging of reactive oxygen species leads to diminished peritoneal tumor recurrence. Cancer Res 2000;60:5625-9.
28. Schlaeppi M, Ruegg C, Tran-Thang C, Chapuis G, Tevaearai H, Lahm H, Sordat B. 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;4:439-55.
29. Ziprin P, Ridgway PF, Peck DH, Darzi AW. Laparoscopic enhancement of tumour cell binding to the peritoneum is inhibited by anti-intercellular adhesion molecule-1 monoclonal antibody. Surg Endosc 2003;17:1812-7.
30. Hosono J, Narita T, Kimura N, Sato M, Nakashio T, Kasai Y, Nonami T, Nakao A, Takagi H, Kannagi R. Involvement of adhesion molecules in metastasis of SW1990, human pancreatic cancer cells. J Surg Oncol 1998;67:77-84.
31. Hill AD, Redmond HP, Naama HA, Bouchier-Hayes D. Granulocyte-macrophage colony-stimulating factor inhibits tumor growth during the postoperative period. Surgery 1996;119:178-85.
32. Roh JL, Sung MW, Park SW, Heo DS, Lee DW, Kim KH. Celecoxib can prevent tumor growth and distant metastasis in postoperative setting. Cancer Res 2004;64:3230-5.

Chapter 3

Surgery Promotes Implantation of Disseminated Tumour Cells, but Does not Increase Growth of Tumour Cell Clusters

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ABSTRACT

Background:

Local recurrence and peritoneal dissemination is common after intentionally curative resection of colorectal carcinoma. It is not yet clear which mechanisms stimulate post-operative intra-abdominal tumour development. Enhanced adhesion or growth of tumour cells and/or post-operative immune suppression may influence tumour recurrence.

In the present study, we evaluated effects of local and remote surgery on intra-abdominal tumour development.

Methods:

A standardized intra-abdominal trauma was inflicted by rubbing both uterus horns in laparotomy groups, while a dorsolateral thoracotomy was performed in thoracotomy groups (on day -1, 0, or +3). To induce tumour development rats were injected intra-peritoneally with the coloncarcinoma cell line CC531s on day 0 and evaluated after 21 days.

Results:

Rats undergoing laparotomy and injection on day 0 showed significantly higher tumour load than control rats (195mm $\pm$ 20 vs. 47mm $\pm$ 29, $p < 0.001$). When a laparotomy was performed, the day before tumour inoculation even higher tumour load was seen (245mm $\pm$ 37 vs. 195mm $\pm$ 20, $p < 0.01$). Strikingly, performing a thoracotomy on the day before or on the same day as tumour inoculation resulted in enhanced tumour load compared to controls as well (135 $\pm$ 84 vs. 47 $\pm$ 29; $p < 0.001$ and 88 $\pm$ 38 vs. 47 $\pm$ 29; $p < 0.05$, respectively). Either laparotomy or thoracotomy 3 days after tumour cell inoculation did not affect growth of pre-existing tumour cell clusters.

Conclusions:

The (post) surgical intra-peritoneal microenvironment enhances successful implantation of spilled tumour cells, whereas growth of adhered tumour cell clusters is not affected. The inflammatory response as a result of remote surgery promotes successful tumour development as well.

INTRODUCTION

The course of gastro-intestinal malignancies after potentially curative resection is often compromised due to local or regional recurrences, mostly presenting within the first two years after operation.[1,2] Especially, the local resection area and peritoneal sites after pancreatic, gastric, and colorectal resections are preferential regions for local tumour recurrence.[3] The relation between surgical trauma, wound healing, and tumour recurrence has been acknowledged since early observations of recurrent malignancies in operative wounds.[4,5] An association between surgical trauma and tumour development was furthermore supported by a number of experimental studies.[6–11] The amount of surgical trauma was found to correlate with the extensiveness of tumour recurrence.[10,11] Minimizing surgical trauma through the use of laparoscopy has shown to reduce tumour recurrence in experimental models.[12,13] Additionally, solid tumour development required less tumour cells in wounded locations compared to non-wounded sites.[14] The reported increased risk of tumour recurrence that correlates with perioperative blood transfusions may be caused by the circumstances that necessitate them, such as extensive tissue trauma.[15]

Aetiology of local recurrent disease after surgical resection and the role of the wound environment have only been partly elucidated. The presence of tumour cell clusters at the time of surgery as well as seeding of tumour cells due to surgical handling or clamping may result in macrometastatic disease. Furthermore, the post-operative wound microenvironment provides advantageous conditions for tumour recurrence as tumour development was influenced by locally produced growth factors, inflammatory cytokines, and reactive oxygen species produced by inflammatory cells.[14,16] Additionally, surgery induced immune suppression resulting in impaired immunological defence against disseminated tumour cells might affect tumour development as well.[17,18]

Successful development of metastases depends on several successive processes, such as adhesion, implantation, and proliferation of seeded tumour cells. Experimental data suggest that wound healing mediators affect both tumour cell adhesion and growth, though involved mechanisms are largely unknown.[19,20] However, in most experimental models, tumour cells were introduced during or shortly after surgery. Consequently, effects on tumour cell adhesion and growth of adhered tumour cell clusters might both have contributed to development of the observed tumour load.

We, therefore, investigated the effects of surgery on tumour development in more detail, and aimed to pinpoint the effect of local and remote surgical trauma on outgrowth of seeded tumour cells as well as on established tumour cell clusters.

MATERIALS AND METHODS

Animals

Female WAG/Rij rats of reproductive age weighing 140–180 g were obtained from Charles River, The Netherlands, and kept under standard laboratory conditions. The experimental protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of ‘‘Guidelines on the protection of experimental animals’’ by the Council of Europe (1986), Directive 86/609/EC.

Tumour

The CC531s cell line is a 1,2-dimethylhydrazineinduced, moderately differentiated, and weakly immunogenic colonic adenocarcinoma cell-line, which is transplantable in syngeneic WAG/Rij rats [21]. CC531s cells are maintained in RPMI 1640 medium supplemented with 5% fetal calf serum, 1% penicillin (100 U/ml), 1% streptomycin (100 U/ml), and 1% L-glutamine (200 mM). Medium and supplements were obtained from Life Technologicals BV, Breda, The Netherlands. Cells were passaged once a week using trypsin (0.05%) and EDTA (0.02%). Prior to use in vivo, tumour cells were harvested by trypsinization (5 min, 37°C), and resuspended in RPMI 1640 (without supplements), providing cell suspensions with viability greater than 95%. To induce tumour development, 0.5×10^6 CC531s tumour cells suspended in 1 ml RPMI 1640 were injected intra-peritoneally (i.p.) through a 19-G injection needle under brief Isoflurane anaesthesia.

Operative Procedures

Laparotomy group. Laparotomy was performed under Isoflurane anaesthesia and aseptic conditions using a lower midline incision of 5 cm. Both uterus horns were exposed, rubbed 10 times over their total length with surgical gauze and sutured to the lateral peritoneum both proximally and distally, using Surgilene 6-0 sutures. Rubbing was performed with a device that enabled application of a constant pressure of 120 g/cm2. In this way, a standardized amount of peritoneal trauma was inflicted. The abdomen was closed in two layers with 5-0 polyglycolic acid sutures. The total procedure was accomplished in 25-30 min.

Thoracotomy group. Under Isoflurane anaesthesia rats were intubated and mechanically ventilated with 20 cm H2O positive inspiratory pressure, 6 cm H2O positive end-expiratory pressure, breathing frequency of 30/min, inspiration: expiration is 1:2 and a FiO2 of 0.40. The skin and musculus cutaneous maximus were incised in the left flank. The m. latissimus dorsi was moved to the side while the m. serratus anterior was cut in order to perform a 2 cm dorsolateral thoracotomy through the fourth intercostal space. After spreading the ribs, the thoracotomy was closed using Vicryl 4-0 (sutures) and polyglycolic acid 5-0 sutures for muscle and skin, followed by extubation of the rat. Operating time varied between 25-30 min.

Peritoneal Lavage and Flowcytometry

To determine the time point at which free viable CC531s cells were absent in the peritoneal cavity, peritoneal lavages were performed at different time points after i.p. tumour cell injection (n¼3/time point), and number and viability of tumour cells were analysed with flowcytometry (FACScan, BD). Harvested cells were incubated with anti-CC531 monoclonal antibody CC52 (10 mg/ml, generously provided by Dr. P. Kuppen, LUMC), washed and further incubated with $F(ab')_2$ fragments of FITC-labelled rabbit anti-mouse IgG. Apoptotic and necrotic cells are stained with Annexin V-PE (Pharmingen BD, San Diego, CA) and 7-aminoactinomycin D (Molecular Probes, Leiden, The Netherlands), respectively.

Experimental Design

A total of 90 rats was divided into seven groups and injected with 0.5×10^6 CC531s tumour cells on day 0. The control group received tumour cells under Isoflurane anaesthesia without operation. Group lap 0 and thor 0 were submitted to a laparotomy or a thoracotomy on day 0, respectively, to study influence of local or (remote) systemic effects of surgery on tumour development (fig. 1). In order to study local and/or systemic effects of surgery on growth of tumour cell clusters, groups lap +3 and thor +3 underwent a laparotomy or a thoracotomy on day +3, respectively (when tumour cells had already adhered). To evaluate effects of introducing tumour cells in the early post-operative phase, which is characterized by a prominent surgery-induced immune suppression groups lap -1 and thor -1 underwent a laparotomy or a thoracotomy one day before injection of CC531s, respectively (fig. 1).[22]

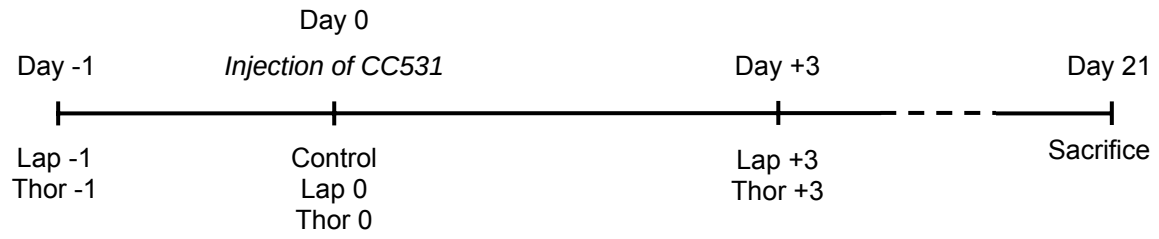

Figure 1. Experimental groups.
Lap = Laparotomy with standardized rubbing of the uterus horns.
Thor = Thoracotomy.

Evaluation of Intra-Peritoneal Tumour Load

Twenty-one days after the injection of tumour cells rats were sacrificed and intra-peritoneal tumour load was scored at the following peritoneal sites: right uterus horn, left uterus horn, subcutaneous (at the site of the operative scar), parietal peritoneum (at the lateral abdominal wall sides), kidney, liver, retroperitoneum, mesentery, and omentum. The scoring was

performed in a blinded fashion by two independent observers and displayed in millimeters (mm) in diameter. The score at all peritoneal sites was summarized for each rat, from which a cumulative total tumor load per rat could be estimated. Additionally, tumour nodules per rat were counted as indication for the number of tumour cells that had implanted and formed nodules.

Statistical Analysis of Data

Statistical analysis was performed using one-way ANOVA tests to determine overall differences. If the ANOVA test was significant at a 5% level, the Fischer LSD post hoc test was carried out to make a comparison between groups. Data are expressed as mean and standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$.

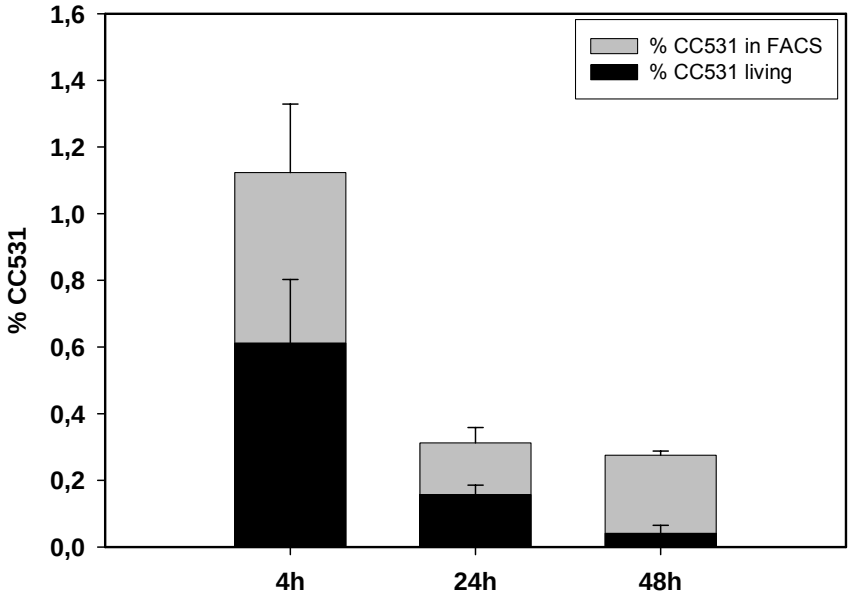

Figure 2. FACS analysis of peritoneal lavage fluid recovered at different time points after i.p. injection and stained for CC531s and apoptosis/necrosis markers. % CC531 of total cell number in peritoneal lavage.

RESULTS

FACS Analysis

Number and viability of CC531s cells after i.p. injection were measured in order to determine the time point at which no viable non-adhered CC531s cells were present in the peritoneal cavity. Percentages of CC531s of total cells in recovered lavage fluids rapidly declined in time from 1.15% (0.64% living) at 4 hr to 0.26% (0.05% living) at 48 hr after i.p. injection (fig. 2). Consequently, we chose 72 hr after i.p. injection of CC531s cells as time point for

performing surgery, so as to exclude possible effects on adhesion of free viable cells and study outcome on growth of established clusters of tumour cells.

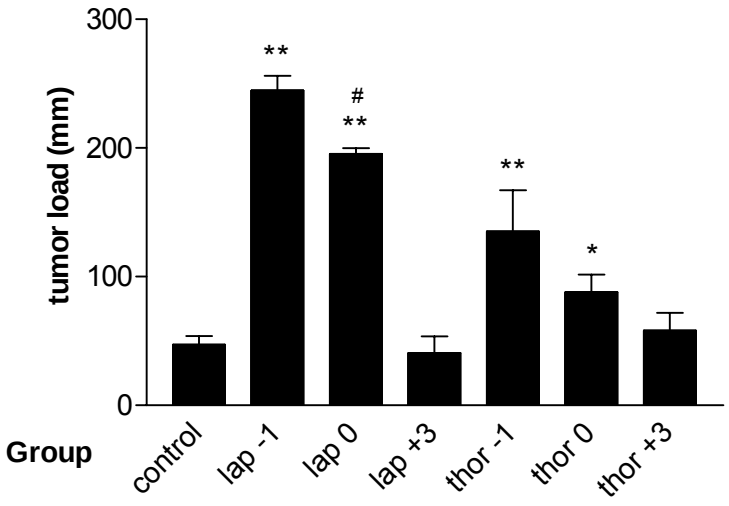

Figure 3. Mean tumour load and SEM 21 days after intra-abdominal instillation of CC531s. All rats received CC531s on day 0; control = no operation; lap = laparotomy; thor = thoracotomy; -1, 0, +3 represent the day of operation.
** = $p < 0{,}001$ vs. control; * = $p < 0{,}05$ vs. control; # = $p < 0{,}01$ vs. lap –1.

In Vivo Experiments

Six rats in the thoracotomy groups, and one rat in the control group died due to ventilation and anaesthesia related problems. No further post-operative complications such as bowel obstructions, wound infections, or peritonitis were observed. Rats gradually gained weight after an initial post-operative weight loss (data not shown). After 21 days, a significant tumour load was found in all rats.

Laparotomized animals enabled us to evaluate tumour outgrowth as a consequence of local and systemic effects of surgery, whereas in thoracotomized animals influences of the systemic response alone might affect intra-abdominal tumour outgrowth. By performing surgery on the same day (0) of injecting CC531s, effects on tumour cell implantation were evaluated. Groups that underwent an operation 1 day before (day 1) injection of CC531s served to evaluate additional effects of surgery induced immune suppression, as previously shown by Allendorf et al.[22]

The lap 0 group showed a significant increased tumour load compared to the control group (195 ± 20 vs. 47 ± 29 respectively, $p < 0.001$) (fig. 3). Strikingly, even higher tumour load was found in the lap -1 group compared to the lap 0 group (245 ± 37 vs. 195 ± 20 respectively, $p < 0.01$). In the lap 0 and lap -1 groups largest tumour load was seen at directly traumatized sites (laparotomy wound and uterus horns) (data not shown). Both

thoracotomized groups, thor -1 and thor 0 showed a significant larger tumour load than controls (135 ± 84 vs. 47 ± 29; $p < 0.001$ and 88 ± 38 vs. 47 ± 29; $p < 0.05$, respectively) (fig. 3).

In the lap +3 and thor +3 groups operations were performed 3 days after i.p. injection of CC531s to evaluate effects of surgery on growth of established tumour cell clusters alone. Tumour load in the lap +3 group found at directly traumatized sites (laparotomy wound and uterus horns) was not different from the control animals (data not shown). Likewise, total tumour load in both groups (lap +3 and thor +3) did not differ from the control group (fig. 3), indicating that growth of already adhered tumour cell clusters was not affected by surgery.

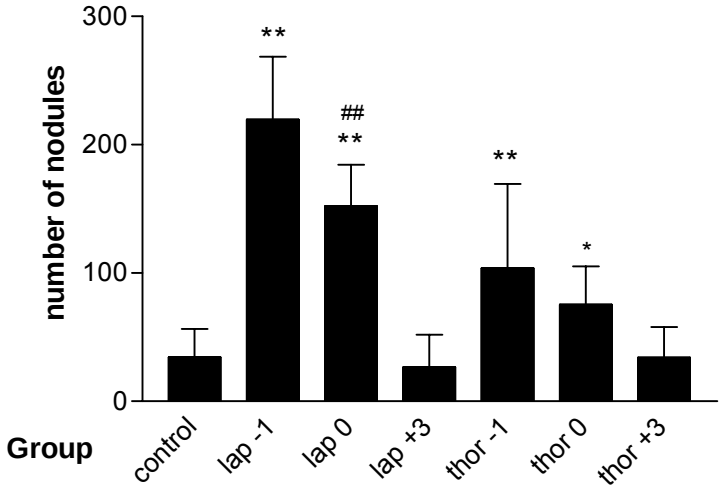

Figure 4. Cumulative total number of tumour nodules 21 days after intra-abdominal instillation of CC531s. All rats received CC531s on day 0; control = no operation; lap = laparotomy; thor = thoracotomy; -1, 0, +3 represent the day of operation.
** = $p < 0{,}001$ vs. control; * = $p < 0{,}01$ vs. control; ## = $p < 0{,}001$ vs. lap –1.

The total number of nodules found after 21 days was determined as an indication of the number of tumour cells that had successfully implanted and grown out (fig. 4). In the lap 0 group significantly more tumour nodules were seen compared to the control group (152 ± 32 vs. 34 ± 22, $p < 0.001$). Even more tumour nodules were counted in lap -1 (lap -1 vs. lap 0; 219 ± 48 vs. 152 ± 32, $p < 0.001$), which is analogous to total tumour size. Groups thor -1 and thor 0 showed increased numbers of nodules compared to control, but no differences amongst both groups were found. Lap +3 and thor +3 groups showed no differences from the control group.

DISCUSSION

Local peritoneal recurrence is a significant cause of morbidity and mortality in patients that have previously been operated on for colorectal cancer with curative intend. There is accumulating evidence that presence of exfoliated tumour cells in the peritoneal cavity after surgery for colorectal cancer, which is considered minimal residual disease, might adversely influence relapse free and overall survival.[23,24] Although surgery remains the only curative option for colorectal cancer, operative trauma to peritoneal surfaces and the subsequent wound healing microenvironment may favour tumour development.[7,8,11,25] Whether the post-surgical wound microenvironment, however, mainly affects tumour cell adhesion, growth of tumour cell clusters, or both processes remained unclear.

At present, few studies have specifically focused on growth of i.p. (syngeneic) tumours in relation to surgery, requiring a study design in which tumours or clusters of implanted tumour cells are present prior to surgery. In a subcutaneous model, Allendorf et al.[12] showed enhanced growth of mammary tumours in mice that had undergone laparotomy 7 days after inoculation. By contrast, no differences in post-surgical tumour growth were observed using Lewis lung carcinoma and F344 colon cancer in subcutaneous models.[6,26] Our data clearly showed enhanced tumour load as well as increased number of tumour nodules when CC531s tumour cells were injected i.p. directly after local or remote surgical trauma. No difference from controls was, however, seen in rats that underwent either laparotomy or thoracotomy 3 days after tumour cell injection. Thus, our experimental data suggest an important role for surgical trauma in facilitating successful tumour cell implantation, but not for enhancing growth of established tumour cell clusters. We consider lap +3 and thor +3 groups accurate to evaluate effects of surgery on growth of tumour cell clusters, since in these groups no difference in tumour load was observed on directly traumatized sites (laparotomy wound and uterus horns) compared to similar sites in non-operated controls, indicating absence of free floating viable tumour cells (in accordance with FACS analysis) that might adhere at these preferred sites.[11,25]

In accordance with other studies, largest tumour load was seen at the site of surgery in the laparotomy groups, indicating facilitation of tumour cell implantation in wounds.[7–11,14] Increased adhesion of tumour cells might represent one explanation since exposed ECM components as a result of mesothelial trauma has been shown to be a preferred site for adherence of tumour cells. In addition, inflammatory mediators produced after intra-abdominal surgery were shown to disrupt the mesothelial monolayer with exposure of ECM, representing a preferential adhesion site as well.[27,28] Furthermore, following surgical trauma a local and systemic reactive inflammatory response is seen in order to initiate the wound healing process. Several cytokines (e.g., IL-8, IL-1b, and TNF-a), which are produced by mesothelial and inflammatory cells induce an upregulation of adhesion molecules such as VCAM-1 and ICAM-1 on mesothelial cells, which might enhance tumour cell adhesion.[29–31]

Van Rossen et al.[20] showed that pre-treatment of mesothelial cells with pro-inflammatory cytokines (especially TNF-a, IL-1b) stimulated adhesion of CC531s to a mesothelial monolayer.

Alternatively, early post-operative immune suppression may also play an important role. Major surgery has been shown to induce immune suppression in the direct post-operative period, hereby transiently downregulating immunological defence mechanisms against disseminated tumour cells, which might facilitate metastasis formation.[17,18,32] Especially, impairment in cellular responsiveness, which is considered to play a pivotal role in anti-CC531s immune responses, is most pronounced in the early post-operative phase.[17,22,32–34] Therefore, survival of free-floating tumour cells could have been improved in the post-surgical i.p. environment as a result of the impaired immune response. The observation that tumour load was enhanced in the lap -1 group compared to the lap 0 group supports the influence of immune suppression on tumour development. It has previously been proposed that basal membrane like structures, composed of matrix proteins, can provide an obstruction in CC531s lung and liver tumours, hereby precluding contact of immune effector cells and tumour cells. As such, surgery-induced down modulation of immune responses may not influence outgrowth of already established tumors.[35]

In conclusion, surgery enhances successful implantation of spilled tumour cells, but does not stimulate growth of already adhered tumour cell clusters. This may be a consequence of enhanced tumour cell adhesion due to exposure of ECM components in the wound or (systemically) circulating inflammatory mediators that upregulate adhesion molecules on mesothelial cells. Alternatively, improved tumour cell survival due to transient (generalized) postoperative immune suppression might explain the observed results. Future studies are required to unravel the precise mechanism in post-operative tumour development. Ultimately this may lead to the rationale design of new therapeutic strategies targeting early (post-operative) tumour development.

REFERENCES

1. Abulafi AM, Williams NS. Local recurrence of colorectal cancer: The problem, mechanisms, management and adjuvant therapy. Br J Surg 1994;81:7–19.
2. Improved survival with preoperative radiotherapy in respectable rectal cancer. Swedish Rectal Cancer rial. N Engl J Med 1997; 336:980–987.
3. Renehan AG, Egger M, Saunders MP, et al. Impact on survival of intensive follow up after curative resection for colorectal cancer: Systematic review and meta-analysis of randomised trials. BMJ 2002;324:813.
4. Jewell WR, Romsdahl MM. Recurrent malignant disease in operative wounds not due to surgical implantation from the resected tumour. Surgery 1965;58:806–809.
5. Dvorak HF. Tumours: Wounds that do not heal. Similarities between tumour stroma generation and wound healing. N Engl J Med 1986;315:1650–1659.
6. Weese JL, Ottery FD, Emoto SE. Do operations facilitate tumour growth? An experimental model in rats. Surgery 1986;100:273–277.
7. Eggermont AM, Steller EP, Sugarbaker PH. Laparotomy enhances intraperitoneal tumour growth and abrogates the antitumor effects of interleukin-2 and lymphokine-activated killer cells. Surgery 1987;102:71–78.
8. Murthy SM, Goldschmidt RA, Rao LN, et al. The influence of surgical trauma on experimental metastasis. Cancer 1989;64: 2035–2044.
9. Lee JY, Murphy SM, Scanlon EF. Effect of trauma on implantation of metastatic tumour in bone in mice. J Surg Oncol 1994;56:178–184.
10. Jacobi CA, Ordemann J, Bohm B, et al. The influence of laparotomy and laparoscopy on tumour growth in a rat model. Surg Endosc 1997;11:618–621.
11. van den Tol PM, van Rossen EE, van Eijck CH, et al. Reduction of peritoneal trauma by using nonsurgical gauze leads to less implantation metastasis of spilled tumour cells. Ann Surg 1998; 227:242–248.
12. Allendorf JD, Bessler M, Horvath KD, et al. Increased tumour establishment and growth after open vs laparoscopic surgery in mice may be related to differences in postoperative T-cell function. Surg Endosc 1999;13:233–235.
13. Bouvy ND, Marquet RL, Hamming JF, et al. Laparoscopic surgery in the rat. Beneficial effect on body weight and tumour take. Surg Endosc 1996;10:490–494.
14. Hofer SO, Shrayer D, Reichner JS, et al. Wound-induced tumour progression: A probable role in recurrence after tumour resection. Arch Surg 1998;133:383–389.
15. Busch OR, Hop WC, Hoynck van Papendrecht MA, et al. Blood transfusions and prognosis in colorectal cancer. N Engl J Med 1993;328:1372–1376.
16. van Rossen ME, Sluiter W, Bonthuis F, et al. Scavenging of reactive oxygen species leads to diminished peritoneal tumour recurrence. Cancer Res 2000;60:5625–5629.
17. Mels AK, Statius Muller MG, van Leeuwen PA, et al. Immunestimulating effects of low-dose perioperative recombinant granulocyte-macrophage colony-stimulating factor in patients operated on for primary colorectal carcinoma. Br J Surg 2001; 88: 539–544.
18. Sietses C, Beelen RH, Meijer S, et al. Immunological consequences of laparoscopic surgery, speculations on the cause and clinical implications. Langenbecks Arch Surg 1999;384: 250–258.
19. Hofer SO, Molema G, Hermens RA, et al. The effect of surgical wounding on tumour development. Eur J Surg Oncol 1999;25: 231–243.
20. van Rossen ME, Hofland LJ, van den Tol MP, et al. Effect of inflammatory cytokines and growth factors on tumour cell adhesion to the peritoneum. J Pathol 2001;193:530–537.

21. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: Importance of tumour site. Int J Cancer 1984;33:689–692.
22. Allendorf JD, Bessler M, Whelan RL, et al. Postoperative immune function varies inversely with the degree of surgical trauma in a murine model. Surg Endosc 1997;11:427–430.
23. Schott A, Vogel I, Krueger U, et al. Isolated tumour 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.
24. Bosch B, Guller U, Schnider A, et al. Perioperative detection of disseminated tumour cells is an independent prognostic factor in patients with colorectal cancer. Br J Surg 2003;90: 882–888.
25. van den TolMP, Haverlag R, van Rossen ME, et al. Glove powder promotes adhesion formation and facilitates tumour cell adhesion and growth. Br J Surg 2001;88:1258–1263.
26. Hill AD, Redmond HP, Naama HA, et al. Granulocytemacrophage colony-stimulating factor inhibits tumour growth during the postoperative period. Surgery 1996;119:178–185.
27. Suematsu T, Hirabayashi Y, Shiraishi N, et al. Morphology of the murine peritoneum after pneumoperitoneum vs. laparotomy. Surg Endosc 2001;15:954–958.
28. Stadlmann S, Raffeiner R, Amberger A, et al. Disruption of the integrity of human peritoneal mesothelium by interleukin- 1beta and tumour necrosis factor-alpha. Virchows Arch 2003; 443:678–685.
29. Klein CN, 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.
30. Slavin J. The role of cytokines in wound healing. J Pathol 1996; 178:5–10.
31. Ziprin P, Ridgway PF, Pfistermuller KL, et al. ICAM-1 mediated mesothelial-mesothelial cell adhesion is modulated by IL-6 and TNF-alpha: A potential mechanism by which surgical trauma increases peritoneal metastases. Cell Commun Adhes 2003;10: 141–154.
32. Ben Eliyahu S, Page GG, Yirmiya R, et al. Evidence that stress and surgical interventions promote tumour development by suppressing natural killer cell activity. Int J Cancer 1999;80: 880–888.
33. Sietses C, Havenith CE, Eijsbouts QA, et al. Laparoscopic surgery preserves monocyte-mediated tumour cell killing in contrast to the conventional approach. Surg Endosc 2000;14: 456–460.
34. Hagenaars M, Ensink NG, Koelemij R, et al. Regional administration of natural killer cells in a rat hepatic metastasis model results in better tumour infiltration and anti-tumour response than systemic administration. Int J Cancer 1998;75:233–238.
35. Hagenaars M, Ensink NG, Basse PH, et al. The microscopic anatomy of experimental rat CC531 colon tumour metastases: Consequences for immunotherapy? Clin Exp Metastasis 2000; 18:189–196.

Chapter 4

The Post-Surgical Inflammatory Response Provokes Enhanced Tumour Recurrence; a Crucial Role for Neutrophils

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ABSTRACT

Background:

Peritoneal trauma activates a cascade of peritoneal defence mechanisms responsible for postoperative intra-abdominal tumour recurrence. After peritoneal trauma, inflammatory cells and soluble factors are present in the abdominal cavity and can be captivated in lavage fluids. The present study evaluated which component enhances intra-abdominal tumour recurrence. Furthermore we evaluated which inflammatory cells were present and studied the influence of anti-neutrophil serum (ANS) on peritoneal tumour recurrence.

Methods:

In a peritoneal trauma model in rats postoperative lavage fluids were collected and separated in cellular and supernatant components. Both components were injected in naïve rats together with CC531s colon carcinoma cells. In a second experiment rats were treated with 1 or 3 doses of ANS.

Results:

Intra-peritoneal injection of naïve recipients with inflammatory cells or supernatant resulted in significant tumour recurrence. Severe peritoneal trauma provoked significant intra-abdominal neutrophil influx, which could be prevented by ANS. Treatment with 1 dose did not affect blood cell counts and significantly reduced tumour recurrence. Treatment with 3 doses ANS decreased blood lymphocytes, monocytes and neutrophils and induced tumour load.

Conclusions:

Neutrophils play a crucial role in postoperative adhesion and growth of spilled tumour cells after surgical peritoneal trauma. Prevention of peritoneal neutrophil influx reduces local tumour recurrence.

INTRODUCTION

Loco-regional tumour recurrence of colorectal carcinomas remains an important complication of potentially curative surgical intra-abdominal interventions. In a clinical study, Busch et al.[1] suggested an association between recurrent tumour disease and the extent of surgical injury. It has also become evident from experimental studies that enhanced tumour cell adherence and tumour growth are inevitable repercussions of surgical peritoneal trauma.[2,3] The pathogenesis of the processes responsible for postoperative intra-abdominal tumour recurrence is only partly clarified. In a previous study we demonstrated that within a few hours after infliction of peritoneal trauma, factors in the abdominal cavity could be captivated in a lavage fluid and enhance tumour recurrence in naive, non-operated recipients.[4] The inflammatory reaction after surgery is not only responsible for the wound healing process, but also induces tumour recurrence. During this inflammatory response peritoneal lymphocytes, sub-mesothelial monocytes, neutrophils and mesothelial cells act under the control of locally expressed cytokines, chemokines and adhesion molecules.[5,6]

In the present study, we focus our attention on the individual capacity of inflammatory peritoneal cells and soluble factors to ascertain which element is mainly responsible for enhanced tumour recurrence. Secondly, assuming that post-traumatic intra-abdominal influx of neutrophils is an important factor in the dynamic cascade of peritoneal defence, possibly responsible for local tumour recurrence, prevention of PMN influx might influence this process. Therefore, a second experiment was performed to evaluate whether post-traumatic intra-abdominal PMN influx could be reduced by treatment with anti-neutrophil serum (ANS), and if so, whether this reduction could influence postoperative tumour development.

MATERIALS AND METHODS

Animals

Female inbred WAG rats of reproductive age weighing 140-180 g were obtained from Harlan-CPB, Austerlitz, The Netherlands. They were bred under specific pathogen-free conditions, kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 hours light and 12 hours dark cycles), and fed with standard rat food and water *ad libitum*. The experimental protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of " Guidelines on the protection of experimental animals " by the Council of Europe (1986), Directive 86/609/EC.

Tumour

Tumour CC531s is a moderately differentiated, weakly immunogenic colonic adenocarcinoma induced in WAG rats by 1,2-dimethylhydrazine.[7] It is transplantable in syngeneic WAG rats. The tumour is maintained as a cell culture in RPMI 1640 medium (RPMI) supplemented with 5% foetal calf serum (virus- and Mycoplasma-screened), 1% penicillin (5000 U/mL), 1% streptomycin (5000 U/mL), and 1% L-glutamine (200 mmol). RPMI 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%). Before use *in vivo*, tumour cells were harvested from stationary cultures by gentle trypsinisation (5 minutes, 37°C), centrifugation (5 minutes, 700 g), and re-suspension in RPMI, providing cell suspensions with a viability greater than 90%. CC531s is relatively insensitive to chemotherapy but is sensitive to the effects of biologic response modifiers.

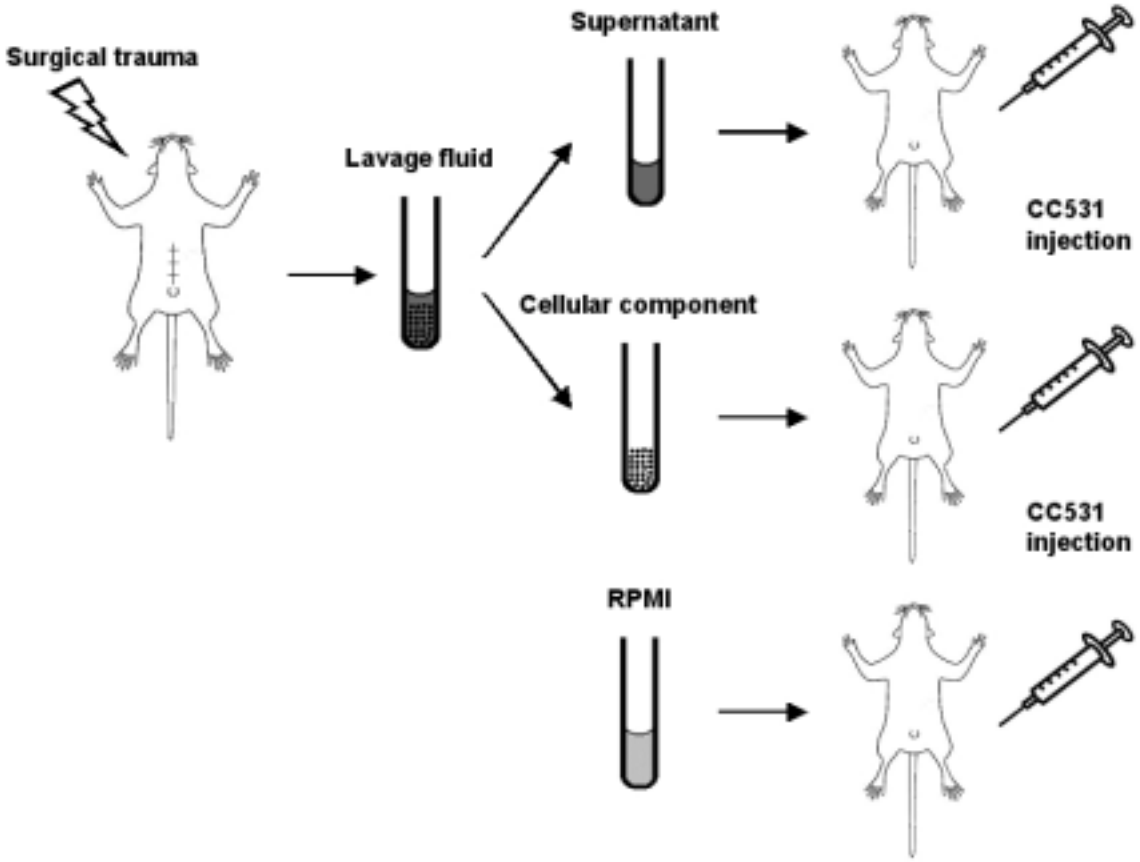

Figure 1. Experimental design of passive transfer experiment.

Gathering of lavage fluid for passive transfer experiment

Under isoflurane anaesthesia, in 14 rats a laparotomy was performed; exposure and rubbing of the uterus horns and a 5-cm long part of the small intestine with surgical Medipress gauze

inflicted subsequent trauma to the peritoneum[4]. Rubbing was performed with a device enabling the application of a constant pressure of 120g/m^2. In this way, a standardized amount of peritoneal trauma can be inflicted. The abdomen was closed in one layer with silk 2-0 sutures. After 5 hours, a second laparotomy was performed during which the abdominal cavity was lavaged with 5ml RPMI. After massaging the abdomen the remaining fluid was aspirated, pooled and kept on ice until further processing.

Experimental design passive transfer (experiment 1)

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. One group served as control group receiving RPMI. The second group was acceptor for the supernatant of the post trauma lavage fluid and the third group was acceptor for the cellular component. Of all three components, 3ml was injected intraperitoneal together with 0.5 million CC531s cells (in 0.5ml 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 (fig. 1).

Rabbit anti-rat neutrophil serum

Polyclonal rabbit anti-rat neutrophil serum (ANS) was purchased from Accurate, Westbury, NY, USA. ANS can deplete blood neutrophils in rats by 99.9% when administered intra-peritoneally at a daily dose of 2 ml per kg bodyweight .[8,9] The number of blood neutrophils remains at this low level until administration of ANS is stopped. In this dose ANS is not specific for neutrophils only, because the number of blood monocytes, lymphocytes and, to a lesser extent, the platelets decrease as well, i.e. by 100%, 80% and 25% respectively.[9] Therefore, a pilot experiment was performed to achieve a dose of ANS in which blood cell counts hardly change, as shown in experiment 2.

Effect of ANS treatment on cell content in peritoneal cavity and blood (experiment 2).

To investigate the influence of ANS treatment on intra-abdominal neutrophil cell count and on the rat immune system the following procedures were performed. Under isoflurane anaesthesia 65 rats underwent a laparotomy. In 5 rats no peritoneal trauma was inflicted. In 20 rats standardised severe peritoneal trauma was inflicted without treatment. In 20 rats severe peritoneal trauma was inflicted (on day 0) in rats treated with 3 intra-peritoneal doses of ANS (1 ml/kg), on day –1, 0 and +1. In 20 rats severe peritoneal trauma was inflicted after a single intra-peritoneal injection of ANS on day –1. After 5 hours, 72 hours, 96 hours and 144 hours 5 rats of each group were operated for the second time. During this second

laparotomy the abdominal cavity was lavaged with 5 ml RPMI 1640 medium. After massaging the abdomen the remaining fluid was aspirated and individually kept on ice until further processing. Blood samples were obtained as well, by cardiac puncture.

The collected lavage fluid samples were separated in a supernatant and a cellular component by centrifugation (1500 rpm, 5 minutes). The cellular component was re-suspended in RPMI medium, total cell amount was determined and HE stained cytocentrifuge slides were made for cell differentiation. At a magnification of 100 x, 100 cells were counted in duplicate and classified into granulocytes (neutrophils, eosinophils, basophils and mast cells) and lymphoid cells (mononuclear phagocytes and lymphocytes). Total blood leukocyte counts were determined with a micro cell counter, and duplicate differential counts were carried out on May-Grünwald and Giemsa-stained blood smears.

Intra-peritoneal tumour cell adhesion and growth after treatment with ANS (experiment 3 and 4)

A reproducible animal experimental model was used.[4] Briefly, under isoflurane anaesthesia and aseptic conditions a laparotomy was performed by the rat using a midline incision of 5 cm. Both uterus horns were exposed, not touched or rubbed with surgical Medipress gauze, and sutured to the lateral peritoneum both proximally and distally using Surgilene 6-0 sutures. In this way a standardised amount of peritoneal trauma was inflicted. To induce peritoneal metastases, 0.5 x 10^6 CC531s tumour cells, in 1 ml RPMI, were injected intra-peritoneally before closing the abdomen. The abdomen was closed in two layers with 5-0 polyglycolic acid and 2-0 silk sutures.

In the first experiment nine rats underwent severe peritoneal trauma without treatment. Nine rats underwent severe peritoneal trauma, which were treated with an intra-peritoneal injection of ANS on day –1. Before closing the abdomen 0.5 x 10^6 CC531s tumour cells, in 1 ml RPMI 1640, were injected intra-peritoneally, in all rats.

In a second experiment similar groups were formed, with the difference that the treated group received intra-peritoneal ANS injection on day –1, 0 and +1. Again before closing the abdomen 0.5 x 10^6 CC531s tumour cells were injected into the abdominal cavity.

Evaluation of tumour load

Three weeks after injection of CC531s all rats were sacrificed and intra-peritoneal tumour load was scored semiquantitatively at the following peritoneal sites: right uterus horn, left uterus horn, subcutaneous (at the site of the operative scar), parietal peritoneum (at the lateral abdominal wall sides where no uterus horns were fixed), kidney, liver, retroperitoneum, and omentum. The scoring was performed by two blinded observers using a tumour scoring system derived from the peritoneal cancer index described by Steller et al.[4,10] and ranging from 0 to 5 per abdominal site. For each rat the score at all peritoneal sites was summarised, from which a mean total tumour load per rat could be estimated.

Statistical analysis

Statistical analysis was performed using one-way ANOVA tests to determine overall differences. If the ANOVA test was significant at a 5% level, the Newman-Keuls *post hoc* test was carried out to make a comparison between groups. Data are expressed as mean and standard error of the mean (SEM). For tumour load, the median and range were calculated and statistical analysis was performed using the non-parametric Kruskal Wallis analysis of variance to determine overall differences followed by the non-parametric Mann Whitney *U* test to compare differences between groups. Statistical significance was defined as $p < 0.05$.

RESULTS

Effect of post trauma lavage fluid on peritoneal tumour load

After intraperitoneal injection of the lavage fluid samples collected after surgical trauma, diffuse peritoneal tumour load was found in all groups (fig. 2). When compared to the control group (RPMI) the cellular factors caused significantly enhanced tumour recurrence ($p < 0.01$) as well as the supernatant ($p < 0.05$). Injection of tumour cells with RPMI alone resulted in a median total Steller score of 0 (0-3) whereas injection with the supernatant or cells resulted in 1 (0-5) and 3 (0-5), respectively. Injection with the cellular component of the lavage fluid also resulted in a significantly higher tumour load compared to the supernatant ($p < 0.05$).

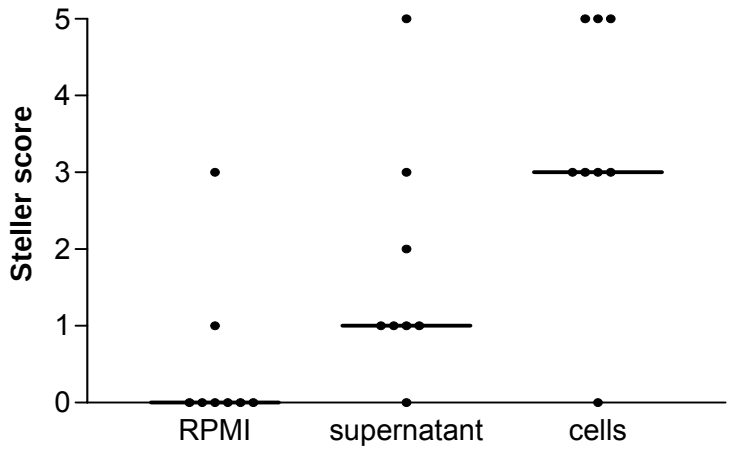

Figure 2. Median intra-peritoneal tumour load and range after passive transfer of RPMI only, the supernatant or the cellular fraction of lavage fluid collected after infliction of severe peritoneal trauma. RPMI vs. cells ($p < 0.01$). RPMI vs. supernatant ($p<0.05$). Supernatant vs. cells ($p < 0.05$).

Cell content in peritoneal cavity and blood after surgery and the effect of ANS treatment

There was a significant increase in total intra-abdominal cell count after infliction of severe peritoneal trauma up to 144 hours after the operation (fig. 3). Figure 3 also shows that intra-peritoneal administration of 3 doses of ANS as well as 1 dose of ANS significantly decreased the total intra-abdominal cell count after infliction of severe peritoneal trauma for at least 96 hours postoperatively ($p < 0.01$). After treatment with 1 dose of ANS the total cell count increased earlier than after treatment with 3 doses, differences were significant at 96 hours ($p < 0.01$) (fig. 3). A marked increase of the percentage of neutrophils was seen in the severely traumatised group ($p < 0.01$). This increase was seen till 96 hours postoperatively (fig. 4). Treating the rats with 3 doses as well as 1 dose of ANS did annul this increase (fig. 4).

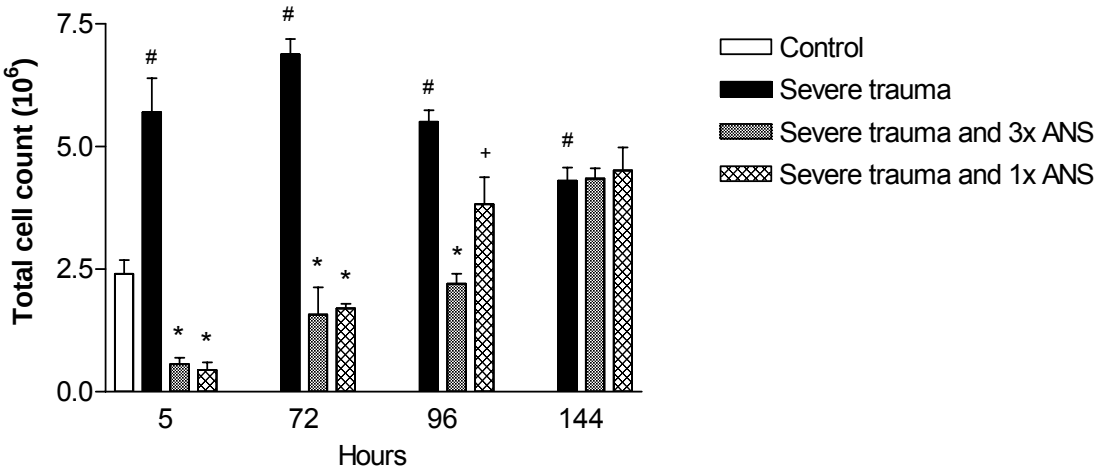

Figure 3. Mean total cell count (SEM) in abdominal lavage fluids after no touch, after infliction of severe peritoneal trauma or after infliction of severe peritoneal trauma in rats treated with 3 or 1 intra-peritoneal doses of ANS. Fluids were collected at 5, 72, 96 and 144 hours postoperatively. * = $p < 0.001$, + = $p < 0.01$ vs. severe peritoneal trauma. # $p < 0.05$ vs. no touch.

Figure 5 indicates the results of blood differential cell counts in the 4 groups at different time points. Similar results were found with lymphocyte, monocyte or neutrophil counts. Treatment with 3 doses of ANS significantly decreased these cell counts for a period of at least 96 hours ($p < 0.01$). This effect was not seen after treatment with 1 dose of ANS.

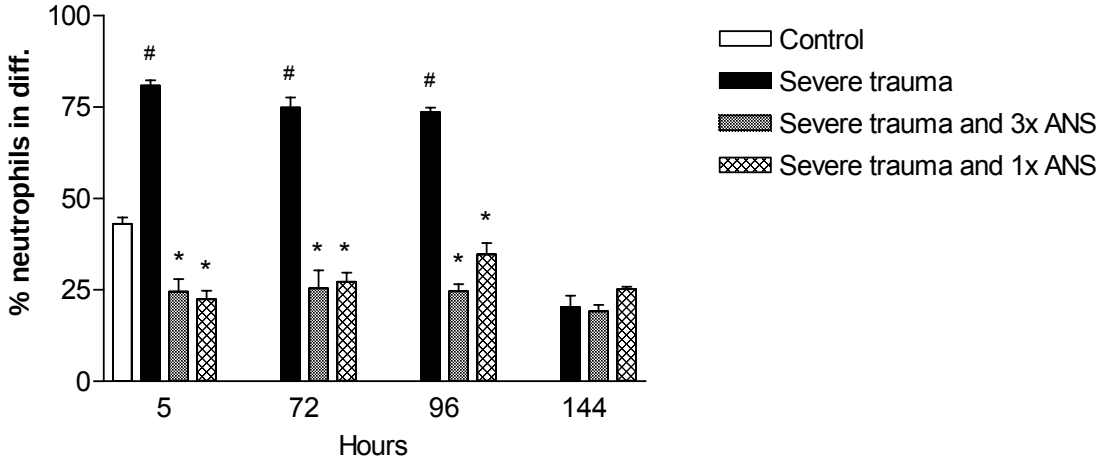

Figure 4. Mean percentage of neutrophils (SEM) in abdominal lavage fluids taken from abdominal cavities after no touch, after infliction of severe peritoneal trauma or after infliction of severe peritoneal trauma in rats treated with 3 or 1 intra-peritoneal doses of ANS. Fluids were collected at 5, 72, 96 and 144 hours postoperatively.
* = $p < 0.001$ vs. severe peritoneal trauma. # = $p < 0.05$ vs. no touch. † = $p < 0.01$ vs. 3x ANS.

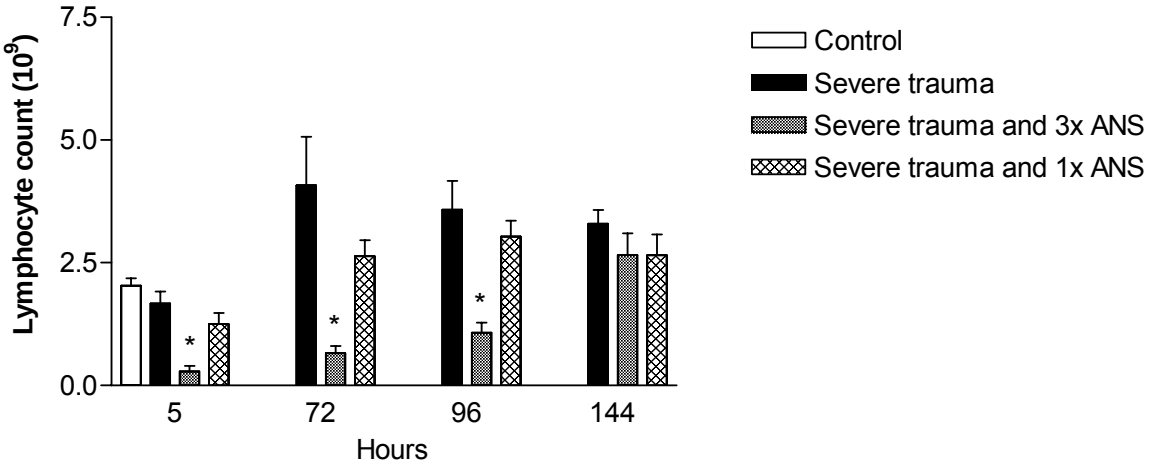

Figure 5. Mean blood lymphocyte count (SEM) after no touch, after infliction of severe peritoneal trauma or after infliction of severe peritoneal trauma in rats treated with 3 or 1 intra-peritoneal doses of ANS. Blood samples were obtained at 5, 72, 96 and 144 hours postoperatively. * = $p < 0.01$ vs. severe peritoneal trauma.

Intra-peritoneal tumour development after treatment with ANS

As shown in figure 6 a marked tumour load is seen after severe trauma. Treatment with a single dose of ANS significantly reduced median tumour load in severely traumatised rats ($p < 0.01$). Surprisingly, intra-peritoneal injection of 3 doses of ANS induced even more tumour load ($p < 0.001$).

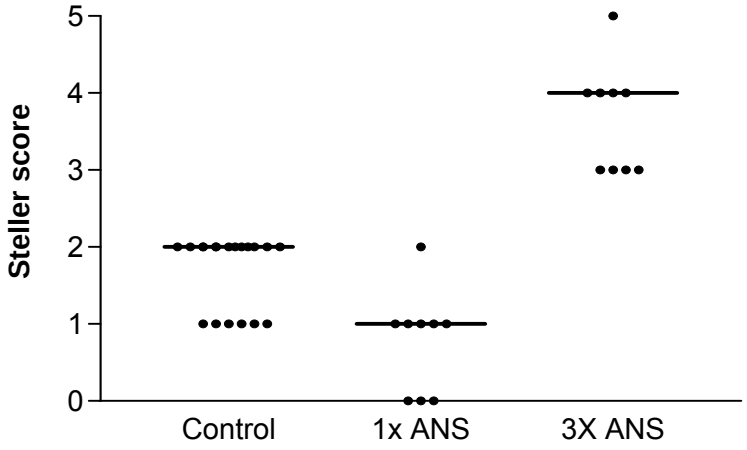

Figure 6. Median intraperitoneal tumour load and range of severely traumatised rats (control), severely traumatised rats treated with ANS on day –1 (1x ANS) and severely traumatised rats treated with ANS on day –1, 0 and +1 (3x ANS). Control vs. 1x ANS ($p < 0.01$). Control vs. 3x ANS ($p < 0.001$).

DISCUSSION

In this study, a cell-seeding model was used to mimic the clinical situation of free intraperitoneal tumour cells and associates the combination of cell adhesion and growth ultimately leading to manifest tumour recurrence. The term tumour recurrence is sometimes used to illustrate tumour load, which is the net result of tumour cell adhesion and tumour growth, because we presume that intra-peritoneal injecting of tumour cells resembles the clinical situation of tumour cell spill during tumour resection.

Following peritoneal trauma, a variety of cytokines, growth factors and other inflammatory mediators are produced by activated mesothelial cells and by stampeded inflammatory cells.[11] The production of mesothelial and inflammatory cell derived chemokines will cause an early post-traumatic migration of PMN and monocytes to the injured peritoneal cavity in order to promote the peritoneal healing process.[12] However, these mediators and recruited cells not only serve peritoneal healing, but as shown in a previous study, could enhance tumour recurrence in naïve recipients as well.[4] The current study demonstrates that the

cellular components of the lavage fluid collected after inflicting surgical trauma i.e. inflammatory cells lead to enhanced tumour recurrence. More detailed analysis of the cellular fraction of these lavage fluids revealed a trauma related influx of PMN in the abdominal cavity (fig. 4). PMN use both oxygen-dependent and oxygen-independent processes in killing micro-organisms, but these processes also (further) damage surrounding host tissue.[13,14] In vitro, adhesion of activated PMN to a mesothelial monolayer has been shown to induce retraction, gap formation and detachment ending with substantial mesothelial cell injury and exposure of extracellular matrix components, hereby already creating a preferential site for adhesion of free tumour cells.[15] 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 and proved promising in rat models.[4,16] In addition, laparoscopic surgery appears to have less impact on the cellular components of the immune response than laparotomy.[3] A recent meta-analysis reported faster postoperative recovery with laparoscopic surgery for colorectal cancer compared to open surgery, though in this meta-analysis no differences were seen in recurrence rates.[17] However, only few studies published long-term results with a favourable trend towards laparoscopy.[18]

The coincidence of post-traumatic intra-abdominal PMN influx with tumour cell adhesion and growth however, is no solid proof for the role of PMN in these pathogenetic processes. Effective inhibition of tissue injury by PMN has been achieved.[19,20] Dovi et al.[21] even showed that wound healing was accelerated in PMN depleted mice. However whether inhibition of PMN affected tumour recurrence has not been investigated. The present study demonstrated that a single intra-peritoneal dose of ANS could prevent the influx of PMN without influencing blood cell count. This experiment showed that a selective reduction of post-traumatic PMN influx, without causing systemic immune suppression, was possible and could indeed significantly lower tumour adhesion and growth. Averting the post-traumatic intra-abdominal PMN influx by intra-peritoneal injection of 3 doses of ANS significantly increased tumour recurrence, at first to our surprise. However treatment with 3 doses of ANS also significantly decreased blood lymphocyte, monocyte and PMN counts, thereby seriously compromising the rat immune system. It is conceivable that this immune suppression promotes tumour growth. We observed earlier that immune suppression leads to enhanced growth of the tumour used in the current experiments.[22]

In conclusion, these studies demonstrated that the early inflammatory sequelae after surgery promote tumour recurrence and that this effect is mainly based on the cellular component of the inflammatory process. Preventing the postoperative influx of PMN without affecting the systemic immune response reduced peritoneal tumour recurrence.

REFERENCES

1. Busch OR, Hop WC, Hoynck van Papendrecht MA, Marquet RL, Jeekel J. Blood transfusions and prognosis in colorectal cancer. N Engl J Med 1993;328:1372-6.
2. Raa ST, Oosterling SJ, van der Kaaij NP, van den Tol MP, Beelen RH, Meijer S, van Eijck CH, van der Sijp JR, van Egmond M, Jeekel J. Surgery promotes implantation of disseminated tumor cells, but does not increase growth of tumor cell clusters. J Surg Oncol 2005;92:124-9.
3. Jacobi CA, Ordemann J, Zieren HU, Volk HD, Bauhofer A, Halle E, Muller JM. Increased systemic inflammation after laparotomy vs laparoscopy in an animal model of peritonitis. Arch Surg 1998;133:258-62.
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;227:242-8.
5. Ellis H, Harrison W, Hugh TB. The healing of peritoneum under normal and pathological conditions. Br J Surg 1965;52:471-76.
6. 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:S17-21.
7. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. Int J Cancer 1984;33:689-92.
8. Sandler H, Hogstorp H, Lundberg C, Gerdin B. Antiserum-induced neutropenia in the rat: characterization of a rabbit anti-rat neutrophil serum. Br J Exp Pathol 1987;68:71-80.
9. de Vree WJ, Essers MC, de Bruijn HS, Star WM, Koster JF, Sluiter W. Evidence for an important role of neutrophils in the efficacy of photodynamic therapy in vivo. Cancer Res 1996;56:2908-11.
10. Steller EP, Ottow RT, Eggermont AM, Marquet RL, Sugarbaker PH. Local conditions in the host influence immunotherapy with interleukin-2 and LAK cells. Cancer Detect Prev 1988;12:81-90.
11. Holmdahl L, Ivarsson ML. The role of cytokines, coagulation, and fibrinolysis in peritoneal tissue repair. Eur J Surg 1999;165:1012-9.
12. Novitsky YW, Litwin DE, Callery MP. The net immunologic advantage of laparoscopic surgery. Surg Endosc 2004;18:1411-9.
13. Weiss SJ. Tissue destruction by neutrophils. N Engl J Med 1989;320:365-76.
14. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. Trends Cell Biol 2005;15:599-607.
15. Andreoli SP, Mallett C, Williams K, McAteer JA, Rothlein R, Doerschuk CM. Mechanisms of polymorphonuclear leukocyte mediated peritoneal mesothelial cell injury. Kidney Int 1994;46:1100-9.
16. Bouvy ND, Marquet RL, Jeekel H, Bonjer HJ. Impact of gas(less) laparoscopy and laparotomy on peritoneal tumor growth and abdominal wall metastases. Ann Surg 1996;224:694-700; discussion 00-1.
17. Reza MM, Blasco JA, Andradas E, Cantero R, Mayol J. Systematic review of laparoscopic versus open surgery for colorectal cancer. Br J Surg 2006;93:921-8.
18. Lacy AM, Garcia-Valdecasas JC, Delgado S, Castells A, Taura P, Pique JM, Visa J. Laparoscopy-assisted colectomy versus open colectomy for treatment of non-metastatic colon cancer: a randomised trial. Lancet 2002;359:2224-9.
19. Zagorski J, Wahl SM. Inhibition of acute peritoneal inflammation in rats by a cytokine-induced neutrophil chemoattractant receptor antagonist. J Immunol 1997;159:1059-62.
20. Wang P, Chen H, Qin H, Sankarapandi S, Becher MW, Wong PC, Zweier JL. Overexpression of human copper, zinc-superoxide dismutase (SOD1) prevents postischemic injury. Proc Natl Acad Sci U S A 1998;95:4556-60.

21. Dovi JV, He LK, DiPietro LA. Accelerated wound closure in neutrophil-depleted mice. J Leukoc Biol 2003;73:448-55.
22. Van de Vrie W, Marquet RL, Eggermont AM. Cyclosporin A enhances locoregional metastasis of the CC531 rat colon tumour. J Cancer Res Clin Oncol 1997;123:21-4.

Chapter 5

The Influence of Reactive Oxygen Species on the Adhesion of Pancreatic Carcinoma Cells to the Peritoneum

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ABSTRACT

Background:

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

Methods:

ROS were produced using the enzymatic reaction of xanthine with xanthine oxidase (X/XO). A reproducible in-vitro assay to study adhesion of human Panc-1 carcinoma tumour cells to a mesothelial cell monolayer of primary human mesothelial cells was used. Mesothelial monolayers were incubated with ROS produced prior to adhesion of the tumour cells.

Results:

Incubation of the mesothelial cells with X/XO resulted in a significant increase (69.5%) in adhesion of Panc-1 in all patients. SOD/catalase, anti-oxidants, could reduce this increase by 56.7%. ROS significantly influenced the expression of the adhesion molecules ICAM-1, VCAM-1 and CD44h on mesothelial cells, but did not influence adhesion molecule expression on Panc-1.

Conclusions:

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

INTRODUCTION

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

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

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

The aim of the current in-vitro study was to evaluate the effect of ROS on pancreatic tumour cell adhesion to a monolayer of human mesothelial cells. And whether anti-oxidants could be used to reduce post-operative enhanced tumour adhesion. Moreover we analyse

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

MATERIALS AND METHODS

Cells

Human mesothelial cells were obtained from the omental tissue of patients undergoing elective abdominal surgery for non-infectious reasons who had given informed consent. The experimental protocol was approved by the Medical Ethical Committee of the Erasmus Medical Centre Rotterdam, the Netherlands. The omental tissue of 10 patients were used, aged 61 ± 9.5 years (6 male and 4 female patients). Five were operated upon for pancreas carcinoma and 5 for oesophagus carcinoma, non had evidence of metastatic disease at the time of surgery. Immediately after opening the abdomen, a specimen of bowel omental tissue (5 x 5 cm) was taken. The specimen was cut in small pieces and washed with HBSS containing 0.5% human serum albumin. After washing three times the specimens were centrifuged for 5 minutes at 580g. The omentum was transferred to fluid containing trypsin (0.05%) and EDTA (0.02%) (Gibco, Breda, the Netherlands) and incubated for 20 minutes at 37°C with continuous shaking. After 20 minutes the detached mesothelial cells were pelleted down by centrifugation at 580g for 10 minutes. After centrifugation the mesothelial cells were resuspended in culture medium (RPMI 1640 containing 10% fetal calf serum, glutamine (2mM) and penicillin (10^5 U/L)). The mesothelial cells were cultured at 37°C, in a fully humidified, 5% CO_2 cabinet in polystyrene 75cm^2 culture flasks pre coated with collagen type I (Roche Diagnostics, Almere, the Netherlands).

The mesothelial origin from the cells was verified by their typical cobblestone appearance in phase-contrast microscopy and by immunohystochemical characterisation with positive staining of keratin (anti-serum Z622; DAKO, Heverlee, the Netherlands), vimentin (anti-serum L1843; DAKO, Heverlee, the Netherlands) and negative staining of von Willebrand factor (F8/86 antibody; DAKO, Heverlee, the Netherlands).

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

Drugs

The cell cultures were exposed to the reactive oxygen species (ROS) superoxide and hydrogen peroxide generated by xanthine oxidase (0.05 U/ml, Sigma-Aldrich Chemie,

Zwijndrecht, the Netherlands) upon the addition of xanthine (0.1 mmol/l, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) (X/XO). The oxygen-free radicals are produced by the following enzymatic reaction: xanthine + O_2 + H_2O → Uric acid + H_2O_2 + O_2^-, catalysed by xanthine oxidase.

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

Ferricytochrome c reduction

Superoxide generation by xanthine/xanthine oxidase (X/XO) was determined using the ferricytochrome c (cytc) reduction assay modified from Leslie et al.[19] In brief, cytc was prepared in HBSS without phenol red and used at a final concentration of 150µM. After filling each well with 100 µl of cytc, cytc + xanthine or cytc + xanthine + SOD/catalase, the reaction was started by adding 100 µl of xanthine oxidase solution (100 µl/well). Wells filled with cytc in HBSS served as a blank. The plate was kept at a constant temperature of 37ºC using a thermostatted microplate reader (Versamax, Molecular Devices), and read every 10 minutes up to 120 minutes. The amount of reduced cytc reduction was calculated from the absorbance at 550 nm with 540 nm as the reference using molar absorbance coefficient of 12.2 x 10^3 M^{-1}.[19]

Apoptosis assay

To assess whether superoxide anions caused apoptosis in mesothelial cells a cell-death detection ELISA^{plus} kit (Roche Applied Science, Almere, The Netherlands) was used for the detection of cytoplasmic histone-associated DNA fragments. In short, mesothelial cells were grown to confluence as described for the adhesion assays in 96-well flat-bottomed micro titre plates. The cells were preincubated with X, XO or X/XO for 12 hours, rinsed (removing detached dead cells) and then lysated, where after 20 µl of the lysate was transferred into Streptavidin-coated microplate wells. Eighty micro litre of immunoreagent containing biotinylated anti-histone and peroxidase-labelled anti-DNA antibodies was added into the wells followed by incubation on a plate shaker under gently shaking (300 rpm) for 2 h at 15-25°C. Then the wells were washed thoroughly with incubation buffer and 100 µl of 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) substrate was added. Plates were incubated for 15 min on a plate shaker at 250 rpm where after photometric analysis at 405 nm was performed.

Adhesion assay

To quantify tumour cell adhesion to mesothelium, a previously standardized and validated cell adhesion assay was used.[20] In short, mesothelial cells were transferred to 96-well plates (Perkin Elmer, Groningen, the Netherlands) pre-coated with collagen type-1 and grown to

confluence as confirmed by light microscopy. In order to determine the effect of ROS on tumour cell adhesion, mesothelial monolayers or Panc-1 were pre-incubated with X/XO or X/XO with SOD/catalase for 12 hours. Untreated cells served as a control.

Tumour cells were labelled with calcein-AM (Molecular Probes, Leiden, NL) by incubating 1 x 106 Panc-1 cells/ml RPMI medium with 1% FCS containing 10 µM calcein-AM for 45 minutes at 37 °C with occasional mixing. After washing the labelled Panc-1 twice with RPMI containing 1% FCS to remove free dye, supernatant from the experimental wells was removed and 30.000 labelled Panc-1 cells in 200 µl RPMI/1% FCS were added. Plates were centrifuged for 1 minute at 80 g and incubated at 37 °C for one hour. After washing away unbound cells, the numbers of adherent tumour cells were assessed by measuring fluorescence at 485 nm excitation and 535 nm emission using a standard curve prepared from various numbers of labelled tumour cells.

Enzyme immuno-assay (EIA) of cellular adhesion molecules

A previously described enzyme immunoassay was used to determine the effect of X/XO on the expression of adhesion molecules.[20] Shortly, mesothelial cells and Panc-1 were grown to confluence in 96-well flat-bottomed multititer plates and pre-incubated with either cell culture medium alone or in combination with X/XO or X/XO and SOD/catalase for 12 hours. Medium from the experimental wells was removed and the cells were fixed in 95% ethanol/ 5% methanol solution for 45 minutes. Subsequently, the cells were pre-incubated for 10 minutes with 1% normal goat serum, diluted in PBS, to block non-specific binding. Mouse anti-human monoclonal antibodies to ICAM-1 (CD54, R&D Systems; diluted 1 : 500), VCAM-1 (CD106, R&D Systems; diluted 1 : 500), LFA-1 (CD11a, Leinco Technologies; diluted 1 : 10), VLA-4 (CD49d, R&D Systems; diluted 1 : 50), CD44H (CD106, R&D Systems; diluted 1 : 500) were added for 60 minutes. Tumour cells or mesothelial cells incubated without one of these primary antibody served as a negative control. After a washing step with phosphate buffered saline (room temperature, pH 7.4) the cells were subsequently incubated with biotinylated goat antimouse antibody (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) in a dilution of 1 : 500 for another 60 minutes. Finally the cells were washed with phosphate buffered saline and incubated with a streptavidin-peroxidase complex for 30 minutes, washed, and substrate (2,2'-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) in 0.05M phosphate-citrate buffer containing 0.03% sodium perborate was added. After 40 minutes, the reaction was stopped using 0.4% sodium fluoride and the absorbance was read at 405 nm. Membrane antigen expression is expressed as OD units.

Statistical analysis

All data were evaluated using analysis of variance (ANOVA). If the ANOVA test was significant at the 5% level, the Tukey-Kramer *post hoc* test was carried out to assess differences between groups. Statistical significance was accepted at $p < 0.05$.

RESULTS

Quantification of ROS generation by xanthine/xanthine oxidase

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

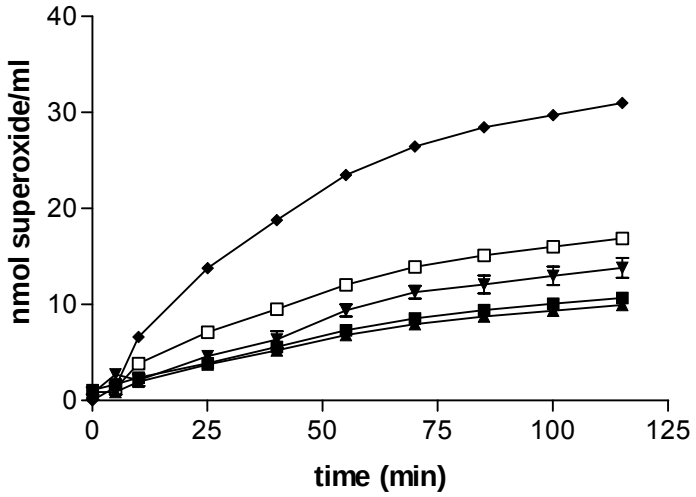

Figure 1. Ferricytochrome C reduction assay. Superoxide production by control (-■-), X (-▲-), XO (-▼-), X/XO (-♦-) or X/XO and SOD/catalase (-□-). Cumulative superoxide production is displayed nmol/ml ± SEM (n=6/condition).

Effect of ROS on mesothelial cell viability

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

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

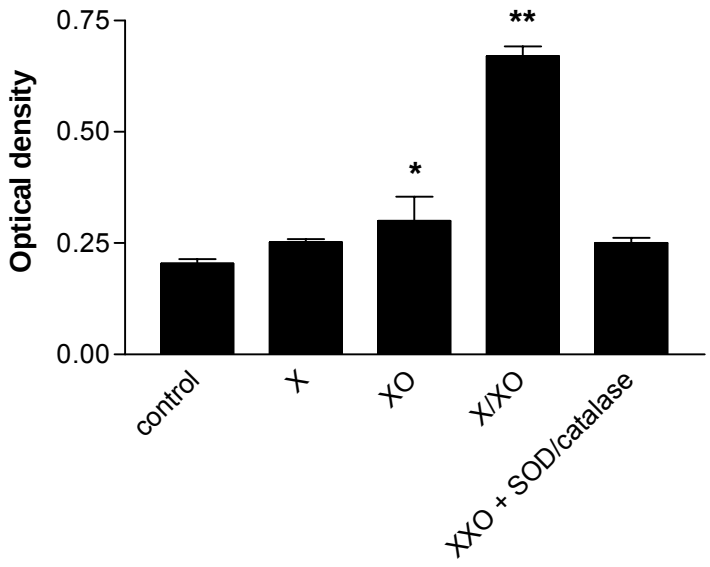

Figure 2. Measurement of apoptosis by ELISA^{plus} in mesothelial monolayers after preincubation with X, XO, X/XO and X/XO with SOD/catalase for 12 hours. Optical density represents mean absorbance values ± SEM of triplate wells. * = $p < 0.05$ vs. control, ** = $p < 0.001$ vs. control.

Effect of ROS on Panc-1 adherence

To examine if ROS affect the adherence of Panc-1 tumour cells to human mesothelium, adhesion of Panc-1 to non-stimulated mesothelial cells was studied first. The results showed that 60 min after addition of Panc-1 cells to mesothelial monolayers obtained from various patients the magnitude of adherence varied between 9 and 36% (fig. 3). The adherence did not increase further with longer incubation times of Panc-1 and mesothelial cells.

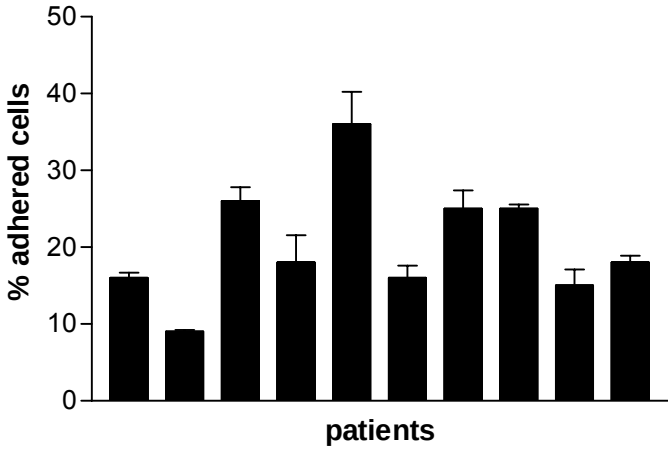

Figure 3. Adhesion of Panc-1 to mesothelial monolayers of 10 different patients. Percentage indicates number of cells adhered of the 30.000 cells added. Data are expressed as the mean percentage (n=6) and SEM.

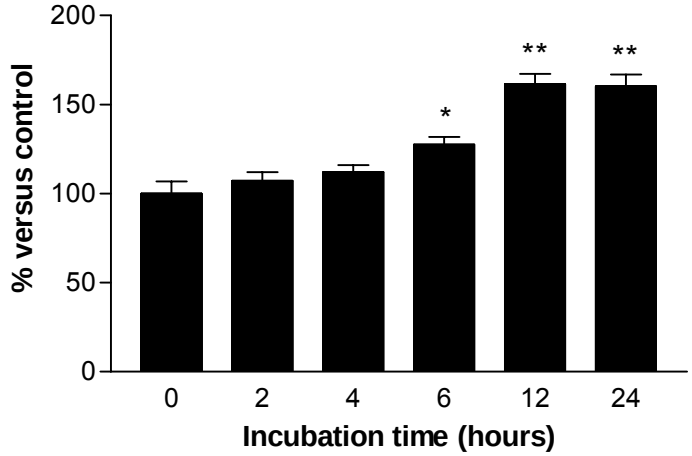

Figure 4. Adhesion of Panc-1 to a mesothelial monolayer after pre-incubation with X/XO at varying time intervals. Data are expressed as the mean (n=6) and SEM. Basal adhesion 20%. * = $p < 0.05$; ** = $p < 0.001$.

After incubation of the mesothelial monolayer with X/XO, the number of adhering Panc-1 cells did not differ from the control up to 6 hr, but increased to 61% stimulation (SD = 14%); $p < 0.001$) after 12 hours of pre-incubation with X/XO (fig. 4). Treatment of mesothelial cells obtained from nine other donors during 12 hr with X/XO led to a mean augmented adhesion with X/XO of 95% (varying from 45 to 166%). If SOD and catalase were added during pre-incubation of the mesothelial monolayer with X/XO, the stimulatory effect of ROS on the adherence of Panc-1 cells to the mesothelial cells could be inhibited almost completely (fig. 5), while pre-incubation of the mesothelium with SOD and catalase alone did not affect tumour cell adhesion. Incubation of Panc-1 with X/XO did not affect adhesion of the tumour cells to the mesothelial monolayer.

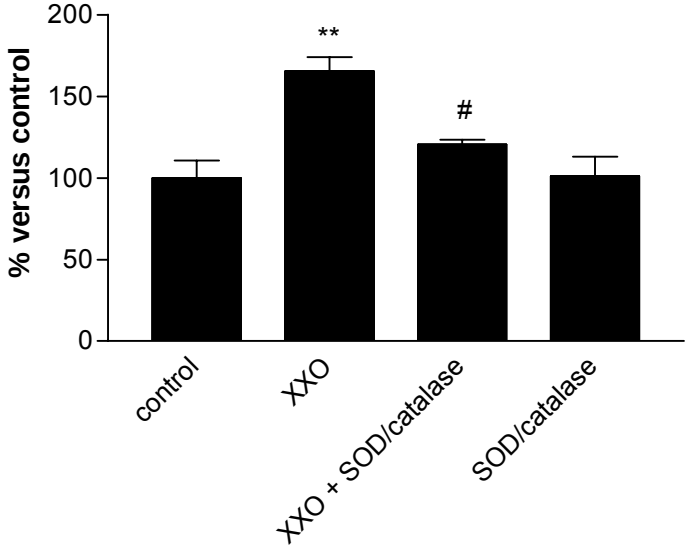

Figure 5. Adhesion of Panc-1 to a mesothelial monolayer after pre-incubation with X/XO with or without SOD/catalase. Data are expressed as the mean (n=6) and SEM. Basal adhesion 18%; * = $p < 0.001$; # = $p < 0.05$.

Expression of cell adhesion molecules

To investigate if an increased expression of cellular adhesion molecules by the mesothelium underlies the increase in adherence of Panc-1 cells to ROS-treated mesothelial cells, the expression of various adhesion molecules was studied. The results showed that normal mesothelial cells express ICAM-1 (141% above the negative control), while the expression of CD44H and VCAM-1 was low (16%) and moderate (56%) (fig. 6). When stimulating the mesothelial cells with X/XO for 12 hours, after which the adhesion of Panc-1 to mesothelial cells was maximal, a significant increase in expression of ICAM-1, VCAM-1 and CD44H could be observed (fig. 6). Adding SOD and catalase to X/XO prevented the increase in expression of these adhesion molecules. Mesothelial cells did not express the adhesion molecules VLA-4 and LFA-1 and this pattern did not change upon stimulation with X/XO (fig. 6).

Panc-1 cells express the adhesion molecules CD44H (89% above neg. control), ICAM-1 (77%) and VCAM-1 (56%), and only low levels of VLA-4 (20%) (fig. 7). Panc-1 does not express LFA-1. The expression levels of these adhesion molecules did not change upon stimulation by X/XO (results not shown).

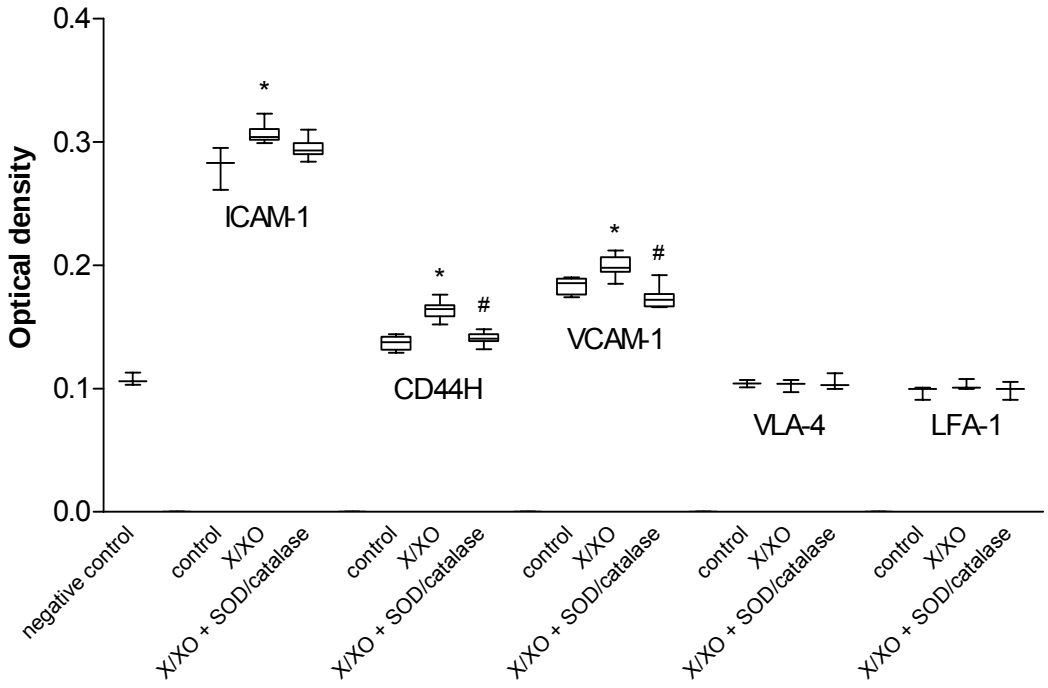

Figure 6. Expression of adhesion molecules on mesothelial cells stimulated with X/XO with or without SOD and catalase. Optical density represents the median absorbance values and range at 405 nm. * = $p < 0.001$ vs. control.

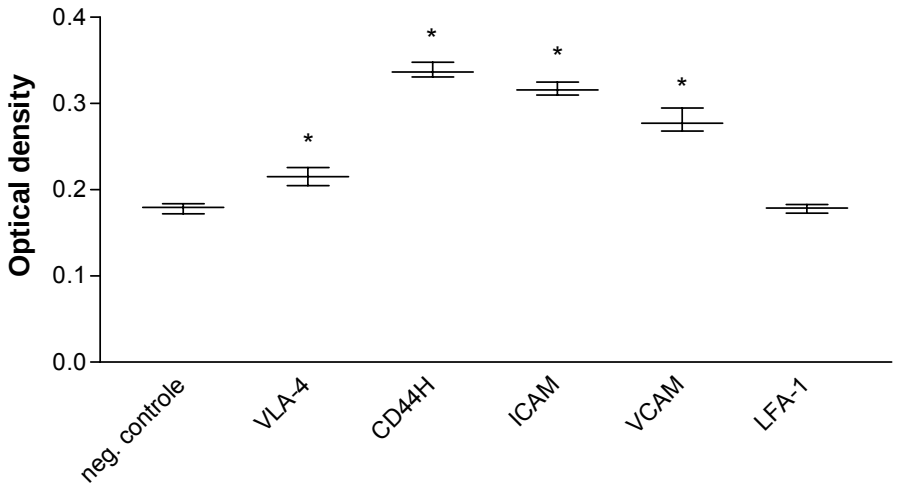

Figure 7. Expression of adhesion molecules on Panc-1. * = $p < 0.01$ vs. negative control. No changes were seen upon stimulation with X/XO. Optical density represents the median absorbance values and range at 405 nm.

DISCUSSION

For local or peritoneal tumour recurrence to develop, adhesion and growth of per-operatively spilled tumour cells is necessary. Cytokines and growth factors produced in great amount after surgical trauma, are known to promote tumour recurrence in in-vivo and in-vitro animal models.[8,11,21]

The present study demonstrates that ROS stimulate tumour cell adhesion to the peritoneum in a human in-vitro model. The mechanism by which ROS are able to enhance tumour cell adhesion is dual. Firstly, we found that ROS are able to directly damage the peritoneal lining (through induction of apoptosis of mesothelial cells). The subsequent exposing of the ECM will create a preferential adhesion site for the tumour cells.[22] Under the present conditions, however, the mesothelial monolayer was still intact as examined by phase-contrast microscopy after 12 hr of stimulation by ROS.

Another mechanism by which ROS could stimulate tumour cell adhesion is by enhancing the expression of adhesion molecules, either on mesothelial cells or pancreatic carcinoma cells. The expression of adhesion molecules on human mesothelial cells under the influence of inflammatory cytokines (TNFα, IL-1β, IL-8) has been analysed. Generally these studies show an increase in ICAM-1, VCAM-1 and CD44 upon stimulation with different pro-inflammatory cytokines.[23-25]

In the present study we found that ROS induced the expression of adhesion molecules VCAM-1, ICAM-1 and CD44H on mesothelial cells as well. ROS did not alter the expression pattern of adhesion molecules on the Panc-1 cell membrane and therefore the observed

increase in Panc-1 tumour cell adhesion to the mesothelial cells is dependent on changes of the mesothelium. Hosono et al.[26] showed that CD44H plays an essential role in the initial attachment of pancreas carcinoma cell-line (SW1990) to mesothelial cells and Ziprin et al.[25] demonstrated that ICAM-1 blockade reduced the ability of pancreas carcinoma cell line PSN-1 to adhere to the mesothelium. These results are supported by findings of van Grevenstein et al.[24] who demonstrated the effect of inflammatory cytokines (TNFα, IL-1β and IL-8) on adhesion of pancreatic carcinoma cell lines (MiaPaCa-2 and BxPc3) to mesothelial cells. Surprisingly, no effects of these cytokines were seen on adhesion of Panc-1 to a monolayer of mesothelial cells in their study. They already discussed the problem of high basal adhesion of Panc-1, which was not seen in the present study. Moreover, no expression of VLA-4 was found on Panc-1 by classical immunohistochemical staining. However, the more sensitive enzyme immunoassay did show expression of VLA-4 on mesothelial cells. Possibly the tripsinisation step used in their experiments influenced the expression of VLA-4. Important is that VLA-4 is the counterpart of VCAM-1 which has shown to be upregulated in mesothelial cells upon stimulation with ROS and inflammatory cytokines. Therefore VCAM-1 might play an important role in the adhesion of Panc-1 to mesothelial monolayers. In the present study ICAM-1 might be of less importance, as the mesothelial cells as well as Panc-1 did not express its natural ligand LFA-1. Although Ziprin et al.[23] showed CD43 as an important ligand for ICAM-1 and mediator in adhesion of pancreas carcinoma as well as colon carcinoma cell-lines to mesothelial cells.

Taking the expression patterns of most pancreas carcinoma cell-lines into account, adhesion to mesothelial cells have shown to be influenced by the adhesion molecules ICAM-1, VCAM-1 or CD44H. ROS upregulate the expression of these adhesion molecules on mesothelial cells and have shown to enhance adhesion of Panc-1 to mesothelial cells. It is very well conceivable that ROS will enhance adhesion of other pancreas carcinoma cell-lines as well, although this was not investigated in the present study.

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

REFERENCES

1. DiMagno EP, Reber HA, Tempero MA. AGA technical review on the epidemiology, diagnosis, and treatment of pancreatic ductal adenocarcinoma. American Gastroenterological Association. Gastroenterology 1999;117:1464-84.
2. Abulafi AM, Williams NS. Local recurrence of colorectal cancer: the problem, mechanisms, management and adjuvant therapy. Br J Surg 1994;81:7-19.
3. Sugarbaker PH. A perspective on clinical research strategies in carcinoma of the large bowel. World J Surg 1991;15:609-16.
4. Schott A, Vogel I, Krueger U, Kalthoff H, Schreiber HW, Schmiegel W, Henne-Bruns D, Kremer B, Juhl H. 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;227:372-9.
5. Meszoely IM, Lee JS, Watson JC, Meyers M, Wang H, Hoffman JP. Peritoneal cytology in patients with potentially resectable adenocarcinoma of the pancreas. Am Surg 2004;70:208-13; discussion 13-4.
6. 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;10:490-4.
7. Busch OR, Hop WC, Hoynck van Papendrecht MA, Marquet RL, Jeekel J. Blood transfusions and prognosis in colorectal cancer. N Engl J Med 1993;328:1372-6.
8. 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;227:242-8.
9. Raa ST, Oosterling SJ, van der Kaaij NP, van den Tol MP, Beelen RH, Meijer S, van Eijck CH, van der Sijp JR, van Egmond M, Jeekel J. Surgery promotes implantation of disseminated tumor cells, but does not increase growth of tumor cell clusters. J Surg Oncol 2005;92:124-9.
10. 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;12:421-9.
11. van Rossen ME, Hofland LJ, van den Tol MP, van Koetsveld PM, Jeekel J, Marquet RL, van Eijck CH. Effect of inflammatory cytokines and growth factors on tumour cell adhesion to the peritoneum. J Pathol 2001;193:530-7.
12. Rodgers KE, diZerega GS. Function of peritoneal exudate cells after abdominal surgery. J Invest Surg 1993;6:9-23.
13. Ellis H, Harrison W, Hugh TB. The Healing of Peritneum under Normal and Pathological Conditions. Br J Surg 1965;52:471-6.
14. Kuraoka S, Campeau JD, Nakamura RM, diZerega GS. Modulation of postsurgical macrophage function by early postsurgical polymorphonuclear leukocytes. J Surg Res 1992;53:245-50.
15. Bulkley GB. The role of oxygen free radicals in human disease processes. Surgery 1983;94:407-11.
16. Weiss SJ. Tissue destruction by neutrophils. N Engl J Med 1989;320:365-76.
17. van Rossen ME, Sluiter W, Bonthuis F, Jeekel H, Marquet RL, van Eijck CH. Scavenging of reactive oxygen species leads to diminished peritoneal tumor recurrence. Cancer Res 2000;60:5625-9.
18. Fried R. Metabolic role of xanthine oxidase as source of superoxide radicals and hydrogen peroxide. In: Bannister, J. V.; Hill, H.A.O., eds Chemical and biochemical aspects of superoxide and superoxide dismutase: Developments in biochemistry. 1979;Vol. 11A. NewYork: Elsevier/North-Holland:65-75.
19. Leslie RG. Evaluation and improvement of a rapid microassay for measuring superoxide anion production by phagocytes. 1. Spectrophotometric aspects. J Immunol Methods 1987;103:253-9.

20. ten Kate M, Hofland LJ, van Grevenstein WM, van Koetsveld PV, Jeekel J, van Eijck CH. Influence of proinflammatory cytokines on the adhesion of human colon carcinoma cells to lung microvascular endothelium. Int J Cancer 2004;112:943-50.
21. 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;133:383-9.
22. Sawai H, Takeyama H, Yamamoto M, Furuta A, Funahashi H, Okada Y, Sato M, Tanaka M, Manabe T. Enhancement of integrins by interleukin-1alpha, and their relationship with metastatic and invasive behavior of human pancreatic ductal adenocarcinoma cells. J Surg Oncol 2003;82:51-6.
23. Ziprin P, Alkhamesi NA, Ridgway PF, Peck DH, Darzi AW. Tumour-expressed CD43 (sialophorin) mediates tumourmesothelial cell adhesion. Biol Chem 2004;385:755-61.
24. van Grevenstein WM, Hofland LJ, Jeekel J, van Eijck CH. The expression of adhesion molecules and the influence of inflammatory cytokines on the adhesion of human pancreatic carcinoma cells to mesothelial monolayers. Pancreas 2006;32:396-402.
25. Ziprin P, Ridgway PF, Pfistermuller KL, Peck DH, Darzi AW. ICAM-1 mediated tumor-mesothelial cell adhesion is modulated by IL-6 and TNF-alpha: a potential mechanism by which surgical trauma increases peritoneal metastases. Cell Commun Adhes 2003;10:141-54.
26. Hosono J, Narita T, Kimura N, Sato M, Nakashio T, Kasai Y, Nonami T, Nakao A, Takagi H, Kannagi R. Involvement of adhesion molecules in metastasis of SW1990, human pancreatic cancer cells. J Surg Oncol 1998;67:77-84.

Chapter 6

Peri-Operative Oral Treatment with Celecoxib Prevents Peritoneal Carcinomatosis in a Rat Model

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ABSTRACT

Background:

Surgical trauma and wounding is associated with (local) cancer recurrence. Yet, for the most intra-abdominal malignancies, surgical excision is the mainstay of treatment and often provides the only chance of cure. The inflammatory environment induced by surgery contributes to the process of tumour recurrence. A key regulator of the inflammatory process is the enzyme cyclooxygenase-2 (COX-2). The present study looked at the effects of treatment with the selective COX-2 inhibitor celecoxib on tumour development in an intra-abdominal surgical model.

Methods:

A reproducible rat model was used in which standardized surgical trauma was inflicted by a laparotomy. At the end of surgery an intra-peritoneal injection of CC531s colon carcinoma cells was performed, resembling peroperative tumour spill. Rats were treated with different oral dosages of celecoxib, daily, during the experiment.

Results:

Surgical trauma provoked significant tumour load. Treatment with a daily oral dose of celecoxib showed a dose dependent reduction in tumour load. Surprisingly, treatment with 50mg/kg celecoxib almost completely inhibited tumour recurrence in the present peritoneal carcinomatosis model.

Conclusions:

High dose celecoxib almost completely inhibits peritoneal tumour recurrence. Peri-operative administration of celecoxib as described in our model might represent a new modality in cancer therapy. The recent concerns on cardiovascular risks of celecoxib might be of less importance when used in selected populations and during a fixed period.

INTRODUCTION

For many solid malignancies, surgical excision is the mainstay of treatment and provides the only chance of cure. However, surgical trauma and wounding has been associated with (local) cancer recurrence or accelerated growth of minimal residual disease[1], which was already suggested by Deelman in 1927.[2] It is becoming increasingly apparent that the tumour environment plays an essential role in development of malignant disease, and correlation between (chronic) inflammation and promotion of carcinogenesis has been demonstrated in several malignancies.[3] The inflammatory environment induced by surgery includes infiltrations of leukocytes (neutrophils, macrophages), release of cytokines/growth factors and stimulation of neovessel formation that might contribute to several steps in the process of tumour recurrence, such as facilitation of tumour cell implantation, proliferation and invasion.[3,4]

A key regulator of the inflammatory process is the enzyme cyclooxygenase-2 (COX-2) that catalyses the conversion from arachidonic acid to prostaglandins, which are potent inflammatory mediators. COX-2 is not expressed in normal tissue, but is induced in response to several pro-inflammatory cytokines, growth factors and tumour promoters.[5] In addition, elevated COX-2 expression has been shown in tumour epithelium from various solid tumours, including colorectal carcinomas, but also in adjacent stromal tumour compartments, localized to both macrophages and vascular endothelial cells.[6-8] Moreover, COX-2 appeared to be induced in tumour neovasculature, but not in normal blood vessels.[9] Inhibition of COX-2 has been shown effective in reducing tumour growth in a nude mice implantation model that involved of COX-2 expressing tumour cells.[10,11] Roh et al. demonstrated that the selective COX-2 inhibitor celecoxib repressed growth of subcutaneous tumours that were implanted adjacent to surgical wounds.[12]

A common site of recurrence after intentionally curative resection for colorectal cancer, besides the liver, is the local peritoneum. Its incidence varies in different series between 4 and 21%, increases parallel to disease stage and generally presents within two years after ‘curative’ resection.[13] Local (peritoneal) recurrences might originate from propagation of spilled tumour cells in the operative field or from minimal residual disease, especially in case of serosa-infiltrating tumours. In our experimental surgical model in the abdominal cavity, representing local recurrence after surgery, we recently showed that surgical trauma (inducing an inflammatory microenvironment) enhances coloncarcinoma cell adhesion to the peritoneum, which eventually resulted in significantly larger tumour load in these operated animals.[4]

In the present study, we investigated the effects of treatment with the selective COX-2 inhibitor celecoxib on tumour development in our intra-abdominal surgical model.

MATERIALS AND METHODS

Animals

WAG/Rij rats weighing 150-190g were obtained from Harlan-CPB, Austerlitz, The Netherlands. The rats were bred under specific pathogen-free conditions. The animals were kept under standard laboratory conditions (temperature 20-24ºC, relative humidity 50-60%, 12 hours light and 12 hours dark cycles), fed with standard rat food and water *ad libitum* and quarantined in our University facilities for at least two days prior to use. The experimental protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC.

Study design

All animals underwent a midline laparotomy of 3cm, simulating surgical trauma on day 0 directly followed by i.p. administration of 0.5 x 10^6 CC531s cells. Subsequently, the abdomen was closed in two layers with 5-0 polyglycolic sutures.

In the first experiment rats received from day 0 (3 hours before surgery) through day +17 after operation daily oral gavages of celecoxib (kindly provided by Pfizer Pharmaceuticals, New York) in a dose of 20mg/kg (n=11) or 10mg/kg (n=11), while controls did not receive (n=10) celecoxib.

In a second experiment rats received from day 0 (3 hours before surgery) through day +17 after operation daily oral gavages of celecoxib (kindly provided by Pfizer Pharmaceuticals, New York) in a dose of 50mg/kg (n=9), while controls did not receive (n=8) celecoxib.

Evaluation of intra-peritoneal tumour load

Tumour load was analysed on day +17 and quantified according to an earlier described semi-quantitative scale based on numbers as well as on size of tumour nodules.[1] The scoring was performed by two independent observers blinded for the treatment groups. For each rat the score at all peritoneal sites was summarized, from which a mean total tumour load per rat could be estimated. Additionally, omenta were excised and analysed for presence of metastases.

Statistics

Statistical analysis was performed using one-way ANOVA tests to determine overall differences. If the ANOVA test was significant at a 5% level, the Fischer LSD *post hoc* test was carried out to make a comparison between groups. Data are expressed as mean and standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$.

RESULTS

General observations

No complications or deaths due to sham laparotomy or administered drugs were observed. Body weights did not significantly differ between groups throughout the study period (data not shown).

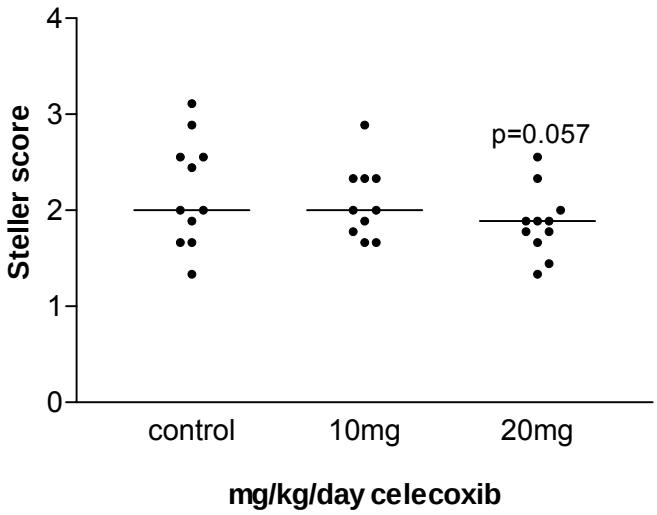

Figure 1. Median intra-peritoneal tumour load and range after intra-abdominal instillation of CC531s and treatment with or without celecoxib.

Tumour load

At sacrifice tumour load was evaluated in all animals. Tumour nodules were primary located on the operation scar, the omentum, liver hilum and gonadal fat streaks.

In figure 1 the results of the first experiment are displayed. A clear tendency in tumour reduction can be seen when comparing the different treatment groups. However the differences seen did not reach statistical significance ($p = 0.057$ when comparing rats treated with a daily oral dose of 20mg/kg celecoxib with the control group). The average number of tumour nodules was evaluated separately as well, since these data could provide an indication for successful adhesion of tumour cell clusters (fig. 2). The number of tumour nodules was lower in the celecoxib treated groups, though a tendency can be noticed, differences did not reach statistical significance.

The second experiment evaluated a higher dose of celecoxib as the previous experiment seemed to show a dose dependent reduction in tumour load. All control rats displayed a significant tumour load, but to our surprise, a daily oral treatment with 50mg/kg celecoxib,

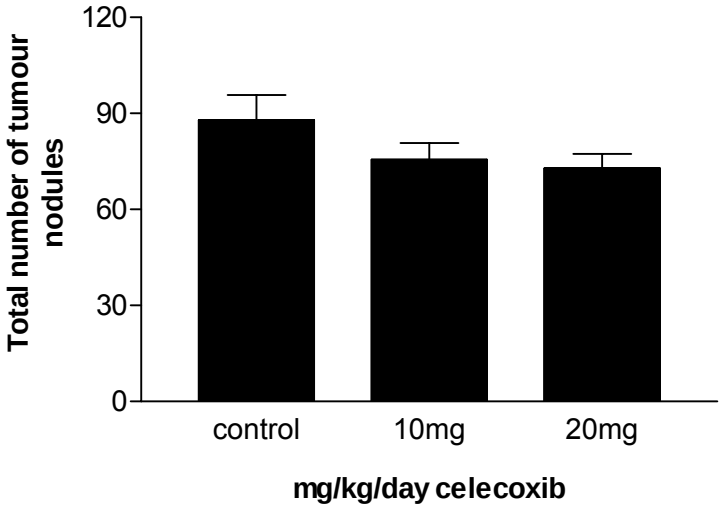

Figure 2. Mean and SEM of cumulative total number of tumour nodules after intra-abdominal instillation of CC531s and treatment with 50mg/kg/day celecoxib. P < 0.01.

completely prevented outgrowth of intra-peritoneal tumours in 6 out of 8 rats, whereas in 2 out of 8 rats, only a few small tumour nodules were found (0 (0 – 0.3) vs. 2.6 (0 – 3.6) compared to control group, p < 0.001) (fig 3). Similar results were seen when evaluating the number of tumour nodules (fig 4, p < 0.01).

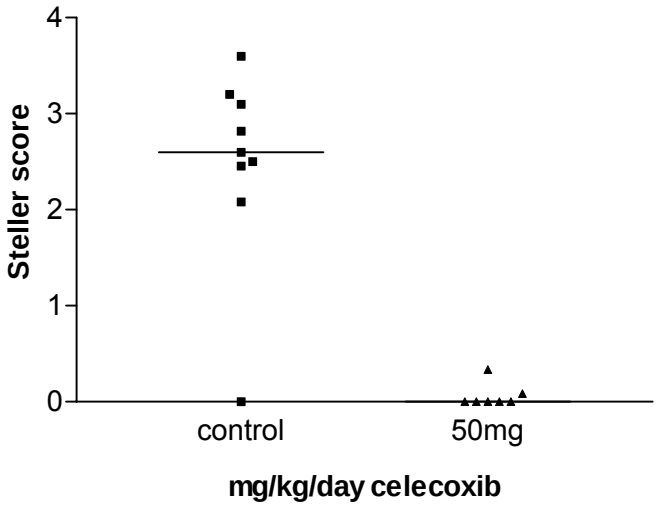

Figure 3. Median intra-peritoneal tumour load and range after intra-abdominal instillation of CC531s and treatment with or without 50mg/kg/day celecoxib. P < 0.0001.

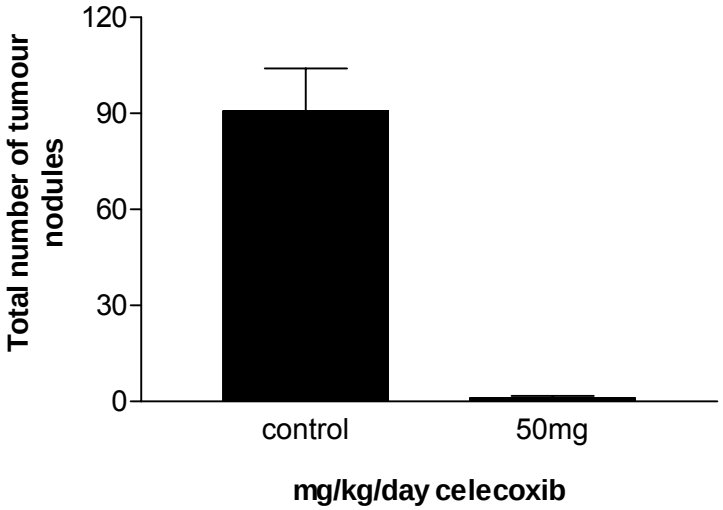

Figure 4. Mean and SEM of cumulative total number of tumour nodules after intra-abdominal instillation of CC531s and treatment with 50mg/kg/day celecoxib. $P < 0.01$.

DISCUSSION

In this study, a cell-seeding model was used to mimic the clinical situation of free intraperitoneal tumour cells, as we presume that intra-peritoneal injecting of tumour cells resembles the clinical situation of tumour cell spill during tumour resection. Experimental studies have shown that wound healing provided a microenvironment favourable for tumour recurrence. The surgical wounds causes recruitment of inflammatory cells into the fields and production of growth factors and cytokines that results in enhanced tumour recurrence after surgery.

The present study showed a remarkable dose dependent reduction in post-operative tumour recurrence by celecoxib. A daily oral high dose celecoxib treatment even almost completely prevented post-operative peritoneal tumour cell adhesion and growth. Several mechanisms might explain the anti-cancer properties of selective COX-2 inhibitors such as celecoxib.

Incubation with a “high-dose” celecoxib has been shown to induce apoptosis in coloncarcinoma cell lines in vitro, which was independent of expression level of COX-2.[11] In vivo however, induction of apoptosis seems a less relevant mechanism, indicating that additional mechanisms are likely to be involved.[14] The role of host (micro)environment COX-2 levels in relation to carcinoma growth was exemplified by the finding that Lewis lung carcinoma cells grew more slowly when injected subcutaneous in Cox2 -/- mice compared to wild-type controls.[14] This implicates that COX-2 expression in host tissue or tumour microenvironment might contribute to advantageous circumstances for tumour cells to thrive.

Roh et al.[12] showed a suppressive effect of the selective COX-2 inhibitor celecoxib on growth of several subcutaneous implanted tumours after surgical wounding. The tumours used expressed different levels of COX-2, with no relation to the inhibitory effect of celecoxib. It was demonstrated that SCC VII (mouse squamous carcinoma cell line) tumours per se did not contain COX-2 proteins, but the peri-tumoural infiltrate did, which peaked on day 3 after wounding. This leukocyte infiltrate was not further characterised, but in agreement with the COX-2 level showed a peak at 3 days after surgical wounding.[12] Therefore, infiltrating leukocytes might well be involved and influenced by COX-2 levels and the inhibitory effect seen with celecoxib might largely be explained by its effect on the inflammatory response induced by surgery.

Interestingly, in a study by Oosterling et al.[15] COX-2 mRNA levels were significantly higher in CC531s (intraperitoneal coloncarcinoma) control tumours than in tumours isolated from animals, in which macrophages had been depleted. This indicates that macrophages might well contribute to the expression of COX-2 in peri-tumoural tissue. Macrophages that generally constitute a major component of the tumour stroma have conventionally been described as anti-tumour effector cells.[16] However, they are increasingly regarded as promoters of angiogenesis, matrix breakdown and remodelling, and tumour progression.[17,18] Additionally, fibroblasts have been shown to contribute to neovessel formation by producing angiogenic factors. Capacity to produce vascular endothelial growth factor (VEGF) was reduced in Cox2 -/- fibroblasts or wild-type mouse fibroblasts that had been treated with a selective COX-2 inhibitor.[14] Thus selective COX-2 inhibitors might (also) limit the ability of macrophages and/or fibroblasts to support neovascularization. In agreement, celecoxib treatment has been shown to suppress neovascularization in tumors.[12,19] However, inhibition of vascularization might also implicate that celecoxib treatment interferes with wound healing. Roh et al.[12] showed a delay in re-epithelialization until day 7 after surgical injury, which thereafter recovered and not differed from controls at day 14 and 28. Wound healing problems were not identified in the present study at day 17.

Selective COX-2 inhibitors have recently come under enquiry because of reported association with cardiovascular harm in long-term studies in which prevention of premalignant colorectal adenomas by selective COX-2 inhibitors/celecoxib was investigated. Indeed, a recent large, randomised, placebo-controlled double-blind trial showed a dose-related enhanced risk of cardiovascular events in long-term users.[20] However, short-term clinical trials with selective COX-2 inhibitors in patients with for example arthritis and adenomatous polyposis did not increase cardiovascular risk.[21,22]

Concluding, high dose celecoxib almost completely inhibits peritoneal tumour recurrence in our rat model. Peri-operative administration as described in our model might represent a new modality in cancer therapy, which is (presumably) not accompanied by cardiovascular hazards. Further investigation should be aimed at illuminating exact mechanisms behind these promising results, especially with focus on the role of infiltrating leucocytes.

REFERENCES

1. 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 tumour cells. Ann Surg 1998; 227(2): 242-8.
2. Deelman HT. The part played by injury and repair in the development of cancer. Br Med J 1927; 1: 872.
3. Balkwill F, Mantovani A. Inflammation and cancer: Back to Virchow? Lancet 2001; 357(9255): 539-45
4. ten Raa S, Oosterling SJ, van der Kaaij NP, van den Tol MP, Beelen RH, Meijer S, van Eijck CH, van der Sijp JR, van Egmond M, Jeekel J. Surgery promotes implantation of disseminated tumour cells, but does not increase growth of tumour cell clusters. J Surg Oncol 2005; 92(2): 124-9.
5. Gupta RA, DuBois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer 2001; 1(1): 11-21.
6. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-Regulation of Cyclooxygenase 2 Gene Expression in human colorectal adenomas and adenocarcinomas. astroenterology 1994; 107(4): 1183-8.
7. Chapple KS, Cartwright EJ, Hawcroft G, Tisbury A, Bonifer C, Scott N, Windsor AC, Guillou PJ, Markham AF, Coletta PL, Hull MA. Localization of cyclooxygenase-2 in human sporadic colorectal adenomas. Am J Pathol 2000; 156(2): 545-53.
8. Masferrer JL, Leahy KM, Koki AT, Zweifel BS, Settle SL, Woerner BM, Edwards DA, Flickinger AG, Moore RJ, Seibert K. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res 2000; 60(5): 1306-11.
9. Leahy KM, Ornberg RL, Wang Y, Zweifel BS, Koki AT, Masferrer JL. Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. Cancer Res 2002; 62(3): 625-31.
10. Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, Beauchamp RD, DuBois RN. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J Clin Invest 1997; 99(9): 2254-9.
11. Williams CS, Watson AJ, Sheng H, Helou R, Shao J, DuBois RN. Celecoxib prevents tumour growth in vivo without toxicity to normal gut: Lack of correlation between in vitro and in vivo models. Cancer Res 2000; 60(21): 6045-51.
12. Roh JL, Sung MW, Park SW, Heo DS, Lee DW, Kim KH. Celecoxib can prevent tumour growth and distant metastasis in postoperative setting. Cancer Res 2004; 64(9): 3230-5.
13. Oosterling SJ, van der Bij GJ, van Egmond M, van der Sijp JR. Surgical trauma and peritoneal recurrence of colorectal carcinoma. Eur J Surg Oncol 2005; 31(1): 29-37.
14. Williams CS, Tsujii M, Reese J, Dey SK, DuBois RN. Host cyclooxygenase-2 modulates carcinoma growth. J Clin Invest 2000; 105(11): 1589-94.
15. Oosterling SJ, van der Bij GJ, Meijer GA, Tuk CW, van Garderen E, van Rooijen N, Meijer S, van der Sijp J, Beelen RH, van Egmond M. Macrophages direct tumour histology and clinical outcome in a colon cancer model. J Pathol 2005; 207(2): 147-55.
16. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: Associated-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002; 23(11): 549-55.
17. Condeelis J, Pollard JW. Macrophages: Obligate partners for tumour cell migration, invasion, and metastasis. Cell 2006; 124(2): 263-6.
18. van der Bij GJ, Oosterling SJ, Meijer S, Beelen RH, van Egmond M. The role of macrophages in tumour development. Cell Oncol 2005; 27(4): 203-13.

19. Wei D, Wang L, He Y, Xiong HQ, Abbruzzese JL, Xie K. Celecoxib inhibits vascular endothelial growth factor expression in and reduces angiogenesis and metastasis of human pancreatic cancer via suppression of sp1 transcription factor activity. Cancer Res 2004; 64(6): 2030-8.
20. Solomon SD, McMurray JJ, Pfeffer MA, Wittes J, Fowler R, Finn P, Anderson WF, Zauber A, Hawk E, Bertagnolli M. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. N Engl J Med 2005; 352(11): 1071-80.
21. Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM, Geis GS. gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib long-term arthritis safety study. JAMA 2000; 284(10): 1247-55.
22. Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 2000; 342(26): 1946-52.

Chapter 7

Introduction

The Consequences of Surgical Trauma on Intra-Peritoneal Adhesion Formation

Adapted from:
S. ten Raa, M.P. van den Tol, J. Jeekel. Intra-abdominal adhesions in the experimental setting. *Adhesions: news and views 2003; 2: 22-25*
S. ten Raa, D.J. Swank, J. Jeekel. Treatment of Complications of Abdominal Adhesions: Laparoscopy or Laparotomy? *Adhesions: news and views 2004; 5: 12-15*
H.C. van der Wal, S. ten Raa, J. Jeekel. The impact of irrigants on the peritoneal cavity. Adhesions: news and views *2005; 8: 17-19*

CLINICAL IMPACT

Intra-abdominal adhesions are fibrous or fibrovascular bands between different peritoneal surfaces. Postoperative intra-abdominal adhesion formation remains a major postoperative surgical problem and is still an unavoidable complication of any kind of abdominal surgery, though not widely accepted as a complication.[1] Postoperative adhesions occur in an average of approximately 85% patients after abdominal surgery.[2] Other causes of adhesions are due to inflammation, endometriosis or have a congenital cause. Intra-abdominal adhesions can eventuate in bowel, fertility and abdominal syndromes, which frequently require surgery.

Thompson et al.[3] showed that 36% of patients presenting with post-operative adhesion-induced bowel obstruction had undergone appendicectomy. In European countries adhesion formation causes 30% of all bowel obstructions,[4] and of 15-30% of infertility in women.[5] Adhesion formation has also been implicated as the cause of 13-26% of chronic pelvic pain in women.[6] Though Swank et al.[7] showed that laparoscopic adhesiolysis could not reduce pelvic pain. The cumulative risk of adhesive small-bowel obstruction after (sub)total colectomy is 11-25% within 1 year, increasing to 30% at 10 years.[8,9] The incidence of post-surgical adhesions increases with the number of laparotomies, and the complexity of surgical procedures.[10] Intra-abdominal adhesions increase the morbidity in subsequent surgery. Morbidity involves inadvertent enterotomy during surgery, bowel obstruction, anastomotic leak, wound dehiscence, sepsis and pneumonia.[11] The mortality rate due to adhesion related bowel obstruction is 6-13%.[12] Laparotomy or laparoscopy for adhesiolysis is the only treatment and can be a technical challenging procedure with elevated peroperative complications and significant postoperative morbidity. Besides the risks, adhesiolysis is also complicated by a high recurrence rate of adhesions.[13] Laparotomy has been the only surgical approach to bowel obstruction until 1990 after which laparoscopy was introduced. High complication rates of up to 51% and up to 5% mortality have been reported, and the effect of the adhesiolysis is often temporary.[14] Twenty-nine percent of patients had a recurrence of bowel obstruction after adhesiolysis by laparotomy.[14] Laparoscopy is not always possible because of the risk of visceral injury and because of the bowel distension that would prevent the visualization of the obstruction.[15] The success rate for laparoscopic adhesiolysis for acute small bowel obstruction ranges from 46% to 83%.[16,17] Reformation of adhesions may be minimized when performing laparoscopic adhesiolysis.[18,19]

Clearly the costs of adhesion related health care are significant.[12,20,21] In 1994, in the United States, 303.836 procedures for abdominopelvic peritoneal adhesiolysis were performed at a cost of over 1.3 billion dollars.[21]

PATHOGENESIS

Adhesion formation is a pathophysiological consequence of peritoneal tissue repair. Their development likely result from an imbalance in inflammatory mediators or fibrin degradation during peritoneal wound healing. Surgical trauma to the peritoneal surface induces a sequence of events which effectuates wound healing but which can also lead to adhesion formation.

Damage to the peritoneum causes desquamation of injured mesothelial cells leaving a denuded area, and a local inflammatory reaction, which leads to the formation of a serosanguineous fluid.[3,22] The primary inflammatory response, the acute phase response, is characterised by an increased vascular permeability and the migration of neutrophils (PMNs) and subsequently macrophages and leukocytes to the site of the inflammation.[23,24] Chemoattractants (IL-8, MCP-1), cytokines (TNF-α, IL-1β and IL-6), growth factors (TGF-β, IGF-I and PDGF) and reactive oxygen species, produced and released by damaged mesothelial cells and resident and invading inflammatory cells are important mediators of this inflammatory reaction.[25-29] Excessive or inappropriate production of free radicals is associated with morbidity and mortality after peritoneal trauma and inflammatory diseases and causes ongoing damage to the mesothelial lining. In the resulting serosanguineous fluid matrix deposition will take place through the activated complement- and coagulation systems. When two surfaces contact each other during this process a fibrinous adhesion can appear.[30,31]

Mesothelial cells play an important role in the peritoneal fibrinolytic and coagulant activity. Peritoneal injury upregulates the expression of tissue factor (TF) by mesothelial cells and macrophages.[32] TF leads to the activation of the extrinsic pathway of the coagulation cascade, eventually leading to fibrin formation. Under normal circumstances there is a balance between fibrinogenesis and fibrinolysis; the latter involves degradation from fibrin into its degradation products. This process is driven by the enzyme plasmin, which is derived from its inactive substrate plasminogen by tissue-type plasminogen activator (tPA). On its turn, tPA is inhibited in its reaction by plasminogen activator inhibitor-1 (PAI-1), in order to keep the balance. In the abdominal cavity, tPA is responsible for 95% of the plasminogen conversion.[33] Intra-abdominal surgery disturbs the balance between tPA and PAI-1 resulting in a decreased fibrinolytic activity, an increase in fibrin exudate and eventually an increase in adhesion formation.[34] The matrix deposition itself is not only directly related to the occurrence of fibrinous adhesion through the coagulation cascade, but also plays an important role in the inflammatory response and in the development of fibrinous adhesions to organized fibroblast populated adhesions.[35] The main components (fibronectin, glycosaminoglycans, proteoglycans, collagenases, matrix metalloproteinases) of the matrix deposition function as binding sites for inflammatory cells and fibroblasts, participate in presenting growth factors and provide chemotacic signals to various cell types (e.g. fibroblasts, macrophages). After

the initial response to the peritoneal trauma the fibrin matrix is either degraded or is gradually replaced by collagen, concurrent with its population by fibroblastic and mesothelial cells.

The results of the inflammatory and fibrinogenetic response to peritoneal trauma should result in peritoneal wound healing, but unfortunately can also result in post-surgical adhesion formation.

PREVENTION

As shown above surgery for treatment of complications of intra-abdominal adhesions carries an increased peroperative complication risk with a high recurrence rate, without effect on pain and does not prevent future small bowel obstruction.[1,4,36] Prevention of adhesion formation should therefore be the focus of attention. Peritoneal trauma is the basis of adhesion formation after intra-abdominal surgery. The extent of adhesion formation correlates significantly with the degree of peritoneal damage.[37]

Many chemical agents and procedures to prevent or reduce the formation of postoperative adhesions have been studied. The purpose of all studies has been to interfere with one or several of the pathogenetic steps in the process of peritoneal healing and adhesion formation.

Attempts at decreasing adhesion formation by specifically altering macrophage function or reducing the influx of anti-inflammatory cells have been considered. However the role of the macrophage in adhesion formation is still not fully clarified. Zeyneloglu et al.[38] reduced adhesion formation by reducing the number of macrophages infiltrating the traumatised peritoneal surface by using neutralising antibodies to monocyte chemotactic protein-1 (MCP-1). In contrast Ar'Rajab et al.[39] showed that enhancement of peritoneal macrophages reduce postoperative peritoneal adhesion formation. A role of neutrophils in adhesion formation has also been suggested.[40-42] Mast cells have also been investigated.[43-45] In general the experiments have shown a decrease in adhesion formation when mast cell function was influenced, either by stimulating or blocking the production of mast cells, stabilising the mast cell or blocking its products. It is too early to know whether these experiments will lead to a treatment option in humans, but to focus on the cellular constituents of the inflammatory reaction and their role during wound healing appears to be a worthwhile avenue of investigation.

Efforts to influence the inflammatory reaction by mastering the mediators of this process have also been suggested as an adhesion-abating alternative.[46-48] Experimental studies showed a decrease of adhesion formation after administration of antibodies against the cytokines TGF-β, IL-1 and IL-10.[49-52] A significant correlation between the severity of adhesion formation and levels of TNF-α in serum and peritoneal fluid were found in a rat-experiment.[53] Again, it is not certain that these results can be repeated in clinical studies, but

selective immune suppression, at a molecular level, might be part of future solutions to the problem of postoperative adhesion formation.

Attempts at modifying the components of the matrix deposition have resulted in various results. Unlike expected, inhibiting matrix metalloproteinases did not effect adhesion formation,[54] though an association was found between the expression of matrix metalloproteinases and adhesion formation.[55,56] Other approaches included local absorbable barriers (e.g. seprafilm) preventing damaged peritoneal surfaces to oppose. Seprafilm is a bioresorbable membrane consisting of hyaluronic acid (a member of the glycosaminoglycans) and carboxymethylcellulose. The use of seprafilm in animal as well as in human studies could reduce post-surgical adhesions. Although the incidence of adhesions or the incidence of small bowel obstruction did not change when using seprafilm in the clinical setting.[57,58] However the severity of adhesions was less and seprafilm is the only anti-adhesive agent, which has shown to be able to reduce the number of operations for small bowel obstruction in a randomised controlled trial.[57,58] The main disadvantage of local absorbable barriers is that surgeons have to predict the sites at which they consider adhesions are likely to develop.

More easily and less user dependent would be liquid solutions. Liquids are thought to have their anti-adhesive properties through two mechanisms; a barrier function through hydroflotation or coating of the peritoneal surface and/or through dilution of fibrin and its products in the peritoneal cavity. Main concerns on this topic are peritoneal absorption rate and side effects. Crystalloid solutions including lactated Ringer's solution, saline 0.9% solution and phosphate buffered saline solution showed ineffective in preventing adhesion formation.[59] Mainly because these crystalloid solutions are absorbed within 24 hours of instillation. Macromolecular solutions showed more promising results. 32% dextran 70 (Hyskon) was marginally effective, but clinical side effects such as ascites, pleural effusion and liver function abnormalities, rendered it useless as anti-adhesive agent.[59,60] Sepracoat and intergel significantly decreased post-surgical adhesion formation in animal studies as well as in human.[61-63] Unfortunately intergel was withdrawn from use, because of unacceptable side effects. Icodextrin is an α-1,4 glucose polymer of high molecular weight, which is rapidly metabolised to glucose by the α-amylase present in the systemic circulation, but is absorbed only slowly from the peritoneal cavity. Adept is an iso-osmolar 4% solution of icodextrin, having an intraperitoneal residence time of at least 4 days. Animal studies demonstrated significant reduction in post-surgical adhesion formation and re-formation.[64-66] A pilot study in human did also show promising results.[67,68]

The ideal anti-adhesive agent should be a material that persists during the critical remesothelialisation phase, remains active in the presence of blood and should be completely biodegradable. In addition it should not interfere with healing nor promote infection, it should have carrier qualities and easy to use, without depending on the prediction

of the surgeon. At present icodextrin seems to be most promising as liquid anti-adhesive agent.

REFERENCES

1. Holmdahl L. Making and covering of surgical footprints. Lancet 1999;353:1456-7.
2. Menzies D, Ellis H. Intestinal obstruction from adhesions--how big is the problem? Ann R Coll Surg Engl 1990;72:60-3.
3. Thompson J. Pathogenesis and prevention of adhesion formation. Dig Surg 1998;15:153-7.
4. Menzies D. Peritoneal adhesions. Incidence, cause, and prevention. Surg Annu 1992;24:27-45.
5. Milingos S, Kallipolitis G, Loutradis D, Liapi A, Mavrommatis K, Drakakis P, Tourikis J, Creatsas G, Michalas S. Adhesions: laparoscopic surgery versus laparotomy. Ann N Y Acad Sci 2000;900:272-85.
6. Rapkin AJ, Mayer EA. Gastroenterologic causes of chronic pelvic pain. Obstet Gynecol Clin North Am 1993;20:663-83.
7. Swank DJ, Swank-Bordewijk SC, Hop WC, van Erp WF, Janssen IM, Bonjer HJ, Jeekel J. Laparoscopic adhesiolysis in patients with chronic abdominal pain: a blinded randomised controlled multi-centre trial. Lancet 2003;361:1247-51.
8. Parker MC, Ellis H, Moran BJ, Thompson JN, Wilson MS, Menzies D, McGuire A, Lower AM, Hawthorn RJ, O'Briena F, et al. Postoperative adhesions: ten-year follow-up of 12,584 patients undergoing lower abdominal surgery. Dis Colon Rectum 2001;44:822-29; discussion 29-30.
9. Nieuwenhuijzen M, Reijnen MM, Kuijpers JH, van Goor H. Small bowel obstruction after total or subtotal colectomy: a 10-year retrospective review. Br J Surg 1998;85:1242-5.
10. DeCherney AH, diZerega GS. Clinical problem of intraperitoneal postsurgical adhesion formation following general surgery and the use of adhesion prevention barriers. Surg Clin North Am 1997;77:671-88.
11. Van Der Krabben AA, Dijkstra FR, Nieuwenhuijzen M, Reijnen MM, Schaapveld M, Van Goor H. Morbidity and mortality of inadvertent enterotomy during adhesiotomy. Br J Surg 2000;87:467-71.
12. Jeekel H. Cost implications of adhesions as highlighted in a European study. Eur J Surg Suppl 1997;579:43-5.
13. Barkan H, Webster S, Ozeran S. Factors predicting the recurrence of adhesive small-bowel obstruction. Am J Surg 1995;170:361-5.
14. Landercasper J, Cogbill TH, Merry WH, Stolee RT, Strutt PJ. Long-term outcome after hospitalization for small-bowel obstruction. Arch Surg 1993;128:765-70; discussion 70-1.
15. Bonjer HJ, Hazebroek EJ, Kazemier G, Giuffrida MC, Meijer WS, Lange JF. Open versus closed establishment of pneumoperitoneum in laparoscopic surgery. Br J Surg 1997;84:599-602.
16. Navez B, Arimont JM, Guiot P. Laparoscopic approach in acute small bowel obstruction. A review of 68 patients. Hepatogastroenterology 1998;45:2146-50.
17. Sato Y, Ido K, Kumagai M, Isoda N, Hozumi M, Nagamine N, Ono K, Shibusawa H, Togashi K, Sugano K. Laparoscopic adhesiolysis for recurrent small bowel obstruction: long-term follow-up. Gastrointest Endosc 2001;54:476-9.
18. Tittel A, Treutner KH, Titkova S, Ottinger A, Schumpelick V. New adhesion formation after laparoscopic and conventional adhesiolysis: a comparative study in the rabbit. Surg Endosc 2001;15:44-6.
19. Tittel A, Treutner KH, Titkova S, Ottinger A, Schumpelick V. Comparison of adhesion reformation after laparoscopic and conventional adhesiolysis in an animal model. Langenbecks Arch Surg 2001;386:141-5.
20. Ellis H, Moran BJ, Thompson JN, Parker MC, Wilson MS, Menzies D, McGuire A, Lower AM, Hawthorn RJ, O'Brien F, et al. Adhesion-related hospital readmissions after abdominal and pelvic surgery: a retrospective cohort study. Lancet 1999;353:1476-80.
21. Ray NF, Denton WG, Thamer M, Henderson SC, Perry S. Abdominal adhesiolysis: inpatient care and expenditures in the United States in 1994. J Am Coll Surg 1998;186:1-9.
22. Milligan DW, Raftery AT. Observations on the pathogenesis of peritoneal adhesions: a light and electron microscopical study. Br J Surg 1974;61:274-80.

23. Ward PA, Lentsch AB. The acute inflammatory response and its regulation. Arch Surg 1999;134:666-9.
24. Raftery AT. Regeneration of parietal and visceral peritoneum. A light microscopical study. Br J Surg 1973;60:293-9.
25. Lanfrancone L, Boraschi D, Ghiara P, Falini B, Grignani F, Peri G, Mantovani A, Pelicci PG. 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;80:2835-42.
26. Topley N. The cytokine network controlling peritoneal inflammation. Perit Dial Int 1995;15:S35-9; discussion S39-40.
27. Li FK, Davenport A, Robson RL, Loetscher P, Rothlein R, Williams JD, Topley N. Leukocyte migration across human peritoneal mesothelial cells is dependent on directed chemokine secretion and ICAM-1 expression. Kidney Int 1998;54:2170-83.
28. 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:S17-21.
29. Badia JM, Ayton LC, Evans TJ, Carpenter AJ, Nawfal G, Kinderman H, Zografos G, Uemoto S, Cohen J, Habib NA. Systemic cytokine response to hepatic resections under total vascular exclusion. Eur J Surg 1998;164:185-90.
30. Thompson JN, Whawell SA. Pathogenesis and prevention of adhesion formation. Br J Surg 1995;82:3-5.
31. diZerega GS. Biochemical events in peritoneal tissue repair. Eur J Surg Suppl 1997;577:10-6.
32. Sitter T, Toet K, Fricke H, Schiffl H, Held E, Kooistra T. Modulation of procoagulant and fibrinolytic system components of mesothelial cells by inflammatory mediators. Am J Physiol 1996;271:R1256-63.
33. Holmdahl L, Falkenberg M, Ivarsson ML, Risberg B. Plasminogen activators and inhibitors in peritoneal tissue. Apmis 1997;105:25-30.
34. Scott-Coombes D, Whawell S, Vipond MN, Thompson J. Human intraperitoneal fibrinolytic response to elective surgery. Br J Surg 1995;82:414-7.
35. Boland GM, Weigel RJ. Formation and prevention of postoperative abdominal adhesions. J Surg Res 2006;132:3-12.
36. Menzies D, Parker M, Hoare R, Knight A. Small bowel obstruction due to postoperative adhesions: treatment patterns and associated costs in 110 hospital admissions. Ann R Coll Surg Engl 2001;83:40-6.
37. van den Tol MP, van Stijn I, Bonthuis F, Marquet RL, Jeekel J. Reduction of intraperitoneal adhesion formation by use of non-abrasive gauze. Br J Surg 1997;84:1410-5.
38. Zeyneloglu HB, Seli E, Senturk LM, Gutierrez LS, Olive DL, Arici A. The effect of monocyte chemotactic protein 1 in intraperitoneal adhesion formation in a mouse model. Am J Obstet Gynecol 1998;179:438-43.
39. Ar'Rajab A, Dawidson I, Sentementes J, Sikes P, Harris R, Mileski W. Enhancement of peritoneal macrophages reduces postoperative peritoneal adhesion formation. J Surg Res 1995;58:307-12.
40. 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;61:143-6.
41. Braslow L. Neutrophilia, oxygen free radicals, and abdominal adhesions. Arch Surg 1992;127:747.
42. Vural B, Canturk NZ, Esen N, Solakoglu S, Canturk Z, Kirkali G, Sokmensuer C. The role of neutrophils in the formation of peritoneal adhesions. Hum Reprod 1999;14:49-54.
43. Xu X, Pappo O, Garbuzenko E, Bischoff SC, Rivkind A, Levi-Schaffer F. Mast cell dynamics and involvement in the development of peritoneal adhesions in the rat. Life Sci 2002;70:951-67.
44. Yao YL, Ishihara T, Takai S, Miyazaki M, Mita S. Association between the expression of mast cell chymase and intraperitoneal adhesion formation in mice. J Surg Res 2000;92:40-4.
45. Okamoto Y, Takai S, Miyazaki M. Chymase inhibitor suppresses adhesion formation in a hamster experimental model. Eur J Pharmacol 2002;435:265-7.

46. 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;165:1024-30.
47. Holmdahl L, Ivarsson ML. The role of cytokines, coagulation, and fibrinolysis in peritoneal tissue repair. Eur J Surg 1999;165:1012-9.
48. Badia JM, Whawell SA, Scott-Coombes DM, Abel PD, Williamson RC, Thompson JN. Peritoneal and systemic cytokine response to laparotomy. Br J Surg 1996;83:347-8.
49. Lucas PA, Warejcka DJ, Young HE, Lee BY. Formation of abdominal adhesions is inhibited by antibodies to transforming growth factor-beta1. J Surg Res 1996;65:135-8.
50. Saba AA, Kaidi AA, Godziachvili V, Dombi GW, Dawe EJ, Libcke JH, Silva YJ. Effects of interleukin-6 and its neutralizing antibodies on peritoneal adhesion formation and wound healing. Am Surg 1996;62:569-72.
51. Kaidi AA, Nazzal M, Gurchumelidze T, Ali MA, Dawe EJ, Silva YJ. Preoperative administration of antibodies against tumor necrosis factor- alpha (TNF-alpha) and interleukin-1 (IL-1) and their impact on peritoneal adhesion formation. Am Surg 1995;61:569-72.
52. Montz FJ, Holschneider CH, Bozuk M, Gotlieb WH, Martinez-Maza O. Interleukin 10: ability to minimize postoperative intraperitoneal adhesion formation in a murine model. Fertil Steril 1994;61:1136-40.
53. Kaidi AA, Gurchumelidze T, Nazzal M, Figert P, Vanterpool C, Silva Y. Tumor necrosis factor-alpha: a marker for peritoneal adhesion formation. J Surg Res 1995;58:516-8.
54. Mirastschijski U, Johannesson K, Jeppsson B, Agren MS. Effect of a matrix metalloproteinase activity and TNF-alpha converting enzyme inhibitor on intra-abdominal adhesions. Eur Surg Res 2005;37:68-75.
55. Chegini N, Kotseos K, Bennett B, Diamond MP, Holmdahl L, Burns J. Matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP in peritoneal fluids and sera and correlation with peritoneal adhesions. Fertil Steril 2001;76:1207-11.
56. Chegini N, Kotseos K, Zhao Y, Ma C, McLean F, Diamond MP, Holmdahl L, Burns J. Expression of matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP in serosal tissue of intraperitoneal organs and adhesions. Fertil Steril 2001;76:1212-9.
57. Fazio VW, Cohen Z, Fleshman JW, van Goor H, Bauer JJ, Wolff BG, Corman M, Beart RW, Jr., Wexner SD, Becker JM, et al. Reduction in adhesive small-bowel obstruction by Seprafilm adhesion barrier after intestinal resection. Dis Colon Rectum 2006;49:1-11.
58. Vrijland WW, Tseng LN, Eijkman HJ, Hop WC, Jakimowicz JJ, Leguit P, Stassen LP, Swank DJ, Haverlag R, Bonjer HJ, et al. Fewer intraperitoneal adhesions with use of hyaluronic acid-carboxymethylcellulose membrane: a randomised clinical trial. Ann Surg 2002;235:193-9.
59. Wiseman DM, Trout JR, Diamond MP. The rates of adhesion development and the effects of crystalloid solutions on adhesion development in pelvic surgery. Fertil Steril 1998;70:702-11.
60. diZerega GS. Contemporary adhesion prevention. Fertil Steril 1994;61:219-35.
61. Burns JW, Skinner K, Colt J, Sheidlin A, Bronson R, Yaacobi Y, Goldberg EP. Prevention of tissue injury and postsurgical adhesions by precoating tissues with hyaluronic acid solutions. J Surg Res 1995;59:644-52.
62. Diamond MP. Reduction of de novo postsurgical adhesions by intraoperative precoating with Sepracoat (HAL-C) solution: a prospective, randomised, blinded, placebo-controlled multicenter study. The Sepracoat Adhesion Study Group. Fertil Steril 1998;69:1067-74.
63. Johns DB, Keyport GM, Hoehler F, diZerega GS. Reduction of postsurgical adhesions with Intergel adhesion prevention solution: a multicenter study of safety and efficacy after conservative gynecologic surgery. Fertil Steril 2001;76:595-604.
64. Rodgers KE, Verco SJ, diZerega GS. Effects of intraperitoneal 4% icodextrin solution on the healing of bowel anastomoses and laparotomy incisions in rabbits. Colorectal Dis 2003;5:324-30.

65. Muller SA, Treutner KH, Anurov M, Titkova S, Oettinger AP, Schumpelick V. Experimental evaluation of phospholipids and icodextrin in re-formation of peritoneal adhesions. Br J Surg 2003;90:1604-7.
66. Verco SJ, Peers EM, Brown CB, Rodgers KE, Roda N, diZerega G. Development of a novel glucose polymer solution (icodextrin) for adhesion prevention: pre-clinical studies. Hum Reprod 2000;15:1764-72.
67. diZerega GS, Verco SJ, Young P, Kettel M, Kobak W, Martin D, Sanfilippo J, Peers EM, Scrimgeour A, Brown CB. A randomised, controlled pilot study of the safety and efficacy of 4% icodextrin solution in the reduction of adhesions following laparoscopic gynaecological surgery. Hum Reprod 2002;17:1031-8.
68. Menzies D, Pascual MH, Walz MK, Duron JJ, Tonelli F, Crowe A, Knight A. Use of icodextrin 4% solution in the prevention of adhesion formation following general surgery: from the multicentre ARIEL Registry. Ann R Coll Surg Engl 2006;88:375-82.

Chapter 8

The Role of Neutrophils and Oxygen Free Radicals in Post-Operative Adhesions

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ABSTRACT

Background:

Postoperative intra-abdominal adhesion formation remains a major surgical problem. Surgery induces an inflammatory reaction, which is responsible for adhesion formation. Neutrophils and their oxygen-free radicals are key mediators in the early post-operative inflammatory response. The present study evaluates the effect of either blocking the influx of neutrophils or its products by scavenging oxygen-free radicals on adhesion formation.

Materials and methods:

Reproducible rat models were used to induce post-surgical intra-abdominal adhesions. In the first experiment anti-neutrophil serum (ANS) was used to prevent neutrophils from entering the peritoneal cavity after surgery. In a second experiment superoxide dismutase (SOD), catalase and mannitol were tested, to scavenge the superoxide, hydrogen peroxide and hydroxyl radicals, respectively.

Results:

In positive control groups 69-76% of the area of interest contained adhesions. In all experimental groups, except for mannitol, a significant reduction in post-surgical adhesion formation could be achieved. ANS reduced adhesion formation by 38% ($p<0.001$) and SOD/catalase by 42% ($p<0.01$). Mannitol could not reduce adhesion formation.

Conclusions:

Intra-abdominal influx of neutrophils after surgical peritoneal trauma plays an important role in post-operative adhesion formation. Preventing the intra-abdominal influx of neutrophils in the early post-operative inflammatory reaction can reduce adhesion formation, but an even more selective approach, by scavenging its products, proved as efficient.

INTRODUCTION

Intra-abdominal adhesions are abnormal unions between different peritoneal surfaces. Postoperative intra-abdominal adhesion formation remains a major postoperative surgical problem and is still an unavoidable complication of any kind of abdominal surgery, though not widely recognized.[1] Postoperative adhesions occur in an average of approximately 85% (55-100%) of patients undergoing abdominal surgery.[2-4] The mortality rate from adhesion related bowel obstruction is 6-15%.[5,6] Clearly the costs of adhesion related health care are significant.[6-8] Until now there is no clinically relevant cost effective method available to prevent adhesion formation and further research is needed.

Adhesion formation is a physiological consequence of peritoneal tissue repair. Surgical trauma of the peritoneal surfaces induces a sequence of events which effectuates wound healing but which can also ultimately lead to adhesion formation.[9] Surgery induces an inflammatory reaction which is characterized by migration of neutrophils, macrophages and other leukocytes to the site of the inflammation during the first 48 to 72 hours.[10] Chemoattractants (IL-8, MCP-1), cytokines (TNF-α, IL-1β and IL-6), growth factors (TGF-β, IGF-I and PDGF) and reactive oxygen species (ROS), produced and released by resident and invading inflammatory cells and subsequent damaged mesothelial cells are the key mediators of this inflammatory reaction.[11,12]

Attempts at decreasing adhesion formation by reducing the influx of inflammatory cells has been considered.[13-16] Vural et al.[16] showed beneficial effects of cyclophosphamide on adhesion formation, by significant reduction of neutrophil counts and already discussed the bioacceptance and usefulness of these factors in the formation of post-operative abdominal adhesions. Efforts to influence the inflammatory reaction by mastering the mediators of this process have also been suggested as an adhesion-abating alternative with promising results.[17-20]

The present study will further explore the role of neutrophils and ROS in the post-operative intra-abdominal formation of adhesions. Although at present it cannot be predicted whether these experiments will lead to a treatment option in humans, focusing on the cellular constituents of the inflammatory reaction and their products appears to be a worthwhile avenue of investigation.

MATERIALS AND METHODS

Animals

Adult female inbred WAG/Rij rats weighing 145-190g were obtained from Harlan-CPB, Austerlitz, The Netherlands. The rats were bred under specific pathogen-free conditions. The animals were kept under standard laboratory conditions (temperature 20-24ºC, relative

humidity 50-60%, 12 hours light and 12 hours dark cycles), fed with standard rat food and water *ad libitum* and quarantined in our University facilities for at least two days prior to use. The experimental protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC.

Mesothelial cell culture

Human mesothelial cells were isolated from omental tissue of patients undergoing elective abdominal surgery for non-infectious reasons. A specimen of bowel omental tissue was cut in smaller pieces and washed with Hanks' Balanced Salt Solution (HBSS) containing 0.5% human serum albumin. After washing three times the specimens were centrifuged for 5 minutes at 580 xg. The omentum was transferred to a solution containing trypsin (0.05%) and EDTA (0.02%) and incubated for 20 minutes at 37°C under continuous shacking. After 20 minutes the detached mesothelial cells were pelleted down by centrifugation at 580 xg for 10 minutes. After centrifugation the mesothelial cells were resuspended in culture medium (RPMI 1640 containing 10% fetal calf serum, glutamine (2mM) and penicillin (10^5 U/L)). The mesothelial cells were cultured at 37°C, in a fully humidified, 5% CO_2 cabinet in polystyrene 75cm^2 culture flasks pre coated with collagen type I (Roche Diagnostics, Almere, the Netherlands). The mesothelial origin of the cells was verified by their typical cobblestone appearance by phase-contrast microscopy and by immunohystochemical characterization with positive staining of keratin (anti-serum Z622; DAKO, Heverlee, the Netherlands) and vimentin (anti-serum L1843; DAKO, Heverlee, the Netherlands) and negative staining of von Willebrand factor (F8/86 antibody; DAKO, Heverlee, the Netherlands).

Neutrophil isolation

Neutrophils were isolated using PolymorphPrep™ (Nycomed, Oslo, Norway). Briefly, blood was obtained from healthy donors by vena puncture using 7 ml heparin-coated tubes (BD Vacutainer Systems, Plymouth, United Kingdom). 5 ml of anticoagulated blood was layered on top of 5 ml of PolymorphPrep™ (Nycomed, Oslo, Norway) in a 15 ml polypropylene tube that was then spun at 460 x*g* for 32 min at room temperature. The layer of neutrophils was removed using a Pasteur pipette and placed with an equal volume of hypertonic sodium chloride solution (0.45% w/v) in another 15 ml tube to reduce viscosity. Following another spin, the supernatant was aspirated and the pellet resuspended in red cell lysis buffer, to remove excess erythrocytes, and centrifuged again. The pellet was resuspended in a small volume of culture medium, and the cells were counted and checked for viability by the exclusion of 0.02% (v/v) trypan blue and used directly. The degree of purity of neutrophils was 98% and the viability was greater than 95%. Next, the neutrophil concentration was adjusted with culture medium to 1 x 10^6/ml. Formyl-methionyl-leucyl phenylalanine (fMLP)

was added to the culture medium in a final concentration of 1 x 10^{-6} M to stimulate the neutrophils, just before the start of the experiments. One ml was added to a monolayer of mesothelial cells for 12 hours to examine the effects of fMLP-stimulated neutrophils on the mesothelial monolayer.

Drugs

For in-vitro experiments, the mesothelial cell cultures were exposed to the reactive oxygen species (ROS) superoxide and hydrogen peroxide generated by xanthine oxidase (0.05 U/ml, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) upon the addition of xanthine (0.1 mmol/l, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) (X/XO). Amounts used were calculated to equal the production of superoxide as produced by 1 x 10^6 PMN stimulated with fMLP (using the ferricytochrome C reduction technique (ferricytochrome C assay).

Superoxide dismutase (SOD) (5000 U/mg) and catalase (2350 U/mg) from Roche Diagnostics BV, Almere, the Netherlands, and d-mannitol (M-4125) from Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands, were dissolved in phosphate buffered saline (PBS) to the appropriate concentration and kept on ice to scavenge the ROS in vivo. SOD (400U/ml) and catalase (200U/ml) were also added to the mesothelial cell cultures (Non-toxic concentrations were used, achieved by proliferation assay).

Polyclonal rabbit anti-rat neutrophil serum (ANS) (Accurate, Westbury, NY, USA) was used to deplete blood neutrophils. It has been shown that ANS leads to a decrease in blood neutrophil numbers of 99.9% when administered intra-peritoneally in a dose of 2 ml per kg bodyweight.[21,22] Since this dose ANS is not specific for neutrophils only, but decreased the number of blood monocytes, lymphocytes and, to a lesser extent, the platelets decrease as well,[22] in our experiments rats were given a dose of 1 ml ANS per kg bodyweight by intra-peritoneal injection 1 day before laparotomy (day –1).

Ferricytochrome C Assay

Superoxide generation by PMN and xanthine/xanthine oxidase (X/XO) was determined using the ferricytochrome C reduction techniques modified from Leslie et al.[23] In brief, ferricytochrome C (cytc) was prepared in HBSS without phenol red and used at a final concentration of 150µM. All solutions were pre-warmed to 37ºC. The assay was performed as six-fold replicates by filling the wells in vertical rows. Cytc with X/XO (0.1 mmol/l xanthine with 0.05 U/ml xanthine oxidase) or cytc with 1 x 10^6 PMN stimulated with fMLP (both conditions in 200 µl/well) were distributed in the rows. Immediately after adding the xanthine oxidase and fMLP the plate was read t=0 with t=360 minutes as endpoint. The plate was kept at a constant temperature of 37ºC. The amount of cytc reduction at 37°C was determined by taking kinetic measurements of absorbance at 540 and 550 nm using a plate reader. The

cumulative superoxide production is shown as optical density (OD) by deducting the OD at 550 nm from the OD measured at 540 nm.

Proliferation assay

To establish the protective effect of scavengers to ROS, X/XO with or without SOD/catalase was added to growing mesothelial cell cultures (n = 4 wells/condition). Growth curves were made using a DNA measurement. Mesothelial cells were trypsinized and seeded in 24 well plates in a concentration of 50.000 cells per well. X/XO with or without SOD/catalase were added directly after seeding of the mesothelial cells. At 1, 2 and 3 days the plates were washed twice with saline and stored at -20°C until further analysis. The cells were extracted with ammonia solution (1mmol/L) – Triton X100 (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 mixed with Hoechst dye H33258 (100µg/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 was referenced to a standard curve of calf thymus DNA (type II, no D-3636; Sigma, Zwijndrecht, the Netherlands).

PAI-1 and tPA assays

To investigate the influence of ROS on the fibrinolytic properties of human mesothelial cells, plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (tPA) levels were measured in resting mesothelial cells (control) and after 12 hours of stimulation with X/XO with or without SOD/catalase (n = 6 wells/condition). The PAI-1 elisa kit from Technoclone, Surrey, United Kingdom and the tPA Tintelize from Biopool, Trinity biotech, Ireland were used.

Measurement of lactate dehydrogenase release

Mesothelial cell toxicity was assessed by measurement of lactate dehydrogenase (LDH) release. Mesothelial cells were seeded into 24-well culture plates and grown to confluent monolayers. Thereafter, the medium was removed, and the cells were incubated for 12 hours with culture medium with or without X/XO (n = 6 wells/condition). After incubation the medium was collected and centrifuged (800g) to remove dead cells. LDH activity in this medium and in cell lysate was assayed spectrophotometrically using the ELAN autoanalyser (Merck & Co.). The LDH-release was expressed as percentage of total cellular enzyme activity liberated from the cells by 1% Triton-X100.

Apoptosis assay

To establish whether overnight incubation of the mesothelial monolayer with culture medium with or without X/XO could induce apoptosis, a cell death detection ELISA-kit (Roche

Diagnostics BV, Almere, the Netherlands), was used (n = 6 wells/condition). The assay is based on a quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against human DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates.

Adhesion experiment I

To investigate the influence of ANS treatment on intra-abdominal neutrophil cell count and on the rat immune system the following procedures were performed. Under anaesthesia 50 rats underwent a laparotomy. In 25 rats standardised severe peritoneal trauma was inflicted by rubbing both exposed uterus horns and a 5 cm long part of the small intestine with surgical Medipres gauze. In 25 rats (group B) severe peritoneal trauma was inflicted after a single intra-peritoneal injection of ANS on day -1. After 5 hours, 72 hours, 96 hours, 144 hours and 192 hours 5 rats of each group were operated for the second time. During this second laparotomy the abdominal cavity was lavaged with 5 ml RPMI 1640 medium. After massaging the abdomen the remaining fluid was aspirated and individually kept on ice until further processing.

Blood samples were obtained by cardiac puncture.

Adhesion experiment II and III

To investigate whether treatment with ANS or scavengers interferes with adhesion formation previously described reproducible rat adhesion models were used[24,25] In experiment II a standardised amount of minimal peritoneal trauma was inflicted in 10 rats (mild trauma group). Severe peritoneal trauma was inflicted in 20 rats (of which 10 received an intraperitoneal (i.p.) injection of ANS the day before surgery) (severe-trauma group). Severe trauma was created by rubbing the peritoneum with a device enabling the application of a constant pressure of 120 g/cm^2.[24] The abdomen was closed in two layers with 5-0 polyglycolic acid sutures (Safil®, Braun, Melsungen, Germany).

To investigate whether treatment with the scavengers SOD and catalase or mannitol interferes with adhesion formation a third experiment was performed. In all rats standardized peritoneal trauma was inflicted. At the end of the operation rats were randomly assigned to one of the treatment groups; in 8 rats 2 ml of PBS i.p. (control), in 8 rats 2 ml of a combination of SOD and catalase i.p. (1250 U/ml and 2500 U/ml respectively) (SOD/catalase) or in 10 rats 2 ml of a 5% mannitol solution i.p. (mannitol).

Two weeks after surgery, the rats were sacrificed for assessment of intra-abdominal adhesion formation. Macroscopically the adhesions were scored according to their extent (quantity) by two independent observers.[24,25]

Statistical analysis

Means and standard error of the mean (SEM) were calculated. Because the data in all experiments were normally distributed, they were statistically analysed using the one-way ANOVA test to determine overall differences. If the ANOVA test was significant on a 5% level, the Student Newman Keuls *post hoc* test was carried out to make a comparison between groups. Statistical significance was defined as $p < 0.05$.

RESULTS

Ferricytochrome C assay

The assay is based on the turnover of ferric(Fe^{3+}) iron to ferrous (Fe^{2+}) iron by superoxide. The turnover is a measure of the cumulative production of superoxide and is expressed as the optical density. The optical density was measured over a period of 360 minutes. Similar OD were reached with X/XO compared to 1 x 10^6 PMN stimulated with fMLP (0.61 (SD 0.02) and 0.59 (SD 0,02), respectively after 360 minutes). Indicating a similar superoxide production in both conditions (fig. 1).

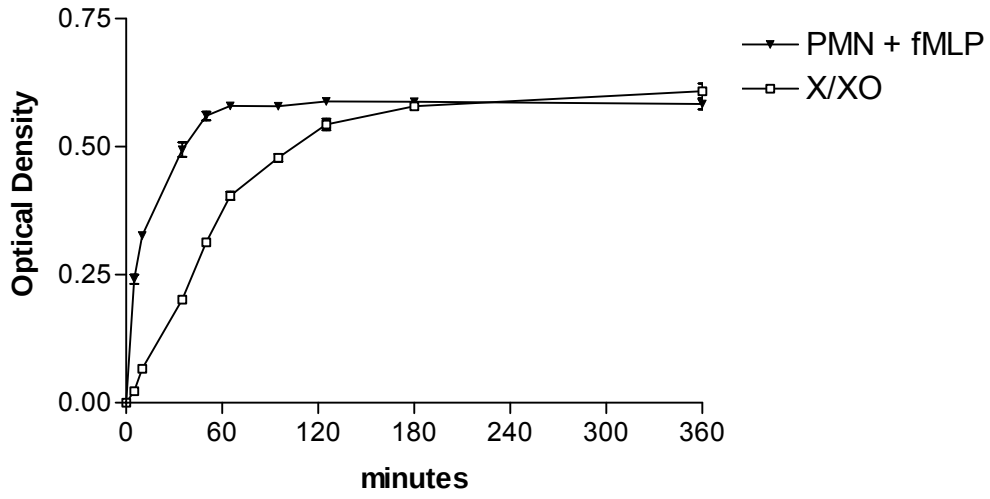

Figure 1. Superoxide production by X/XO (-□-) or 1 x 10^6 PMN stimulated by 1 x 10^{-6} M fMLP (-▲ -). Cumulative superoxide production is displayed by the optical density ± SEM (n=6/condition).

Proliferation assay

Based on the results (data not shown) of growth curves made using different seeding densities of mesothelial cells and different concentrations of FCS the ideal culture conditions for studying growth stimulation and inhibition was established. In the non-stimulated control

group the amount of DNA gradually increased from day 0 to day 3 of culture ($p < 0,001$). Indicating that the mesothelial cells are actively proliferating during this period (fig. 2). Adding X/XO to the growing mesothelial cells resulted in a markedly reduced number of mesothelial cells compared to the control group ($p < 0,0001$ at all time points). But when the scavengers SOD and catalase are added to the cultures together with X/XO the number of mesothelial cells is not disturbed (fig. 2), indicating that X/XO itself is not responsible for the reduction, but the ROS it produces. The effect of X/XO on the number of growing mesothelial cells will partially be due to the toxic and apoptotic effect of X/XO.

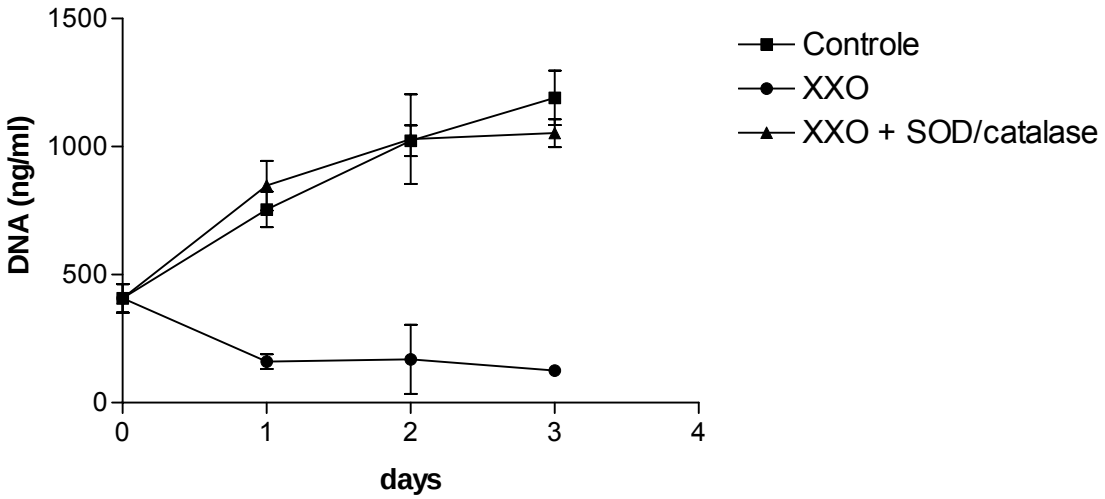

Figure 2. Effect of ROS on the growth rate of mesothelial cells. The mesothelial cells were seeded at 50,000 cells per well in quadruple wells/condition/day and cultured during 3 days in complete culture medium only (-■-), or in the presence of X/XO (-●-) or X/XO with SOD/catalase (-▲ -).

PAI-1 and tPA assays

Figure 3 shows a marked increase in PAI-1 after stimulation of human mesothelial cells with X/XO, which could be reduced by adding SOD/catalase. No significant change in release of tPA by mesothelial cells was seen upon stimulation with X/XO (fig. 3). These results suggest a decrease in the fibrinolytic capacity of mesothelial cells when X/XO is added, which can be reduced by adding SOD/catalase.

LDH-release

Cell toxicity was assessed by measuring LDH-release after exposing the mesothelial cells to X/XO or its components. As shown in figure 4 X/XO has a direct toxic effect on the mesothelial cells after 12 hours of exposure.

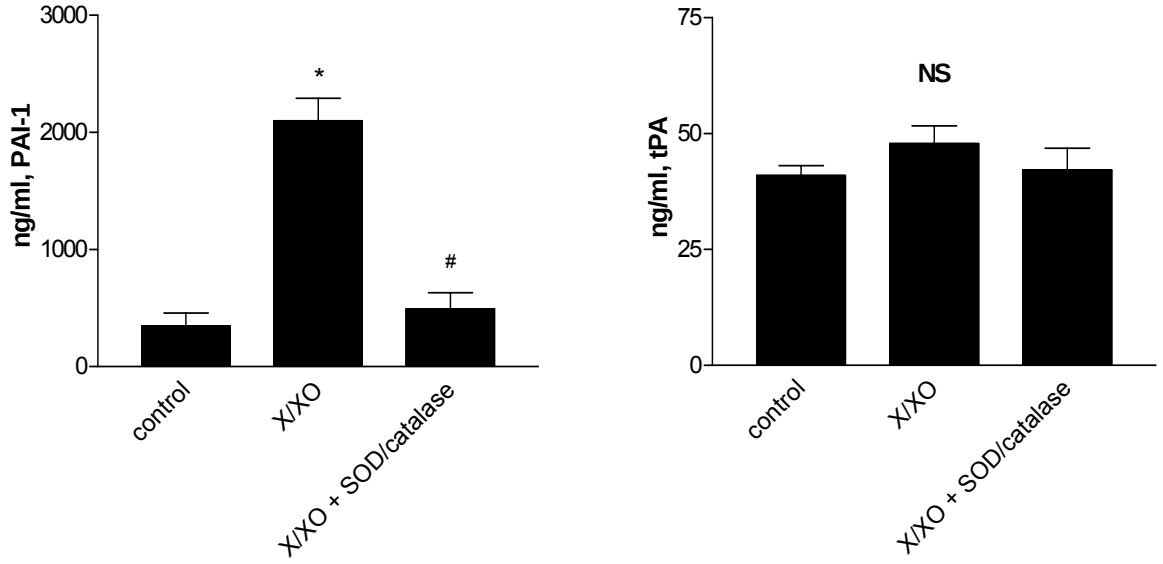

Figure 3. PAI-1 or tPA production by mesothelial cells stimulated with X/XO with or without SOD/catalase. Bars represent the mean values ± SEM (n=6). * = $p < 0.01$ vs. control; # = $p < 0.01$ vs. X/XO. NS = No significant difference.

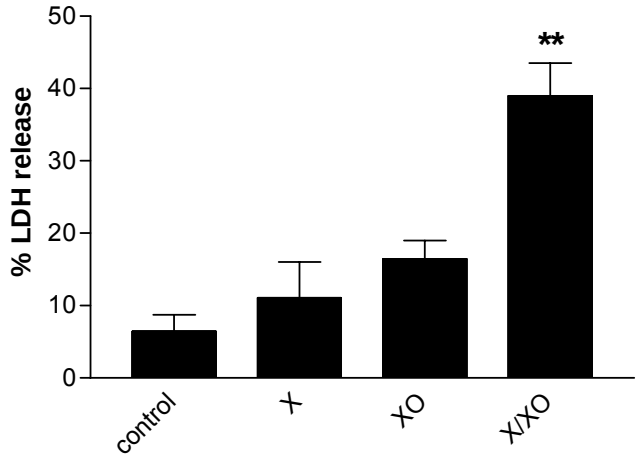

Figure 4. LDH-release by mesothelial cells exposed to X/XO. LDH-release is expressed as mean (SEM) percentage of total cellular enzyme activity liberated from the cells by 1% Triton-X100

Apoptosis assay

The apoptosis data of the mesothelial cells showed that a significant part of the mesothelial cells turned apoptotic when stimulated with X/XO for 12 hours. Xanthine oxidase already showed a slight induction of apoptosis, xanthine alone did not induce apoptosis (fig. 5).

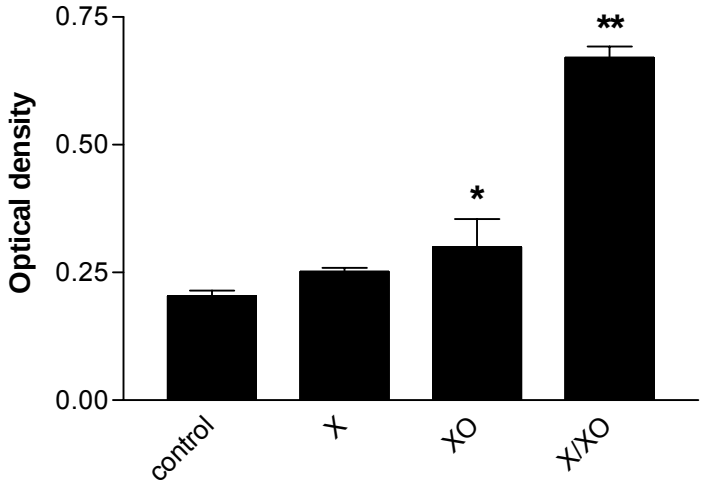

Figure 5. Apoptosis of mesothelial cells stimulated with X/XO. Bars represent the mean absorbance values (Optical density at 405nm) ± SEM. * = $p < 0.05$ vs. control, ** = $p < 0.001$ vs. control.

Adhesion experiments:

Gross animal findings

No postoperative complications such as bowel obstructions, marked delayed wound healing, wound infections or peritonitis were observed. All rats gradually gained weight after an initial postoperative weight loss.

Experiment I

The total number of neutrophils found in abdominal lavage fluids at different time points is depicted in figure 6. Up until 96 hours after laparotomy severely traumatised peritoneal cavities contained significantly higher numbers of neutrophils than severely traumatised peritoneal cavities treated with ANS ($p \leq 0.0001$).

In blood, lymphocyte, monocyte and neutrophil counts did not differ significantly in severely rats. This effect was not seen after treatment with 1 dose of ANS.

Experiment II

Figure 7 shows the mean adhesion percentages (SD) of found adhesions, after infliction of peritoneal trauma. The infliction of severe peritoneal trauma induced significantly more

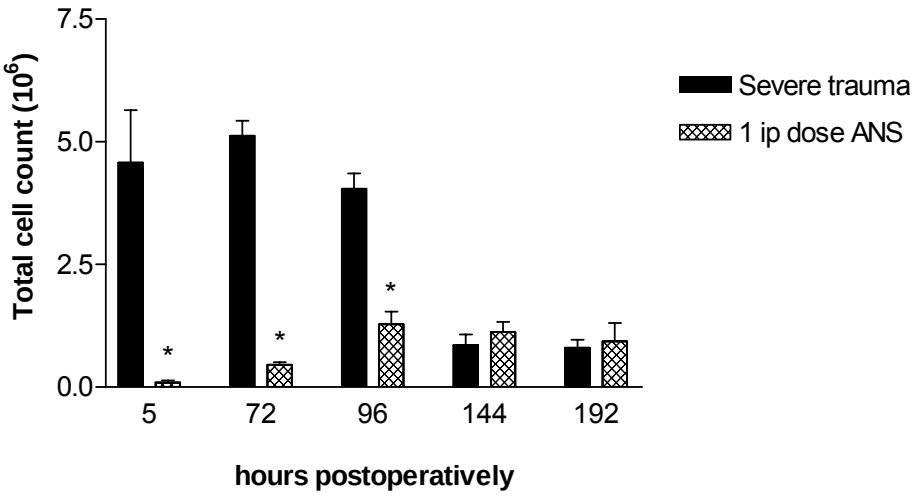

Figure 6. Mean neutrophil count (SEM) in abdominal lavage fluids after infliction of severe peritoneal trauma (filled bars) and after infliction of severe peritoneal trauma in rats treated with 1 intra-peritoneal dose of ANS (cross-hatched bars).
* = p < 0,0001: Student's t-test. N = 5 per bar.

adhesions than infliction of minimal peritoneal trauma (76% vs. 23%; p < 0.001). A significant reduction of adhesion formation after infliction of severe peritoneal trauma was seen after the injection of ANS to decrease the influx of neutrophils into the lesion (47%; p < 0.001), although the mean adhesion percentage was still significantly higher than after infliction of minimal trauma (p < 0.001).

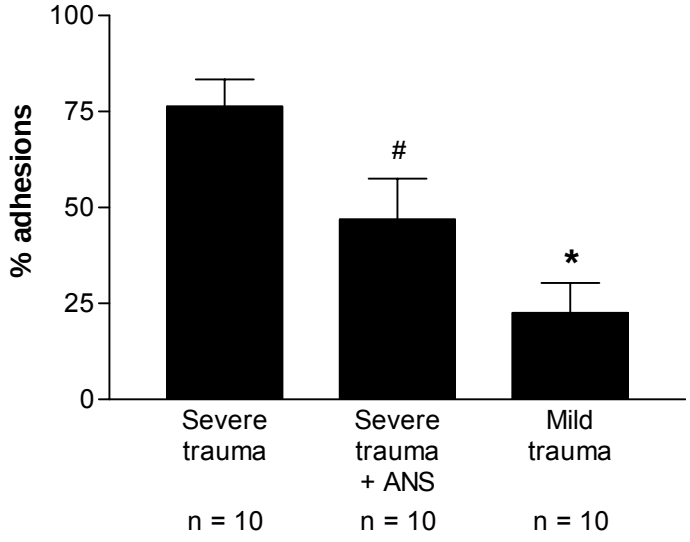

Figure 7. Mean adhesion percentages (SEM) after infliction of severe peritoneal trauma (severe trauma), after infliction of severe peritoneal trauma and treatment with ANS (severe trauma + ANS) and minimal peritoneal trauma (mild trauma).
= p < 0.001 vs. control; * = p < 0.001 vs. control.

Experiment III

To prevent any generation of ROS by the neutrophils entering at the site of trauma the combination of SOD with catalase was administered, since the total amount of ROS will not be reduced by SOD only. SOD converts the superoxide anion into hydrogen peroxide, but next catalase converts hydrogen peroxide into molecular oxygen and water. A mean of 71% (SD= 16%) of the length of the surgical scar was covered by adhesions in the control group. This percentage could be reduced to 40% (SD= 13%) by a single i.p. dose of SOD/catalase ($p < 0.01$) directly post-operative (fig. 8). A well-known scavenger of the hydroxyl radical, mannitol, on its own could not reduce adhesion formation compared to control (58% ± 20% vs. 69% ± 17%, respectively; fig. 8).

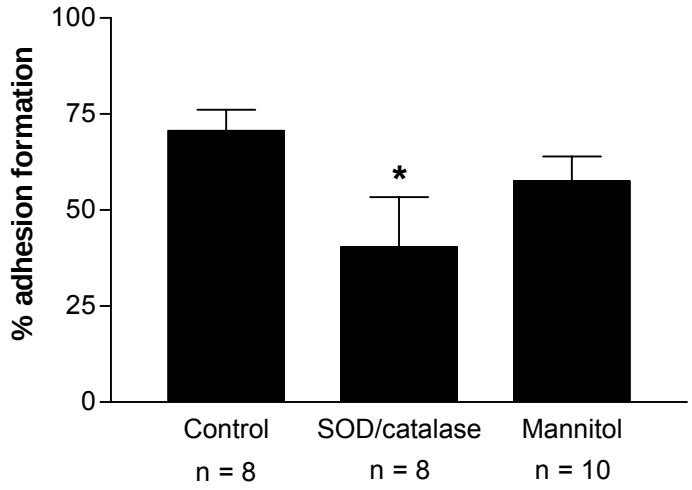

Figure 8. Mean adhesion percentages (SEM) after infliction of peritoneal trauma (control), after infliction of severe peritoneal trauma and treatment with SOD/catalase (SOD/catalase) or mannitol (Mannitol). * = $p < 0.01$ vs. control.

DISCUSSION

The pathogenesis of the processes leading to postoperative adhesion formation is multifactorial and only partly clarified. The production of mesothelial and inflammatory cell derived chemokines such as IL-8 (CINC in rats), MCP-1 and IL-1β will cause post-traumatic migration of neutrophils to the injured peritoneal cavity in order to promote the peritoneal healing process.[26-28] However, these mediators and recruited cells not only serve peritoneal healing, but are believed to be responsible for adhesion formation as well.[15,29]

Upon addition, neutrophils generate, amongst other mediators, reactive oxygen metabolites and discharge contents of granular organelles into either phagocytic vacuoles or the local environment during ingestion of foreign particles or microorganisms. Both oxygen-

dependent and oxygen-independent processes participate in the killing of bacteria but also may (further) damage surrounding host tissue.[30,31] The coincidence of post-traumatic intra-abdominal influx of neutrophils with adhesion formation is, however, no solid proof for the role of neutrophils in these pathogenetic processes. Effective inhibition of tissue injury caused by neutrophils has been achieved by neutralising neutrophils chemo attractants,[32] blocking of neutrophil adhesion molecules[33,34] and scavenging of reactive oxygen species,[35] but these approaches have thus far not been investigated thoroughly to prevent adhesion formation in a surgical peritoneal trauma model. A recent study by Roy et al.[29] demonstrated enhanced nitrotyrosine levels (the footprints of ROS) in samples of human peritoneal adhesions, providing more evidence for a crucial role of ROS in adhesion formation.

Vural et al.[16] pioneered the role of neutrophils in the formation of peritoneal adhesions. In their model cyclophosphamide, administered intraperitoneal two days before surgery, decreased peritoneal adhesions significantly, indicating that neutrophils play an important role in the dynamic cascade of adhesion formation. Cyclophosphamide is known to suppress humoral as well as the cellular immune responses at the bone marrow level.[36] The known side effects of cyclophosphamide make it unavailable in clinical practice. The present study evaluated a more selective blockage of neutrophils to the peritoneal cavity, which enables a more accurate evaluation of the role of the neutrophil in post-surgical adhesion formation. Reducing the post-traumatic intra-abdominal influx of neutrophils by intra-peritoneal injection of ANS, significantly reduced adhesion formation. Hereby confirming the important role of the neutrophil in the pathogenesis of adhesion formation.

Selectively reducing the number of neutrophils remains a troublesome procedure with possible systemic effects on the number of blood neutrophils, resulting in an immune compromised patient in the direct post-operative period. Therefore, examining the mechanism by which the neutrophils are possibly responsible for adhesion formation is of importance. Neutrophils generally act by generating ROS, especially superoxide radicals, although it cannot be excluded that these phagocytes use other tools of their detrimental armamentarium, e.g., the enzyme myeloperoxidase to generate hypochlorous acid.

Scavenging ROS in vivo with SOD and catalase significantly reduced adhesion formation in our surgical peritoneal excision model. Similar results were shown by Tsimoyiannis et al.[37], though using a rat ileal ischaemia/ reperfusion model, and by Portz et al.[38] using a rabbit endometriosis model. SOD and catalase are scavengers for superoxide radical and hydrogen peroxide. Another extremely reactive, unstable and powerful free radical, the hydroxyl radical, which could be even more important is not yet taken into account.[30,39] Catalysed by ferric iron, hydrogen peroxide is converted to the hydroxyl radical in the Fenton reaction. Next, superoxide will recycle ferric iron into ferrous iron thereby establishing a chain reaction (Haber-Weiss reaction). In an ileal ischaemia/reperfusion model Tsimoyiannis et al.[37] did show a reduction of adhesion formation using dimethyl sulphoxide (DMSO) indicating that hydroxyl radicals play an important role. Mannitol is a known scavenger of the

hydroxyl radical and as it is already clinically used, in transplant perfusion, it could have been an ideal treatment modality. However, mannitol could not reduce adhesion formation in our model. In the ischaemia/reperfusion model blood is a large source for iron and hydroxyl radicals. During general surgery, however, ischaemia/reperfusion is less pronounced and perioperative blood loss is much less nowadays. In our peritoneal excision model the blood loss is negligible and no ischaemia/reperfusion injury is inflicted, creating a more realistic surgical model. This might explain why no reduction was found in our model, when using mannitol. It is well-known that hydrogen peroxide can act as a second messenger upregulating the expression of cellular adhesion molecules[40] and transiently increasing the endothelial permeability barrier.[41,42] Therefore, it is likely that in post-surgical adhesion formation the generation of hydrogen peroxide directly, or indirectly via superoxide plays an important role in mediating the postoperative inflammatory reaction. Myeloperoxidase and hypochlorous acid might also play an important role by which neutrophils induce adhesion formation and can be addressed in future research to elucidate the mechanism by which neutrophils play a role in postoperative adhesion formation. The present studies indicate an important role of ROS in the complex pathophysiology of postoperative adhesion formation.

In our in-vitro studies we evaluated the influence of the ROS superoxide and hydrogen peroxide produced by X/XO on human mesothelial cells. Hereby further exploring the mechanism by which ROS might induce adhesion formation. As shown in figure 4 ROS have a direct cytotoxic effect on the mesothelial cells indicated by a significant increase in LDH-release. Though not only cytotoxic effects were seen, also apoptosis of the mesothelial cells is induced by ROS (fig. 5). These mechanisms create a further damage of the mesothelial lining extending beyond the damage created during surgery and enhancing the possibility to create postoperative adhesions.

Besides mesothelial cell-death ROS also have a negative effect on the fibrinolytic properties of the mesothelium. ROS inhibit fibrinolysis by increasing the release op PAI-1 by mesothelial cells. Holmdahl et al.[43] already emphasized the important role of PAI-1 produced by the mesothelium in their studies.

In conclusion, these studies demonstrate the role of neutrophils and their products in post-operative intra-abdominal adhesion formation. Preventing tissue damage by reduction of inflammatory cells like neutrophils, by prohibiting their intra-abdominal influx or by scavenging their products might provide a novel strategy in averting adhesion formation. Wound healing processes rely on the same biological mechanisms as adhesion formation, thus manipulation of neutrophils has to be done selectively and in moderation to prevent unwanted side effects.

REFERENCES

1. Holmdahl L. Making and covering of surgical footprints. Lancet 1999;353:1456-7.
2. Menzies D. Peritoneal adhesions. Incidence, cause, and prevention. Surg Annu 1992;24:27-45.
3. Ellis H. Internal overhealing: the problem of intraperitoneal adhesions. World J Surg 1980;4:303-6.
4. Luijendijk RW, de Lange DC, Wauters CC, Hop WC, Duron JJ, Pailler JL, Camprodon BR, Holmdahl L, van Geldorp HJ, Jeekel J. Foreign material in postoperative adhesions. Ann Surg 1996;223:242-8.
5. Menzies D, Parker M, Hoare R, Knight A. Small bowel obstruction due to postoperative adhesions: treatment patterns and associated costs in 110 hospital admissions. Ann R Coll Surg Engl 2001;83:40-6.
6. Jeekel H. Cost implications of adhesions as highlighted in a European study. Eur J Surg Suppl 1997;579:43-5.
7. Ellis H, Moran BJ, Thompson JN, Parker MC, Wilson MS, Menzies D, McGuire A, Lower AM, Hawthorn RJ, O'Brien F, et al. Adhesion-related hospital readmissions after abdominal and pelvic surgery: a retrospective cohort study. Lancet 1999;353:1476-80.
8. Ray NF, Denton WG, Thamer M, Henderson SC, Perry S. Abdominal adhesiolysis: inpatient care and expenditures in the United States in 1994. J Am Coll Surg 1998;186:1-9.
9. Thompson JN, Whawell SA. Pathogenesis and prevention of adhesion formation. Br J Surg 1995;82:3-5.
10. Ward PA, Lentsch AB. The acute inflammatory response and its regulation. Arch Surg 1999;134:666-9.
11. Lanfrancone L, Boraschi D, Ghiara P, Falini B, Grignani F, Peri G, Mantovani A, Pelicci PG. 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;80:2835-42.
12. Topley N. The cytokine network controlling peritoneal inflammation. Perit Dial Int 1995;15:S35-9; discussion S39-40.
13. Zeyneloglu HB, Seli E, Senturk LM, Gutierrez LS, Olive DL, Arici A. The effect of monocyte chemotactic protein 1 in intraperitoneal adhesion formation in a mouse model. Am J Obstet Gynecol 1998;179:438-43.
14. Ar'Rajab A, Dawidson I, Sentementes J, Sikes P, Harris R, Mileski W. Enhancement of peritoneal macrophages reduces postoperative peritoneal adhesion formation. J Surg Res 1995;58:307-12.
15. 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;61:143-6.
16. Vural B, Canturk NZ, Esen N, Solakoglu S, Canturk Z, Kirkali G, Sokmensuer C. The role of neutrophils in the formation of peritoneal adhesions. Hum Reprod 1999;14:49-54.
17. 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;165:1024-30.
18. Holmdahl L, Ivarsson ML. The role of cytokines, coagulation, and fibrinolysis in peritoneal tissue repair. Eur J Surg 1999;165:1012-9.
19. Saba AA, Kaidi AA, Godziachvili V, Dombi GW, Dawe EJ, Libcke JH, Silva YJ. Effects of interleukin-6 and its neutralizing antibodies on peritoneal adhesion formation and wound healing. Am Surg 1996;62:569-72.
20. Kaidi AA, Nazzal M, Gurchumelidze T, Ali MA, Dawe EJ, Silva YJ. Preoperative administration of antibodies against tumor necrosis factor- alpha (TNF-alpha) and interleukin-1 (IL-1) and their impact on peritoneal adhesion formation. Am Surg 1995;61:569-72.
21. Sandler H, Hogstorp H, Lundberg C, Gerdin B. Antiserum-induced neutropenia in the rat: characterization of a rabbit anti-rat neutrophil serum. Br J Exp Pathol 1987;68:71-80.
22. de Vree WJ, Essers MC, de Bruijn HS, Star WM, Koster JF, Sluiter W. Evidence for an important role of neutrophils in the efficacy of photodynamic therapy in vivo. Cancer Res 1996;56:2908-11.

23. Leslie RG. Evaluation and improvement of a rapid microassay for measuring superoxide anion production by phagocytes. 1. Spectrophotometric aspects. J Immunol Methods 1987;103:253-9.
24. van den Tol MP, van Stijn I, Bonthuis F, Marquet RL, Jeekel J. Reduction of intraperitoneal adhesion formation by use of non-abrasive gauze. Br J Surg 1997;84:1410-5.
25. Holmdahl L, al-Jabreen M, Risberg B. Experimental models for quantitative studies on adhesion formation in rats and rabbits. Eur Surg Res 1994;26:248-56.
26. Ribeiro RA, Flores CA, Cunha FQ, Ferreira SH. IL-8 causes in vivo neutrophil migration by a cell-dependent mechanism. Immunology 1991;73:472-7.
27. Slavin J. The role of cytokines in wound healing. J Pathol 1996;178:5-10.
28. Rollins BJ, Walz A, Baggiolini M. Recombinant human MCP-1/JE induces chemotaxis, calcium flux, and the respiratory burst in human monocytes. Blood 1991;78:1112-6.
29. Roy S, Clark CJ, Mohebali K, Bhatt U, Wallace WA, Nahman NS, Ellison EC, Melvin WS, Sen CK. Reactive oxygen species and EGR-1 gene expression in surgical postoperative peritoneal adhesions. World J Surg 2004;28:316-20.
30. Weiss SJ. Tissue destruction by neutrophils. N Engl J Med 1989;320:365-76.
31. 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;174:445-7.
32. Zagorski J, Wahl SM. Inhibition of acute peritoneal inflammation in rats by a cytokine-induced neutrophil chemoattractant receptor antagonist. J Immunol 1997;159:1059-62.
33. Walzog B, Scharffetter-Kochanek K, Gaehtgens P. Impairment of neutrophil emigration in CD18-null mice. Am J Physiol 1999;276:G1125-30.
34. Andreoli SP, Mallett C, Williams K, McAteer JA, Rothlein R, Doerschuk CM. Mechanisms of polymorphonuclear leukocyte mediated peritoneal mesothelial cell injury. Kidney Int 1994;46:1100-9.
35. Shandall AA, Williams GT, Hallett MB, Young HL. Colonic healing: a role for polymorphonuclear leucocytes and oxygen radical production. Br J Surg 1986;73:225-8.
36. Otterness IG, Chang YH. Comparative study of cyclophosphamide, 6-mercaptopurine, azathiopurine and methotrexate. Relative effects on the humoral and the cellular immune response in the mouse. Clin Exp Immunol 1976;26:346-54.
37. Tsimoyiannis EC, Tsimoyiannis JC, Sarros CJ, Akalestos GC, Moutesidou KJ, Lekkas ET, Kotoulas OB. The role of oxygen-derived free radicals in peritoneal adhesion formation induced by ileal ischaemia/reperfusion. Acta Chir Scand 1989;155:171-4.
38. Portz DM, Elkins TE, White R, Warren J, Adadevoh S, Randolph J. Oxygen free radicals and pelvic adhesion formation: I. Blocking oxygen free radical toxicity to prevent adhesion formation in an endometriosis model. Int J Fertil 1991;36:39-42.
39. Grace PA. Ischaemia-reperfusion injury. Br J Surg 1994;81:637-47.
40. van Dam B, van Hinsbergh VW, Stehouwer CD, Versteilen A, Dekker H, Buytenhek R, Princen HM, Schalkwijk CG. Vitamin E inhibits lipid peroxidation-induced adhesion molecule expression in endothelial cells and decreases soluble cell adhesion molecules in healthy subjects. Cardiovasc Res 2003;57:563-71.
41. Berman RS, Martin W. Arterial endothelial barrier dysfunction: actions of homocysteine and the hypoxanthine-xanthine oxidase free radical generating system. Br J Pharmacol 1993;108:920-6.
42. Martin WJ, 2nd. Neutrophils kill pulmonary endothelial cells by a hydrogen-peroxide-dependent pathway. An in vitro model of neutrophil-mediated lung injury. Am Rev Respir Dis 1984;130:209-13.
43. Holmdahl L. The role of fibrinolysis in adhesion formation. Eur J Surg Suppl 1997:24-31.

Chapter 9

The Effects of Icodextrin on Postoperative Adhesion Formation and Peritoneal Tumour Recurrence

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ABSTRACT

Background:

Peroperative peritoneal trauma activates a cascade of peritoneal defence mechanisms responsible for postoperative adhesion formation. The same cascade seems to play a role in the process of intra-abdominal tumour recurrence. Icodextrin is a glucose polymer solution that is absorbed slowly from the peritoneal cavity, allowing prolonged “hydroflotation” of the viscera, hereby preventing adhesion formation.

This study will evaluate the adhesion preventing properties of icodextrin and its effect on peritoneal metastasis.

Materials and Methods:

Reproducible rat models allowing semiquantitative scoring of adhesion formation or tumour load were used. In two experiments peritoneal trauma was inflicted and one group was treated by peroperative intra-abdominal instillation of icodextrin, one by instillation of RPMI (placebo) and in one group there was no instillate (controls). The coloncarcinoma cell line CC531s was injected intraperitoneal to induce tumour load in one experiment.

Results:

Treatment of peritoneally traumatized rats with icodextrin caused a 60% reduction in postoperative adhesion formation ($p < 0.001$). Peroperative intra-abdominal treatment with icodextrin did not affect intra-peritoneal tumour cell adhesion and growth of free intra-abdominal tumour cells in severely traumatized peritoneal cavities. Hydroflotation is the main mechanism by which icodextrin acts.

Conclusions:

Icodextrin is an effective solution in reducing postoperative adhesions without promoting tumour recurrence and therefore can be used safely in oncological surgery.

INTRODUCTION

Postoperative adhesion formation is a major, up till now unavoidable complication of any kind of abdominal surgery. Postoperative adhesions occur in 90-100% of patients undergoing abdominal surgery.[1,2] Clearly, in addition to morbidity and mortality adhesion formation also has major financial consequences.[3-5] A simple cost effective method to reduce or prevent adhesion formation is therefore needed.

Various animal and clinical studies have indicated that placement of absorbable barriers can reduce postoperative adhesion formation.[6-12] A major disadvantage of the site-specific adjuvants is that the surgeon must predict the adhesion formation sites to determine barrier placement. Icodextrin, a glucose polymer solution already successfully and safely used in peritoneal dialysis was further developed into a fluid that is absorbed only slowly, allowing prolonged “hydroflotation” of the peritoneal cavity.[13-15] One aim of this study was to further evaluate the adhesion reducing effect of this new glucose polymer solution, icodextrin, in a well-defined rat adhesion model.

Intra-abdominal, local recurrence and peritoneal dissemination is a common cause for post-surgical tumour recurrence after potentially curative resection of gastro-intestinal carcinoma.[16] Previous studies suggested that the dynamic cascade of peritoneal healing, induced by peritoneal damage, leading to adhesion formation also seems to be important in the process of intra-peritoneal adhesion and growth of tumour cells.[17,18] Previously described clinical and experimental studies showed that surgical trauma promotes intra-abdominal tumour recurrence.[18-20] Therefore we included a study evaluating the effect of icodextrin on intra-abdominal tumour recurrence.

Using a reproducible rat tumour adhesion and growth model we analysed whether icodextrin might reduce or possibly promote the adhesion and growth of intra-peritoneally injected tumour cells.

MATERIALS AND METHODS

Animals

Female inbred WAG/Rij rats of reproductive age weighing 145-190g were obtained from Harlan-CPB, Austerlitz, The Netherlands. The rats were bred under specific pathogen-free conditions. The animals were kept under standard laboratory conditions (temperature 20-24ºC, relative humidity 50-60%, 12 hours light and 12 hours dark cycles), fed with standard rat food and water *ad libitum* and quarantined in our University facilities for at least two days prior to use. The experimental protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this

national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC.

Mesothelial cell culture

Human mesothelial cells were isolated from omental tissue of patients undergoing elective abdominal surgery for non-infectious reasons. A specimen of bowel omental tissue was cut in smaller pieces and washed with Hanks' Balanced Salt Solution (HBSS) containing 0.5% human serum albumin. After washing three times the specimens were centrifuged for 5 minutes at 580xg. The omentum was transferred to a solution containing trypsin (0.05%) and EDTA (0.02%) and incubated for 20 minutes at 37°C under continuous shacking. After 20 minutes the detached mesothelial cells were pelleted down by centrifugation at 580 xg for 10 minutes. After centrifugation the mesothelial cells were resuspended in culture medium (RPMI 1640 containing 10% fetal calf serum, glutamine (2mM) and penicillin (10^5 U/L)). The mesothelial cells were cultured at 37°C, in a fully humidified, 5% CO_2 cabinet in polystyrene 75cm^2 culture flasks pre coated with collagen type I (Roche Diagnostics, Almere, the Netherlands). The mesothelial origin of the cells was verified by their typical cobblestone appearance by phase-contrast microscopy and by immunohystochemical characterization with positive staining of keratin (anti-serum Z622; DAKO, Heverlee, the Netherlands) and vimentin (anti-serum L1843; DAKO, Heverlee, the Netherlands) and negative staining of von Willebrand factor (F8/86 antibody; DAKO, Heverlee, the Netherlands).

Drugs

Icodextrin is a biodegradable, biocompatible, α-1,4 linked glucose polymer, kindly donated by Shire Pharmaceuticals. For the animal experiments a 7.5 % solution of icodextrin was used. The large icodextrin molecule is not digested intra-abdominally but is, via the lymphatic system, gradually absorbed into the bloodstream where it is partitioned sequentially by the enzymes α-amylase and maltase to maltose and glucose. Previous experimental and clinical studies assessed the safety, tolerability and preliminary effectiveness with regard to reducing postoperative adhesion formation. A volume of 20 ml per kg bodyweight has been indicated as the optimal applicable volume.[13,14]

The cell cultures were exposed to the reactive oxygen species (ROS) superoxide and hydrogen peroxide generated by xanthine oxidase (0.05 U/ml, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) upon the addition of xanthine (0.1 mmol/l, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) (X/XO). The oxygen-free radicals are produced by the following enzymatic reaction: xanthine + O2 + H2O → Uric acid + H2O2 + O2-, catalysed by xanthine oxidase.

The anti-oxidant enzymes SOD (5000 U/mg) from Roche Diagnostics BV, Almere, the Netherlands and catalase (2350 U/mg) from Sigma-Aldrich Chemie BV, Zwijndrecht, the

Netherlands were added to the cell cultures in a 400U/ml and 200U/ml concentration respectively to counteract the ROS production specifically.

Tumour

Tumour CC531s is a 1,2-dimethylhydrazine-induced, moderately differentiated, weakly immunogenic colonic adenocarcinoma transplantable in syngenic WAG rats.[21] The tumour is maintained as a cell culture in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) (virus- and Mycoplasma-screened), 1% penicillin (5000 U/mL), 1% streptomycin (5000 U/mL) and 1% L-glutamine (200 mmol). 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%). Before use in vivo, tumour cells were harvested from stationary cultures by gentle trypsinization (5 minutes, 37ºC), centrifugation (5 minutes, 700 g) and re-suspension in RPMI 1640, providing cell suspensions with viability greater than 90%. CC531s is relatively insensitive to chemotherapy but is sensitive to the effects of biologic response modifiers.

Animal experimental design

Adhesion formation

To study the effect of icodextrin on adhesion formation our previously described reproducible rat adhesion model was used[22]. In 30 rats, under isoflurane anaesthesia and aseptic conditions, a laparotomy was performed using a lower midline incision of 5 cm, a standardized amount of peritoneal trauma was inflicted. Before closing the abdominal cavity 10 rats were treated by intra-abdominal instillation of icodextrin, 10 rats with RPMI (placebo) and 10 rats received no further treatment (controls). The abdomen was closed in two layers with 5-0 polyglycolic acid sutures (Braun).

Two weeks after surgery, the rats were sacrificed for assessment of intra-abdominal adhesion formation. Macroscopically the adhesions were scored according to their extent (quantity) and type (quality) by two independent observers.[22]The type of adhesions formed was classified macroscopically using the Zühlke classification.[23]

Peritoneal carcinomatosis

To study the effect of icodextrin on local tumour recurrence our previously designed reproducible tumour adhesion and growth model was used.[18] Under isoflurane anaesthesia and aseptic conditions a laparotomy was performed in 30 rats using a lower midline incision of 5 cm, a standardized amount of peritoneal trauma was inflicted. At the end of the operation 10 rats received icodextrin, 10 rats RPMI (placebo) and in 10 rats nothing (control) was administered intra-abdominally. Before closing the abdomen a tumour cell suspension containing 0.5×10^6 CC531s tumour cells, in 1 ml RPMI, was injected intra-peritoneally. The abdomen was closed in two layers with 5-0 polyglycolic sutures.

Three weeks after surgery, the rats were sacrificed and intra-peritoneal tumour load was scored semiquantitatively at the following peritoneal sites: right uterus horn, left uterus horn, subcutaneous (at the site of the operative scar), parietal peritoneum (at the lateral abdominal wall sides where no uterus horns were fixated), kidney, liver, retroperitoneum, and omentum. The scoring was performed by two independent observers blinded for the treatment groups and ranged from 0 to 5 per site. For each rat the score at all peritoneal sites, except for the uterus horns, was summarized, from which a mean total tumour load per rat could be estimated. In the present study the term “tumour recurrence” is sometimes used to illustrate tumour load, which is the net result of tumour cell adhesion and tumour growth, because we presume that intra-peritoneal injection of tumour cells resembles the clinical situation of tumour cell spill during tumour resection.

Proliferation assay

To establish the effect of icodextrin on growing tumour and mesothelial cell cultures, proliferation curves were made using a DNA measurement. Tumour cells or mesothelial cells were trypsinized and seeded in 24 well plates in a concentration of 10.000 or 50.000 cells per well, respectively. Different concentrations of icodextrin were added directly after seeding of the tumour cells. At 2, 4 and 6 days the plates were washed twice with saline and stored at -20°C until further analysis. The cells were extracted with ammonia solution (1mmol/L) – Triton X100 (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. Sigma-Aldrich, Zwijndrecht, the Netherlands) was added. The remaining solution was mixed with Hoechst dye H33258 (100µg/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 was referenced to a standard curve of calf thymus DNA (type II, no D-3636; Sigma, Zwijndrecht, the Netherlands).

Lactate dehydrogenase release

Mesothelial cell toxicity was assessed by measurement of lactate dehydrogenase (LDH) release. Mesothelial cells were seeded into 24-well culture plates and grown to confluent monolayers. Thereafter, the medium was removed, and the cells were incubated for 15 minutes with different concentrations of icodextrin (n = 6 wells/condition). After incubation the supernatant was collected and centrifuged (800g) to remove dead cells. LDH activity in this medium and in cell lysate was assayed spectrophotometrically using the ELAN autoanalyser (Merck & Co.). The LDH-release was expressed as percentage of total cellular enzyme activity liberated from the cells by 1% Triton-X100.

PAI-1 and tPA assays

To investigate the influence of icodextrin on the fibrinolytic properties of human mesothelial cells, plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (tPA) levels were measured in resting mesothelial cells (control) and after 12 hours of stimulation with different concentrations of icodextrin (n = 6 wells/condition). The PAI-1 elisa kit from Technoclone, Surrey, United Kingdom and the tPA Tintelize from Biopool, Trinity biotech, Ireland were used.

Ferricytochrome *c* reduction

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

Statistical analysis of data

The mean adhesion percentage or tumour load and standard deviation was calculated per group. Data were statistically analysed using the one-way ANOVA test to determine overall differences. If the ANOVA test was significant on a 5% level, the Student Newman Keuls *post hoc* test was carried out to make a comparison between groups.

In all in-vitro experiments means and standard error of the mean (SEM) were calculated. All data was analysed using analysis of variance (ANOVA) to determine differences between treatment groups. If the ANOVA test was significant on a 5% level, the Student Newman Keuls *post hoc* test was carried out to make a comparison between groups.

RESULTS

Gross animal findings

None of the rats were found to have adhesions at the initial operation. There were no postoperative complications e.g. bowel obstructions, peritonitis or tumour overgrowth. No leaking of fluids from the abdominal wounds and no postoperative bulging of abdomens was observed. No remnant fluids were found at necropsy at day 14 or day 21.

Effect of icodextrin on Adhesion Formation

Icodextrin reduced postoperative adhesion formation after severe peritoneal trauma compared to the untreated group (19,3% (SD = 8%) and 69,9% (SD = 14,9%) respectively, $p < 0.001$). The mean adhesion percentage found after peroperative instillation of RPMI did not differ from that of controls (65% (7,3%) and 69,9% (14,9%) respectively, $p > 0,05$). The adhesions formed in the icodextrin group were filmy (Zühlke type 1-2) involving only pelvic fat. Adhesions found after administration of RPMI and in controls were stronger (Zühlke type 2-3), involving pelvic fat, uterine horn and small bowel.

Effect of icodextrin on Tumour Cell Adhesion and Growth

Tumour load is shown in figure 1. Treatment with icodextrin or RPMI did not significantly affect tumour load, mean total tumour scores were similar in the three experimental groups.

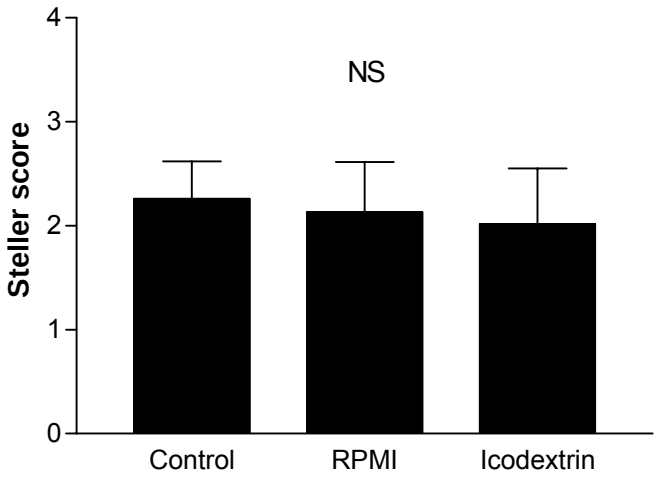

Figure 1. Mean intra-peritoneal tumour load ± SD of severely traumatised rats (control), severely traumatised rats treated with RPMI and severely traumatised rats treated with Icodextrin.

Proliferation assay

Based on the results (data not shown) of growth curves made using different seeding densities and different concentrations of FCS the ideal culture conditions for studying growth stimulation and inhibition of CC531s and mesothelial cells was established.

For CC531s, the control group showed an increasing amount of DNA from day 0 and 2 to day 4 and 6 of culture ($p < 0,001$), indicating that the cells are actively growing during this period (fig. 2). In vitro there was no growth stimulating effect of icodextrin on CC531s (fig. 2). Icodextrin 4% even reduced tumour growth after 4 and 6 days ($p < 0,001$). Lower concentrations of icodextrin had no effect on tumour growth ($p > 0,05$).

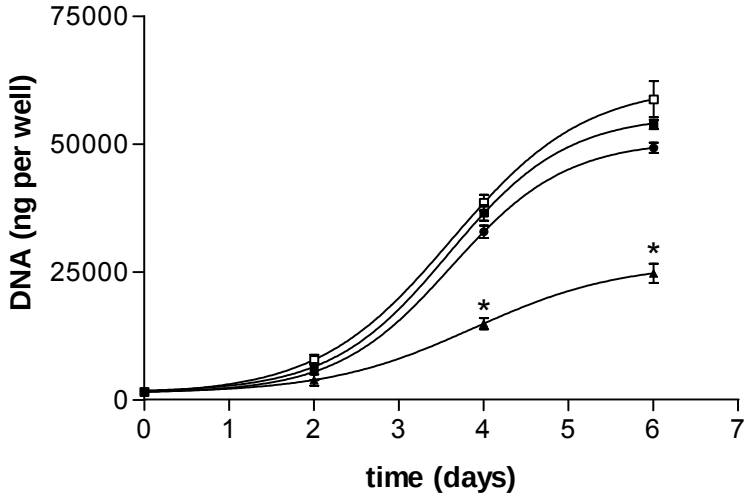

Figure 2. Proliferation curves of 10.000 CC531s tumour cells □ RPMI with 5% FCS (control); ▲ Icodextrin 4%; ● Icodextrin 2%; ■ Icodextrin 1%. The values are expressed as the mean of four replicates with SEM. * = p < 0,001 Icodextrin 4% versus control.

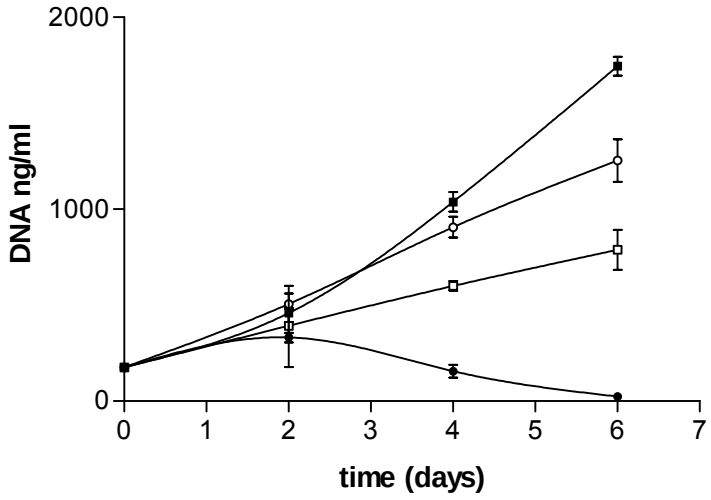

Figure 3. Proliferation curves of 50.000 mesothelial cells. ■ RPMI with 10% FCS (control); ● Icodextrin 4%; □ Icodextrin 2%; ○ Icodextrin 1%. The values are expressed as the mean of four replicates with SEM.

Figure 3 shows the results for mesothelial cells. The control group showed an increasing amount of DNA throughout the experiment ($p < 0.001$). Already in a low concentration the addition of icodextrin during seeding inhibited mesothelial cell proliferation. At addition of 4% icodextrin during seeding no mesothelial cells survived at all, implicating a cytotoxic effect of icodextrin on ex-vivo actively proliferating mesothelial cells.

LDH assay

Cell toxicity was assessed by measuring LDH-release after exposing mesothelial cell monolayers to different concentrations of icodextrin. As shown in figure 4 icodextrin had no significant effect on LDH release. Though a tendency to enhanced LDH release is seen with 4% icodextrin.

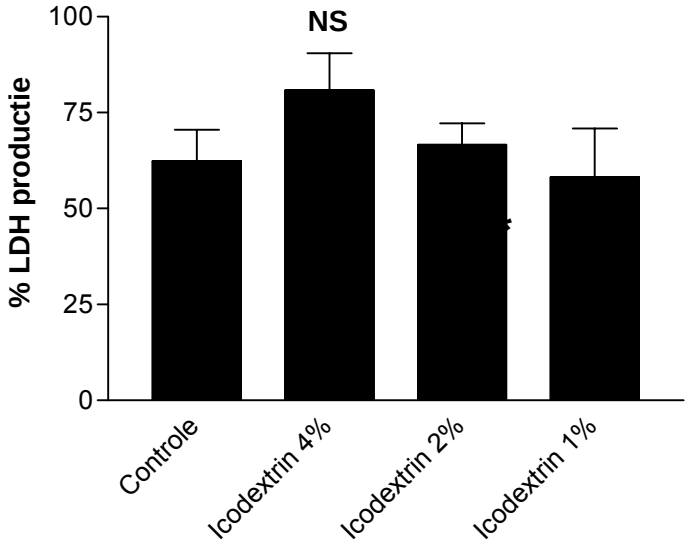

Figure 4. Lactate dehydrogenase release by mesothelial monolayer exposed to Icodextrin or culture medium (control). NS: not significant.

tPA and PAI-1 assays

Figure 5 shows a marked increase in tPA after incubation of human mesothelial cells with different concentrations of icodextrin. No significant change in release of PAI-1 by mesothelial cells was seen upon stimulation (fig. 5). These results suggest tPA mediated decrease in the fibrinolytic capacity of mesothelial cells when stimulated with icodextrin.

Quantification of ROS generation

To appreciate the scavenging effects of icodextrin on the xanthine/xanthine oxidase (X/XO) system, the amount of ROS produced by X/XO was determined over time. The results showed that X and XO alone did not produce significant amounts of ROS. However when combining X and XO superoxide was produced at a rate of 0.4097 nmol/ml/min during the observation period of 60 min (fig. 6). After that the amount of superoxide did not increase much showing that the rate of superoxide production approached zero. In the presence of SOD, the superoxide generated by X/XO was almost completely inactivated (fig. 7). Adding

Icodextrin showed a slight concentration dependent decrease in superoxide production (fig. 7).

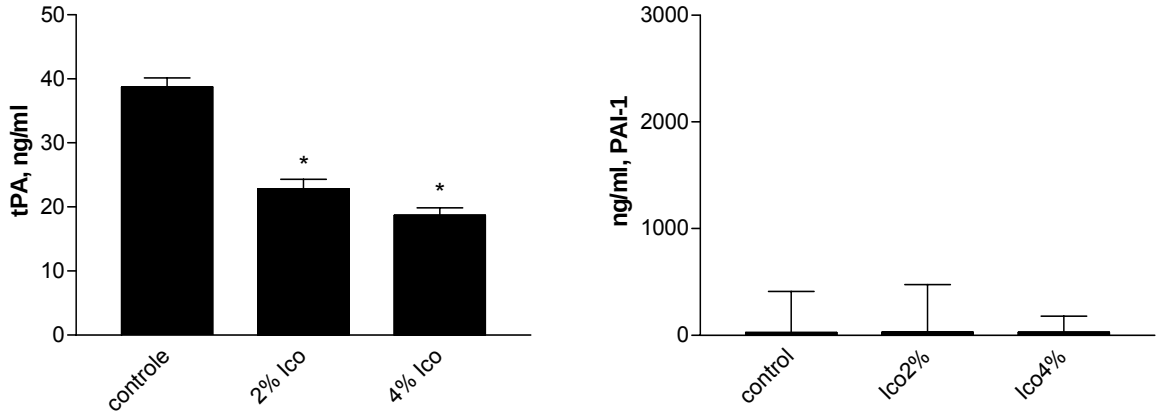

Figure 5. Levels of tissue plasminogen activator (tPA) or plasminogen activator inhibitor-1 (PAI-1) in conditioned media. Results are illustrated as mean and SEM. * = p < 0,001 compared to control.

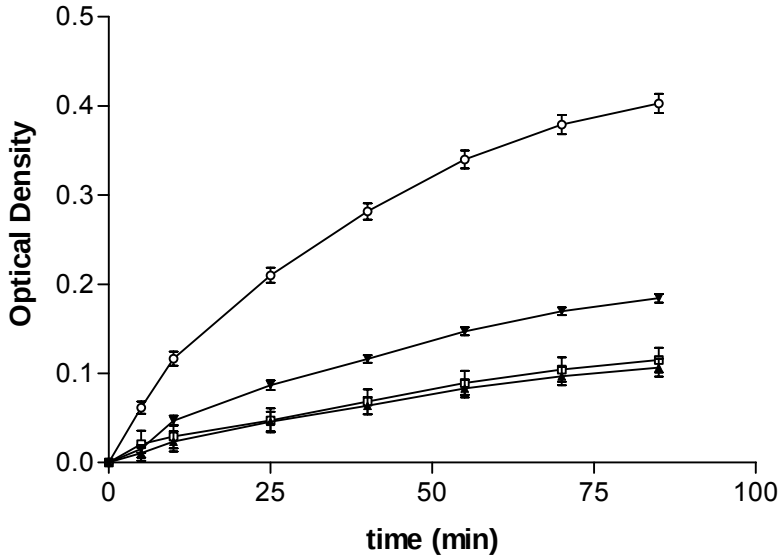

Figure 6. Ferricytochrome C reduction. □ control (ferricytochrome C alone); ▲ Xanthine; ▼ Xanthine oxidase; ○ Xanthine and Xanthine oxidase. The values are expressed as mean and SEM

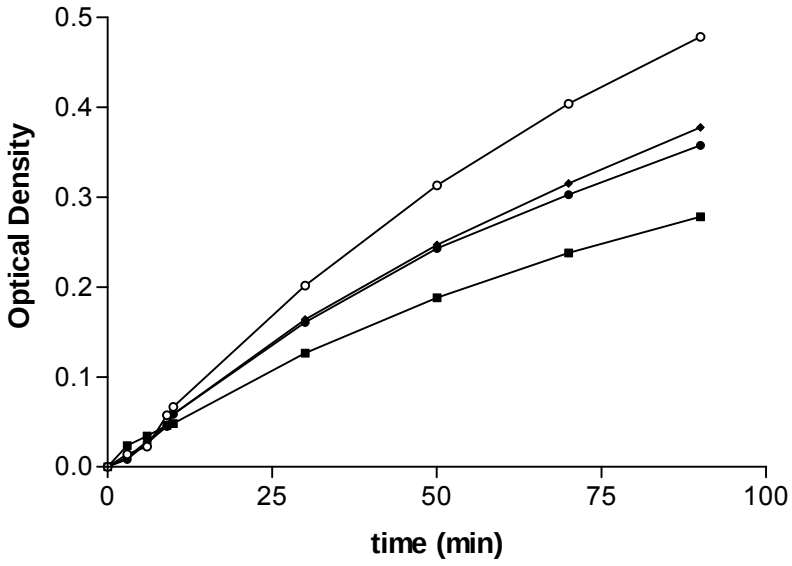

Figure 7. Ferricytochrome-C reduction. ○ Xanthine and Xanthine oxidase (XXO) ■ XXO with superoxide dismutase; ♦ XXO with 2% Icodextrin; ● XXO with 4% Icodextrin. The values are expressed as mean and SEM.

DISCUSSION

The formation of an adhesion commences with injury to two layers of the peritoneum, one opposite the other. The resulting exudation contributes to the deposit of fibrin and adhesion between the two membranes.[24, 25] Whether such adhesions are permanent or will eventually be lysed, is assumed to be dependent on the fibrinolytic capacity of the peritoneum.[25-27] A wide variety of therapeutic modalities affecting different levels in the cascade of fibrinogenesis and fibrinolysis have been studied experimentally and clinically.[28-31] Results are inconsistent and may be associated with side effects such as intra-abdominal haemorrhage and impaired wound healing.

Avoiding per- and postoperative contact between traumatized peritoneal surfaces might bring us closer to reducing postoperative adhesion formation. Increasing the physiological liquid interface between two peritoneal surfaces is liable to reduce the initial adhesion phenomena at its origin. Indeed in a rat model, postoperative peritoneal dialysis significantly reduced adhesion formation, presumably due to a flotation effect and maybe dilution of the fibrin exudates.[32] Obviously postoperative peritoneal dialysis is not feasible in the clinical setting but these results stimulated further investigations. Various liquids have been instilled peroperatively in the peritoneal cavity with the hope that "hydroflotation" would enable traumatized surfaces to be kept separate for a while.[10,11,33] Unfortunately these liquids were absorbed too quickly to bring about clinical successful adhesion prevention[34] or caused unwanted side effects.[35-37] Separating peritoneal surfaces by means of slow absorption

solutions, allowing prolonged postoperative “hydroflotation” of the peritoneal cavity has a more efficient anti-adhesive effect. Icodextrin is a large α-1,4 linked glucose polymer broken down sequentially by the enzymes α-amylase and maltase to maltose and glucose. Amylase is widely distributed throughout the body but is not or hardly present in the peritoneal cavity of humans. When administered peroperatively, icodextrin is largely retained within the abdominal cavity for up to five days during which the polymer is gradually absorbed through the lymphatic system into the systemic circulation.[38] Icodextrin 7.5%, although iso-osmolar, induces ultra filtration through colloid osmosis and the presence of the polymer is presumed to create a constant fluid layer between peritoneal surfaces.[39] Theoretically the fluid acts to reduce the formation of adhesions by a 4-day period of “hydroflotation” of the total abdominal cavity, the time of maximum risk of adhesion formation.[40,41]

This study showed that after a standardized surgical peritoneal trauma icodextrin significantly reduced the extent and severity of adhesion formation in the rat. In humans this effect might even be larger compared to the rat. As mentioned previously icodextrin is degraded by the enzyme α-amylase. α-Amylase is present in the peritoneal cavity of the rat and icodextrin will be degraded more rapidly than in humans.

Further analysis of possible mechanism by which icodextrin reduces adhesion formation showed that icodextrin might act as an anti-oxidant. ROS have shown to be an important factor in post-operative adhesion formation (this thesis). In contrast, icodextrin creates a negative environment for mesothelial cells. A tendency to increased LDH release has been found when exposing mesothelial monolayers to 4% icodextrin and a marked decrease in mesothelial cell proliferation is seen. Indicating a cytotoxic effect of icodextrin on mesothelial cells. Moreover icodextrin seems to be negatively correlated to the fibrinolytic properties of the mesothelial cell. As shown in figure 5 a decreased tPA production of the mesothelial cell is seen when stimulated with icodextrin.

Hydroflotation possibly is the main mechanism of action of icodextrin against post-surgical adhesion formation, aided by a slight anti-oxidant effect.

Previous studies at our laboratory suggest that the peritoneal defence mechanism triggered by surgical trauma to the peritoneum not only promotes adhesion formation but also stimulates tumour recurrence.[18] The fibrin entrapment hypothesis proposes that tumour cells are trapped in fibrin at the resection site and abraded peritoneal surfaces, hereby providing protection from host defence mechanisms.[42]

Whether postoperative “hydroflotation”, could keep free floating intra-abdominal tumour cells from adhering to damaged peritoneum was doubtful. Dilution of the fibrin might result in reduced entrapped tumour cells. Results obtained in our tumour adhesion and growth model showed no reduction in tumour load by peroperative intra-abdominal icodextrin treatment. Apparently it is not possible to keep free floating tumour cells from an injured peritoneal surface by a liquid barrier in which tumour cells suspend and stay alive. Technically

icodextrin does not form a barrier between tumour cells and damaged peritoneal surfaces as it does between two injured peritoneal surfaces.

On the other hand, icodextrin might even be a potential medium in which tumour cells can proliferate and augment motility, increasing their metastatic ability. The results of the in vivo study did not support this hypothesis since total peritoneal tumour load at traumatized and remote peritoneal sites was not increased by icodextrin. The in vitro growth assay even showed a significant reduction in growth of the tumour cells with 4% icodextrin and no effect on growth in lower concentrations (fig. 2). The fact that 7,5% icodextrin did not give any reduction in tumour load in vivo might be because in the peritoneal cavity 7,5% icodextrin will be diluted within a short time limit due to ultra filtration. Intra-abdominal free-floating tumour cells will only be exposed to high concentrations of icodextrin for a short time.

In conclusion the current study showed that the novel glucose polymer solution icodextrin, used as an intra-operative intra-abdominal instillate could significantly reduce postoperative adhesion formation and does not promote or inhibit tumour recurrence in vivo. Taking these results into account and a recent publication by Rodgers et al.[43] showing no effect of icodextrin on bowel anastomotic healing indicates that icodextrin seems to be a good solution to reduce intra-peritoneal adhesion formation and can be used safely in oncological surgery.

REFERENCES

1. Ellis H. Internal overhealing: the problem of intraperitoneal adhesions. World J Surg 1980; 4(3):303-6.
2. Luijendijk RW, de Lange DC, Wauters CC, et al. Foreign material in postoperative adhesions. Ann Surg 1996; 223(3):242-8.
3. Ray NF, Larsen JW, Jr., Stillman RJ, Jacobs RJ. Economic impact of hospitalizations for lower abdominal adhesiolysis in the United States in 1988. Surg Gynecol Obstet 1993; 176(3):271-6.
4. Jeekel H. Cost implications of adhesions as highlighted in a European study. Eur J Surg Suppl 1997; 579:43-5.
5. Ellis H, Moran BJ, Thompson JN, et al. Adhesion-related hospital readmissions after abdominal and pelvic surgery: a retrospective cohort study. Lancet 1999; 353(9163):1476-80.
6. Hellebrekers BW, Trimbos-Kemper GC, van Blitterswijk CA, et al. Effects of five different barrier materials on postsurgical adhesion formation in the rat. Hum Reprod 2000; 15(6):1358-63.
7. Prevention of postsurgical adhesions by INTERCEED(TC7), an absorbable adhesion barrier: a prospective randomized multicenter clinical study. INTERCEED(TC7) Adhesion Barrier Study Group. Fertil Steril 1989; 51(6):933-8.
8. Becker JM, Dayton MT, Fazio VW, et al. Prevention of postoperative abdominal adhesions by a sodium hyaluronate-based bioresorbable membrane: a prospective, randomized, double-blind multicenter study. J Am Coll Surg 1996; 183(4):297-306.
9. Beck DE. The role of Seprafilm bioresorbable membrane in adhesion prevention. Eur J Surg Suppl 1997(577):49-55.
10. Heidrick GW, Pippitt CH, Jr., Morgan MA, Thurnau GR. Efficacy of intraperitoneal sodium carboxymethylcellulose in preventing postoperative adhesion formation. J Reprod Med 1994; 39(8):575-8.
11. Burns JW, Skinner K, Colt J, et al. Prevention of tissue injury and postsurgical adhesions by precoating tissues with hyaluronic acid solutions. J Surg Res 1995; 59(6):644-52.
12. Vrijland WW, Tseng LN, Eijkman HJ, et al. Fewer intraperitoneal adhesions with use of hyaluronic acid-carboxymethylcellulose membrane: a randomized clinical trial. Ann Surg 2002; 235(2):193-9.
13. Verco SJ, diZerega G and the Classic Study Group. Tolerability and effectiveness of 4% icodextrin solution in the reduction of post-surgical adhesions following laparoscopic surgery: a multicenter, pilot, clinical study. J reprod Fertil Abstr Ser 2000; 25:62.
14. Verco SJ, Peers EM, Brown CB, et al. Development of a novel glucose polymer solution (icodextrin) for adhesion prevention: pre-clinical studies. Hum Reprod 2000; 15(8):1764-72.
15. diZerega GS, Verco SJ, Young P, et al. A randomized, controlled pilot study of the safety and efficacy of 4% icodextrin solution in the reduction of adhesions following laparoscopic gynaecological surgery. Hum Reprod 2002; 17(4):1031-8.
16. Abulafi AM, Williams NS. Local recurrence of colorectal cancer: the problem, mechanisms, management and adjuvant therapy. Br J Surg 1994; 81(1):7-19.
17. Eggermont AM, Steller EP, Marquet RL, et al. Local regional promotion of tumour growth after abdominal surgery is dominant over immunotherapy with interleukin-2 and lymphokine activated killer cells. Cancer Detect Prev 1988; 12(1-6):421-9.
18. van den Tol PM, van Rossen EE, van Eijck CH, et al. Reduction of peritoneal trauma by using nonsurgical gauze leads to less implantation metastasis of spilled tumour cells. Ann Surg 1998; 227(2):242-8.
19. Busch OR, Hop WC, Hoynck van Papendrecht MA, et al. Blood transfusions and prognosis in colorectal cancer. N Engl J Med 1993; 328(19):1372-6.
20. Bouvy ND, Marquet RL, Hamming JF, et al. Laparoscopic surgery in the rat. Beneficial effect on body weight and tumour take. Surg Endosc 1996; 10(5):490-4.

21. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumour site. Int J Cancer 1984; 33(5):689-92.
22. van den Tol MP, van Stijn I, Bonthuis F, et al. Reduction of intraperitoneal adhesion formation by use of non-abrasive gauze. Br J Surg 1997; 84(10):1410-5.
23. Zuhlke HV, Lorenz EM, Straub EM, Savvas V. Pathophysiologie und Klassifikation von Adhäsionen. Langenbecks Arch Chir Suppl II Verh Dtsch Ges Chir 1990:1009-16.
24. Montz FJ, Shimanuki T, diZerega G. Post-surgical mesothelial re-epithelialisation. In: DeCherney A.H., Polan Mleds. Reproductive Surgery, Year Book Medical Publishes. Chicago. 1987:31-47.
25. Thompson JN, Whawell SA. Pathogenesis and prevention of adhesion formation. Br J Surg 1995; 82(1):3-5.
26. Raftery AT. Effect of peritoneal trauma on peritoneal fibrinolytic activity and intraperitoneal adhesion formation. An experimental study in the rat. Eur Surg Res 1981; 13(6):397-401.
27. Vipond MN, Whawell SA, Thompson JN, Dudley HA. Peritoneal fibrinolytic activity and intra-abdominal adhesions. Lancet 1990; 335(8698):1120-2.
28. Hockel M, Ott S, Siemann U, Kissel T. Prevention of peritoneal adhesions in the rat with sustained intraperitoneal dexamethasone delivered by a novel therapeutic system. Ann Chir Gynaecol 1987; 76(6):306-13.
29. Rodgers KE. Nonsteroidal anti-inflammatory drugs (NSAIDs) in the treatment of postsurgical adhesion. Prog Clin Biol Res 1990; 358:119-29.
30. Treutner KH, Winkeltau G, Lerch MM, et al. Prevention of postoperative intra-peritoneal adhesions. A new study standardized in rats. Research in Surgery 1992; 4:50-54.
31. Doody KJ, Dunn RC, Buttram VC, Jr. Recombinant tissue plasminogen activator reduces adhesion formation in a rabbit uterine horn model. Fertil Steril 1989; 51(3):509-12.
32. Lindenberg S, Moesgaard F, Vangsted P, Lykkegaard Nielsen M. Peritoneal dialysis reduces intraperitoneal adhesion formation. An experimental study in rats. Eur Surg Res 1982; 14(4):274-8.
33. Burns JW, Skinner K, Colt MJ, et al. A hyaluronate based gel for the prevention of postsurgical adhesions: evaluation in two animal species. Fertil Steril 1996; 66(5):814-21.
34. Hart R, Magos A. Laparoscopically instilled fluid: the rate of absorption and the effects on patient discomfort and fluid balance. Gynaecol Endos 1996; 5:287-291.
35. van Westreenen M, van den Tol PM, Pronk A, et al. Perioperative lavage promotes intraperitoneal adhesion in the rat. Eur Surg Res 1999; 31(2):196-201.
36. Weinans MJ, Kauer FM, Klompmaker IJ, Wijma J. Transient liver function disturbances after the intraperitoneal use of 32% dextran 70 as adhesion prophylaxis in infertility surgery. Fertil Steril 1990; 53(1):159-61.
37. Gauwerky JF, Heinrich D, Kubli F. Complications of intraperitoneal dextran application for prevention of adhesions. Biol Res Pregnancy Perinatol 1986; 7(3):93-7.
38. Gilbert JA, Peers EM, Brown CB. Intra-peritoneal fluid dynamics of 4% icodextrin in non-ESRD patients. Perit Dial Int 1999; 19 (suppl 1):S79.
39. Mistry CD, Gokal R. The use of glucose polymer (icodextrin) in peritoneal dialysis: an overview. Perit Dial Int 1994; 14 Suppl 3:S158-61.
40. Raftery AT. Regeneration of parietal and visceral peritoneum. A light microscopical study. Br J Surg 1973; 60(4):293-9.
41. Harris ES, Morgan RF, Rodeheaver GT. Analysis of the kinetics of peritoneal adhesion formation in the rat and evaluation of potential antiadhesive agents. Surgery 1995; 117(6):663-9.
42. Cunliffe WJ, Sugarbaker PH. Gastrointestinal malignancy: rationale for adjuvant therapy using early postoperative intraperitoneal chemotherapy. Br J Surg 1989; 76(10):1082-90.

43. Rodgers KE, Verco SJ, diZerega GS. Effects of intraperitoneal 4% icodextrin solution on the healing of bowel anastomoses and laparotomy incisions in rabbits. Colorectal Dis 2003; 5(4):324-30.
44. Steller EP, Ottow RT, Eggermont AM, et al. Local conditions in the host influence immunotherapy with interleukin-2 and LAK cells. Cancer Detect Prev 1988; 12(1-6):81-90.

Chapter 10

General Discussion

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The aim of the studies described in this thesis is to examine the mechanisms by which the inflammatory reaction induced by surgical peritoneal trauma can induce both intra-abdominal adhesion formation and peritoneal carcinomatosis. Furthermore, with the knowledge obtained, we tested different treatment options. The present chapter will present a general discussion of our findings in view of the current literature. It includes the background of the thesis, main findings and suggestions for future work.

BACKGROUND

Within the first 2 years after potentially curative surgery for colorectal cancer or pancreatic cancer many patients develop locoregional are/or peritoneal recurrence. Peritoneal carcinomatosis is seen in 5% to 56% and locoregional recurrence in 60% of patients curatively treated for pancreatic cancer. In colorectal cancer locoregional recurrence is seldom encountered but 2 to 19% of patients develop peritoneal metastases. Peri-operatively in up to 42% of patients carcinoma cells can microscopically be found in the peritoneal cavity. These tumour cells are thought to be responsible for intraperitoneal recurrence in patients with colorectal and pancreatic carcinoma.[1-3] Yamamoto et al.[4] and Kanellos et al.[5] found positive cytology to be significantly correlated with the risk of intraperitoneal recurrence. The relative risk of intraperitoneal recurrence in those patients with positive cytology as opposed to patients with negative cytology was 16.5 and 2.9, respectively. Moreover studies have shown that positive cytology is correlated with impaired overall or disease-free survival and increases the risk of relapse post-resection. [3,6-8] Furthermore in up to 40% of patients, with curatively resectable coloncarcinoma, carcinoma cells can be detected in blood samples taken perioperatively.[9] Detection of circulating tumour cells has shown to be related to a significantly higher risk of postoperative metastasis.[10-12] Hepatic metastasis develop in 8% to 30% of patients after curative colon resection and approximately 8% to 10% of patients develop pulmonary metastases after curative resection of the primary colorectal tumour.[13] For pancreatic carcinoma, disseminated tumour cells are detected in up to 47% of patients during potentially curative resection. The detection of disseminated tumour cells was related to disease stage and overall survival.[14]

Another major postoperative problem in abdominal surgery remains the formation of peritoneal adhesions. Complications related to adhesion formation are described to occur in up to 30% of patients after colon surgery.[15,16] Some surgeons report morbidity rates of 51% and mortality rates of 5% in patients operated upon for small bowel obstruction due to adhesion formation.[17] In the USA alone 1.3 billion dollar was spend on procedures related to postoperative intra-abdominal adhesion formation in 1994.[18] Reducing post-operative adhesion formation is socially as well as economically essential.

Previous studies at our laboratory suggested a common denominator for adhesion formation and peritoneal carcinomatosis.[19,20] A relationship was found between the amount of

surgical trauma and the number of post-operative adhesions formed as well as the extend of tumour load found in a peritoneal tumour cell seeding model. Furthermore it was possible to captivate surgery related factors in lavage fluids and if transferred to naïve recipients these factors enhanced peritoneal tumour recurrence in the peritoneal tumour cell-seeding model.[20]

The mechanisms responsible for postoperative adhesion formation and peritoneal tumour recurrence are only partly clarified. To be able to prevent or reduce these surgical "complications" a thorough knowledge of the underlying process is necessary.

MAIN FINDINGS

Peritoneal tumour recurrence

Several studies have suggested that the presence of exfoliated tumour cells in the peritoneal cavity after surgery for colorectal or pancreatic cancer, which is considered minimal residual disease, might adversely influence relapse free and overall survival.[21,22] For a tumour cell, to development into a metastasis, depends on several successive processes, such as adhesion, implantation, proliferation and neo-angiogenesis. Whether the post-surgical wound microenvironment, however, mainly affects tumour cell adhesion, growth of tumour cell clusters, or both processes remained unclear. Experimental data suggest that wound-healing mediators affect both tumour cell adhesion and growth, though involved mechanisms are largely unknown.[23,24] However, in most experimental models, tumour cells were introduced during or shortly after surgery. Consequently, effects on tumour cell adhesion and growth of adhered tumour cell clusters might both have contributed to development of the observed tumour load. The study presented in chapter 3 analysed the presented dilemma. We demonstrated an important role for surgical trauma in facilitating successful tumour cell implantation, but not for enhancing growth of established tumour cell clusters. Based on the observations that tumour load was significantly enhanced when seeding the tumour cells on the day of surgery, rather then if the tumour cells had already adhered prior to surgery (no change in tumour load compared to non-operated rats). When analysing the intra-abdominal sites where the tumour load was found, the main sites were the directly peritoneally traumatized areas. This in accordance to other studies demonstrating the favourable adhesion of tumour cells to traumatized areas where the extra-cellular matrix (ECM) is exposed.[25,26]

Two other remarkable observations were made in this study. Firstly not only peritoneal surgical trauma could enhance tumour cell adhesion/implantation, but also distant trauma could enhance tumour cell adhesion/implantation to the peritoneum. Suggesting that the enhanced tumour cell adhesion/implantation found cannot be explained by exposure of the ECM due to surgical peritoneal trauma alone. Several authors emphasize the importance of adhesion molecules on mesothelial cells.[24,27-29] The presented observations also suggest an important role of tumour cell adhesion directly to the mesothelial cell, stimulated by systemic

inflammatory response. Secondly, the effect of surgical trauma to enhancing peritoneal tumour load was even higher in rats operated upon the day before tumour cell seeding in both laparotomized rats as in the thoracotomized rats. Upon stimulation, enhanced expression of adhesion molecules, especially intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), is partially postponed due to upregulation of the production of adhesion molecules. Hereby delaying the stimulating effect of the surgical inflammatory reaction on mesothelial cell to tumour cell adhesion (also chapter 5). Another explanation might be enhanced survival of free tumour cells due to a generalized immune suppression seen after surgery.[30-32] Hereby offering more tumour cells the opportunity to survive and adhere. This is a realistic opportunity as the tumour cell-line used (CC531s) is weakly immunogenic.[33] Probably a combination of factors will fully address the observations made.

In perspective of earlier studies[20,24] and the observation made in chapter 3 the post-operative inflammatory response plays a mayor role in the development of peritoneal carcinomatosis. After peritoneal trauma, surgery related factors in the abdominal cavity could be captivated in a lavage fluid and if transferred to naïve recipients these factors enhanced peritoneal tumour recurrence.[20] In chapter 4 the lavage fluid is separated in a cellular and supernatant fraction. Especially the cellular fraction showed to enhance tumour load in naïve, non-operated recipients. More detailed analysis of the cellular fraction of these lavage fluids revealed a trauma related influx of PMN in the abdominal cavity. Effective inhibition of tissue injury by PMN has been achieved by neutralising PMN chemoattractants[34], blocking of PMN adhesion molecules[35,36] and scavenging of reactive oxygen species.[37,38] We showed that a selective reduction of post-traumatic PMN influx, without causing immune suppression, was possible and could indeed significantly lower intra-peritoneal tumour load. However when interference was not only confined to the intra-peritoneal influx of PMN, but also compromised blood lymphocyte, monocyte and PMN counts, an enhanced tumour load was found. This result is in accordance with the observations made in chapter 3. With this observation modulating the post-operative influx of PMN might prove to be hazardous, with clear undesirable side effects if handled carelessly. Influencing the mechanism by which the PMN act has shown to be effective as well. Van Rossen et al.[39] were able to reduce post-operative intra-peritoneal tumour recurrence by scavenging the reactive oxygen species (ROS) produced by PMN.

The results of the studies described in chapter 3 and 4 implicate an important role of PMN/ROS and adhesion of tumour cells in the stimulation of post-operative peritoneal carcinomatosis. To further test this hypothesis we used an in-vitro tumour cell adhesion model. With this model we demonstrates that ROS could stimulate tumour cell adhesion to the peritoneum in a human in-vitro model. The mechanism by which ROS are able to enhance tumour cell adhesion most likely is dual. First, ROS are able to directly damage the peritoneal lining with subsequently exposing the ECM (chapter 5; enhanced apoptosis of

mesothelial cells and chapter 8; direct cytotoxic effects).[40,41] The ECM is a preferential site of tumour cell adhesion as demonstrated again in chapter 3. Directly traumatized peritoneal sites, at which the mesothelial lining has been damaged, showed the most tumour load, as shown in the laparotomy groups (lap –1 and lap 0 groups). Several in vitro studies analysed the adhesive properties of colon carcinoma cell lines (CC531, HT29, SW480) and pancreas carcinoma cell lines (BxPc3, SW1990, Panc-1, PaCa-2) to the ECM.[27,42-46] An important role in this process is played by integrins[28], which are expressed on tumour cells (colon as well as pancreatic). Antibodies directed against β1-integrin reduced tumour cell adhesion to the ECM and directly to mesothelial cells in vitro and inhibited peritoneal dissemination in a nude mouse model.[27]

Secondly, in chapter 5, we demonstrated that ROS could enhance the expression of adhesion molecules on mesothelial cells. On stimulation with ROS, the expression of the adhesion molecules VCAM-1, ICAM-1 and CD44H on mesothelial cells were enhanced. Hosono et al.[27] demonstrated the role of CD44H in the initial attachment of pancreas carcinoma cells to mesothelial cells, strengthening the hypothesis that enhanced tumour cell adhesion due to ROS will at least partly be caused by tumour cell to mesothelial cell adhesion. Adhesion of tumour cells to the mesothelium will probably be mediated through several adhesion molecules. Ziprin et al.[47] for instance demonstrated that ICAM-1 blockade only reduced the ability of both pancreatic and colon cancer cell lines to adhere to the mesothelium.

The majority of patients with colorectal cancer present at a stage when the primary cancer can be resected with curative intent. However, despite the high respectability rate, about 30-50% of these patients subsequently develop metastatic disease. As discussed in this thesis 3 – 19% is seen as peritoneal metastasis. But a major role is played by distant metastasis to liver and lung. In accordance to the findings in this thesis, a clear relationship has been shown between surgical trauma and enhanced distant tumour recurrences. Mainly adhesion of circulating tumour cells showed to be stimulated by surgical trauma.[48] Ten Kate et al.[49-51] further explored possible mechanism by which surgery enhances distant metastasis to the lung. A striking analogue can be seen in the mechanisms involved in peritoneal - and endothelial cell to carcinoma cell adhesion. Surgery provokes a local as well as a systemic inflammatory response in which leucocytes are activated with the release of inflammatory mediators and ROS. These factors modulate the adhesive interactions between tumour cells and the peritoneum/endothelium. VCAM-1 and ICAM-1 showed to be upregulated, by TNF-α, IL-1β and ROS, on endothelial cells.[50,51] Similar to those upregulated on mesothelial cells (chapter 5). With the difference that E-selectin possibly is involved in endothelial cell to tumour cell interaction as well and for mesothelial cell to tumour cell interaction, CD44h is involved. Probably a complex of adhesion molecules is responsible for the enhanced adhesion of tumour cells to mesothelial/endothelial cells during surgical trauma.

The crucial role of the inflammatory reaction provoked by surgical trauma in the pathogenesis of peritoneal carcinomatosis (and adhesion formation) is emphasized in literature and several aspects have been studied in this thesis. With the current knowledge treatment modalities have been suggested with modification of components of the inflammatory reaction. A study by van Rossen et al.[39] showed a reduction in peritoneal recurrence using scavengers. An important mechanism by which scavengers might reduce peritoneal recurrence has been explored in chapter 5. In chapter 5 we showed the influence of ROS on the adhesion of tumour cells to mesothelial cells. The enhanced adhesion seen, after stimulating mesothelial cells with ROS, could be reduced by the addition of the scavengers, SOD and catalase. The influence of pro-inflammatory cytokines and ROS on the adhesion and development of metastases have been shown in several in-vitro and in-vivo studies.[24,29,47,49,51] It has been demonstrated that cyclo-oxygenase-2 (COX-2) is highly induced by a number of cytokines, including IL-1 and TNF-α.[52] COX-2 inhibitors have been found to inhibit production of pro-inflammatory cytokines.[53] Selective COX-2 inhibitors have been and still are promising drugs in a variety of research fields. Guvenal et al.[52] and Greene et al.[54] showed that intra-peritoneal as well as oral administration of COX-2 inhibitors were able to reduce adhesion formation. Inhibition of COX-2 has also shown to be effective in reducing tumour growth in a nude mice implantation model that involved of COX-2 expressing tumour cells.[55,56] Roh et al.[57] demonstrated that the selective COX-2 inhibitor celecoxib repressed growth of subcutaneous tumours that were implanted adjacent to surgical wounds. In chapter 6 we analysed the effect of, the selective COX-2 inhibitor, celecoxib in our tumour cell-seeding model. Orally administered high dose celecoxib could almost abolish peritoneal carcinomatosis in our experiments. Much publicity has recently been addressed to the unwanted side effects of selective COX-2 inhibitors. Specifically long term use of COX-2 inhibitors increases the risk for adverse cardiovascular events.[58,59] However, short-term clinical trials with selective COX-2 inhibitors in patients with for example arthritis and adenomatous polyposis did not increase cardiovascular risk.[60,61] If proven to be equally effective in reducing peritoneal carcinomatosis (and adhesion formation) in humans, the selective COX-2 inhibition could be used as peri-operative prophylaxis for surgical patients. The likelihood of cardiovascular events (if any in short-term use) must be weighed against the beneficial effects.

Adhesion formation

For post-operative tumour recurrence we showed an important role of the post-operative influx of PMN and their products, ROS, in the post-operative inflammatory response and the effect on peritoneal carcinomatosis. As the post-operative inflammatory response is also correlated to adhesion formation, we analysed the role of PMN and ROS on post-operative intra-abdominal adhesion formation (chapter 8). Attempts at decreasing adhesion formation by reducing the influx of inflammatory cells has been considered previously with variable

results.[62-65] Vural et al.[65] showed beneficial effects of cyclophosphamide on adhesion formation. Cyclophosphamide suppresses humoral as well as cellular immune responses at bone marrow level and the known side effects of cyclophosphamide renders it unavailable in general clinical practice.[66] In our experiments we demonstrated a reduction in post-operative adhesion formation by selectively inhibiting the influx of PMN. Still, selectively reducing the number of PMN remains a troublesome procedure with possible systemic effects on the number of blood PMN, resulting in an immune compromised patient in the direct post-operative period, which have shown to be detrimental to peritoneal carcinomatosis (chapter 4). PMN generally act by generating ROS, especially when activated. Per-operative treatment with the anti-oxidants superoxide dismutase and catalase significantly reduced adhesion formation in our surgical model. The effects of ROS on adhesion formation were considered. In-vitro ROS showed to have a direct cytotoxic effects and apoptosis of the mesothelial cells was induced. Upon surgical trauma to the peritoneum the subsequent influx of PMN with their ROS induce a further damage of the mesothelial lining extending beyond the damage created during surgery and enhancing the possibility to create postoperative adhesions, even at intra-abdominal site where no direct peritoneal trauma was inflicted. Furthermore ROS influence the fibrinolytic properties of the mesothelial cells by inhibiting fibrinolysis. ROS stimulate the release of PAI-1 by mesothelial cells. Holmdahl et al.[67] already emphasized the important role of PAI-1 produced by the mesothelium in their studies.

Adept has recently be shown to be a save agent to use in humans in the AREAL study.[68] Adept is a 4% icodextrin solution, which has proved to be an effective anti-adhesive agent in animal models and is currently being analysed in the clinical setting. The working mechanism of icodextrin is said to be mainly due to hydroflotation and no medicinal properties are ascribed to icodextrin. In chapter 9 we tried to get an impression of the influence of icodextrin on, the previously accounted important factor in adhesion formation, ROS and on the mesothelial cell. Icodextrin did show to have medicinal properties as well. Icodextrin showed to have beneficial anti-oxidant properties, but an adverse effect of icodextrin on the fibrinolytic properties of the mesothelial cell was seen. Reverting these results to the in-vivo findings, the anti-oxidant properties seem to be too delicate to influence post-operative peritoneal tumour development and although the fibrinolytic ability of the mesothelial cell is disturbed a reduction in postoperative adhesion formation is seen. The hydroflotation is of more relevance in-vivo.

At present Seprafilm is the only anti-adhesive agent available that has shown to be able to reduce the number of complications due to adhesion formation. Although the incidence of adhesions or the incidence of small bowel obstruction did not change when using seprafilm in the clinical setting, the severity of adhesions was less and seprafilm is the only anti-adhesive agent, which has shown to be able to reduce the number of operations for small bowel obstruction in a randomised controlled trial.[69,70] With the use of the knowledge

obtained in this thesis and studies performed by other research groups, new randomised controlled clinical trials should be performed.

FUTURE PERSPECTIVES

Surgical trauma induces intra-abdominal adhesion formation and provides favourable circumstances for locoregional tumour recurrence. This thesis emphasized and further explored the importance of the peritoneal and systemic inflammatory response to surgical trauma. Locoregional tumour recurrence and adhesion formation have both shown to be related to the early post-operative inflammatory response, creating the possibility to cross use obtained knowledge in tackling these postoperative “complications”. Unfortunately, when evaluating current general clinical practice the peri/post-operative period is hardly used as therapeutic window to prevent/reduce tumour recurrence or adhesion formation. By exploring different components of the early post-operative inflammatory response, well-founded interventions can be made to reduce adhesion formation and locoregional tumour recurrence.

In order to reduce tissue trauma and tumour recurrence, minimal invasive surgery is promoted and proved promising in rat models.[20,71] In the clinical setting though, the expected reduction in recurrences and benefit in survival has not yet been shown. A recent meta-analysis reported faster postoperative recovery with laparoscopic surgery for colorectal cancer compared to open surgery, though in this meta-analysis no differences were seen in recurrence rates.[72] However, a few studies publishing long-term results did show a favourable trend towards laparoscopy, looking at recurrence rates.[73] Lezoche et al.[74] could not show a difference in recurrence rate and survival between laparoscopic or open surgery at a mean follow-up of 5 years. Interestingly, it did show a significant delay in the time at which recurrences presented in the laparoscopic group. Taking the experimental results and the clinical trials concerning laparoscopic surgery into account, preventing unnecessary surgical trauma should have high priority for every surgeon, especially in oncological surgery.

For a tumour cell, to development into a metastasis, depends on several successive processes, such as adhesion, implantation, proliferation and neo-angiogenesis. As shown in chapter 3 an essential role in the *post-surgical* tumour recurrence is enhanced adhesion of tumour cells either to ECM components or directly to the mesothelial cell. Taking the results of chapter 3 into account our focus of attention directed to prevention of *adhesion* of tumour cells in the peri-operative period. In vitro studies have shown upregulation of adhesion molecules by pro-inflammatory cytokines (TNF-α, IL-1β) and in chapter 5 ROS enhanced expression of adhesion molecules on mesothelial cells as well. It is conceivable that prevention/reduction of tumour cell adhesion after surgical trauma can be achieved by

maintaining the mesothelium in a refractory state. Scavenging ROS proved valuable in protecting mesothelial cells and preventing activation of mesothelial cells, concerning adhesion molecule expression and apoptosis. As the importance of ROS has been demonstrated, clinically applicable scavengers should be explored and eventually evaluated in the clinical setting. For instance, by the use of high dose L-ascorbic acid (readily available and cheap), peri-operatively. Another venue would be selectively blocking adhesion molecules or integrins, although the consequences on the immune system should be extensively tested in in-vivo experimental models, first. Furthermore, the currently available antibodies will proof to be a very expensive venue.

Directly influencing the PMN influx has shown to be a hazardous pathway (chapter 4). A delicate balance exists between trying to selectively influencing the post-operative inflammatory response in order to reduce tumour recurrence and creating troublesome immune suppression in the organism. For extending our knowledge on the evolution of post-operative tumour recurrence it showed to be of importance and therefore further exploration, by influencing different immune cells is important. But clinical implications should be made with care.

Another key inflammatory regulator is COX-2, which showed to be of influence in tumour development (chapter 6). Although, highly selective COX-2 inhibitors have been topic of discussion in recent literature, as it carries the risk of cardiovascular side effects in long-term users. Still, its effect on tumour development and tumour recurrence (chapter 6), and as short-term use of COX-2 inhibitors has not shown to be associated with major side effects, grants further exploration. Focus of attention should also be on the mechanism by which COX-2 inhibitors influences tumour development/recurrence. The tumour cell itself has shown to be able to produce COX-2. COX-2 catalyses the conversion of arachidonic acid to prostaglandin, which in turn facilitates tumour progression by stimulating cellular proliferation and angiogenesis, inhibiting apoptosis, enhancing cellular invasiveness, and modulation of immunosuppression[75]. Different carcinomas have shown to produce different levels of COX-2, indicating that COX-2 inhibitors have a possible direct effect on tumour development. In this thesis COX-2 inhibitors were used because of its anti-inflammatory properties, influencing the post-operative inflammatory response. Celecoxib (used in chapter 6), in specific, not only influences COX-2 expression, but also possesses anti-angiogenic properties that might influence tumour recurrence. In the current experiments no anti-angiogenic effect could be seen, but long-term experiments might proof otherwise.

For post-surgical intra-abdominal adhesion formation, careful surgical technique including gentle tissue handling; meticulous haemostasis; excision of necrotic tissue; minimizing ischaemia and desiccation; the use of fine, nonreactive suture materials and prevention of foreign-body reaction and infection can help to reduce, but cannot prevent. Adhesion prevention might be reduced by preventing the introduction of reactive foreign bodies, by reducing the local inflammatory response, by inhibiting the coagulation cascade and/or

promoting fibrinolysis, or by creating barriers between damaged peritoneal surfaces. This thesis explored two different pathways in reducing adhesion formation. By the use of scavengers, modulating/down-tuning the local inflammatory response and by creating a physical barrier between damaged peritoneal sites (hydroflotation).

Influencing the local inflammatory response was successfully evaluated in chapter 8 (and 4 and 5), by studying ROS in the peri-operative period. Not only a reduction in tumour adhesion/recurrence can be seen with the use of scavengers, but also a reduction in adhesion formation can be achieved. Further exploration should include the use of readily available scavengers, e.g. L-ascorbic acid in the clinical setting, possibly combined with another anti-adhesive agent.

Influencing the coagulation cascade and/or promoting fibrinolysis have been studied by the use of heparin, streptokinase or urokinase.[76] However, peritoneal irrigation with these solutions strokes with clinical practice, as possible side effects like bleeding are feared. Low molecular weight heparins are currently being explored as side effects are expected to be less. Results of animal experimental studies show promising results.[77]

The main area of interest has been in developing local barriers. Hyaluronic acid (Sepracoat), polyethylene glycol (Spraygel), fibrin glue (Tissuecol), oxidized regenerated cellulose (Interceed), expanded polytetrafluoroethylene (ePTFE) and hyaluronic acid and carboxymethylcellulose film (Seprafilm) have been developed and explored. Seprafilm is the only currently available barrier that has shown to reduce the number of operations needed for small bowel obstruction.[69,70] The main disadvantage of local barriers remains the fact that the surgeon has to predict the areas where the adhesions will be formed. Therefore barrier solutions like adept or sepracoat should be more the centre of interest.

Future research should focus on developing a carrier solution with barrier properties that is able to locally apply chemicals which carefully modulates overshoots in the inflammatory response and stimulates re-mesothelialization of the damaged peritoneal surfaces. The solution will probably not be found in an agent attacking only one aspect of adhesion formation.

REFERENCES

1. Koppe MJ, Boerman OC, Oyen WJ, Bleichrodt RP. Peritoneal carcinomatosis of colorectal origin: incidence and current treatment strategies. Ann Surg 2006;243:212-22.
2. Nakao A, Fujii T, Sugimoto H, Kanazumi N, Nomoto S, Kodera Y, Inoue S, Takeda S. Oncological problems in pancreatic cancer surgery. World J Gastroenterol 2006;12:4466-72.
3. Vogel I, Kruger U, Marxsen J, Soeth E, Kalthoff H, Henne-Bruns D, Kremer B, Juhl H. Disseminated tumor cells in pancreatic cancer patients detected by immunocytology: a new prognostic factor. Clin Cancer Res 1999;5:593-9.
4. Yamamoto S, Akasu T, Fujita S, Moriya Y. Long-term prognostic value of conventional peritoneal cytology after curative resection for colorectal carcinoma. Jpn J Clin Oncol 2003;33:33-7.
5. Kanellos I, Demetriades H, Zintzaras E, Mandrali A, Mantzoros I, Betsis D. Incidence and prognostic value of positive peritoneal cytology in colorectal cancer. Dis Colon Rectum 2003;46:535-9.
6. Lloyd JM, McIver CM, Stephenson SA, Hewett PJ, Rieger N, Hardingham JE. Identification of early-stage colorectal cancer patients at risk of relapse post-resection by immunobead reverse transcription-PCR analysis of peritoneal lavage fluid for malignant cells. Clin Cancer Res 2006;12:417-23.
7. Guller U, Zajac P, Schnider A, Bosch B, Vorburger S, Zuber M, Spagnoli GC, Oertli D, Maurer R, Metzger U, et al. Disseminated single tumor cells as detected by real-time quantitative polymerase chain reaction represent a prognostic factor in patients undergoing surgery for colorectal cancer. Ann Surg 2002;236:768-75; discussion 75-6.
8. Wind P, Norklinger B, Roger V, Kahlil A, Guin E, Parc R. Long-term prognostic value of positive peritoneal washing in colon cancer. Scand J Gastroenterol 1999;34:606-10.
9. Weitz J, Kienle P, Lacroix J, Willeke F, Benner A, Lehnert T, Herfarth C, von Knebel Doeberitz M. Dissemination of tumor cells in patients undergoing surgery for colorectal cancer. Clin Cancer Res 1998;4:343-8.
10. Koch M, Kienle P, Kastrati D, Antolovic D, Schmidt J, Herfarth C, von Knebel Doeberitz M, Weitz J. Prognostic impact of hematogenous tumor cell dissemination in patients with stage II colorectal cancer. Int J Cancer 2006;118:3072-7.
11. Koch M, Kienle P, Hinz U, Antolovic D, Schmidt J, Herfarth C, von Knebel Doeberitz M, Weitz J. Detection of hematogenous tumor cell dissemination predicts tumor relapse in patients undergoing surgical resection of colorectal liver metastases. Ann Surg 2005;241:199-205.
12. Wang JY, Wu CH, Lu CY, Hsieh JS, Wu DC, Huang SY, Lin SR. Molecular detection of circulating tumor cells in the peripheral blood of patients with colorectal cancer using RT-PCR: significance of the prediction of postoperative metastasis. World J Surg 2006;30:1007-13.
13. Shah SA, Haddad R, Al-Sukhni W, Kim RD, Greig PD, Grant DR, Taylor BR, Langer B, Gallinger S, Wei AC. Surgical resection of hepatic and pulmonary metastases from colorectal carcinoma. J Am Coll Surg 2006;202:468-75.
14. Soeth E, Grigoleit U, Moellmann B, Roder C, Schniewind B, Kremer B, Kalthoff H, Vogel I. Detection of tumor cell dissemination in pancreatic ductal carcinoma patients by CK 20 RT-PCR indicates poor survival. J Cancer Res Clin Oncol 2005;131:669-76.
15. Parker MC, Ellis H, Moran BJ, Thompson JN, Wilson MS, Menzies D, McGuire A, Lower AM, Hawthorn RJ, O'Briena F, et al. Postoperative adhesions: ten-year follow-up of 12,584 patients undergoing lower abdominal surgery. Dis Colon Rectum 2001;44:822-29; discussion 29-30.
16. Nieuwenhuijzen M, Reijnen MM, Kuijpers JH, van Goor H. Small bowel obstruction after total or subtotal colectomy: a 10-year retrospective review. Br J Surg 1998;85:1242-5.
17. Landercasper J, Cogbill TH, Merry WH, Stolee RT, Strutt PJ. Long-term outcome after hospitalization for small-bowel obstruction. Arch Surg 1993;128:765-70; discussion 70-1.

18. Ray NF, Denton WG, Thamer M, Henderson SC, Perry S. Abdominal adhesiolysis: inpatient care and expenditures in the United States in 1994. J Am Coll Surg 1998;186:1-9.
19. van den Tol MP, van Stijn I, Bonthuis F, Marquet RL, Jeekel J. Reduction of intraperitoneal adhesion formation by use of non-abrasive gauze. Br J Surg 1997;84:1410-5.
20. 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;227:242-8.
21. Schott A, Vogel I, Krueger U, Kalthoff H, Schreiber HW, Schmiegel W, Henne-Bruns D, Kremer B, Juhl H. 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;227:372-9.
22. Bosch B, Guller U, Schnider A, Maurer R, Harder F, Metzger U, Marti WR. Perioperative detection of disseminated tumour cells is an independent prognostic factor in patients with colorectal cancer. Br J Surg 2003;90:882-8.
23. 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;25:231-43.
24. van Rossen ME, Hofland LJ, van den Tol MP, van Koetsveld PM, Jeekel J, Marquet RL, van Eijck CH. Effect of inflammatory cytokines and growth factors on tumour cell adhesion to the peritoneum. J Pathol 2001;193:530-7.
25. Jacobi CA, Ordemann J, Bohm B, Zieren HU, Liebenthal C, Volk HD, Muller JM. The influence of laparotomy and laparoscopy on tumor growth in a rat model. Surg Endosc 1997;11:618-21.
26. Jansen M, Treutner KH, Jansen PL, Otto J, Schmitz B, Mueller S, Weiss C, Tietze L, Schumpelick V. Phospholipids reduce the intraperitoneal adhesion of colonic tumor cells in rats and adhesion on extracellular matrix in vitro. Int J Colorectal Dis 2004;19:525-32.
27. Hosono J, Narita T, Kimura N, Sato M, Nakashio T, Kasai Y, Nonami T, Nakao A, Takagi H, Kannagi R. Involvement of adhesion molecules in metastasis of SW1990, human pancreatic cancer cells. J Surg Oncol 1998;67:77-84.
28. Schlaeppi M, Ruegg C, Tran-Thang C, Chapuis G, Tevaearai H, Lahm H, Sordat B. 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;4:439-55.
29. van Grevenstein WM, Hofland LJ, Jeekel J, van Eijck CH. The expression of adhesion molecules and the influence of inflammatory cytokines on the adhesion of human pancreatic carcinoma cells to mesothelial monolayers. Pancreas 2006;32:396-402.
30. Allendorf JD, Bessler M, Whelan RL, Trokel M, Laird DA, Terry MB, Treat MR. Postoperative immune function varies inversely with the degree of surgical trauma in a murine model. Surg Endosc 1997;11:427-30.
31. Ben-Eliyahu S, Page GG, Yirmiya R, Shakhar G. Evidence that stress and surgical interventions promote tumor development by suppressing natural killer cell activity. Int J Cancer 1999;80:880-8.
32. 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;12:421-9.
33. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. Int J Cancer 1984;33:689-92.
34. Zagorski J, Wahl SM. Inhibition of acute peritoneal inflammation in rats by a cytokine-induced neutrophil chemoattractant receptor antagonist. J Immunol 1997;159:1059-62.
35. Walzog B, Weinmann P, Jeblonski F, Scharffetter-Kochanek K, Bommert K, Gaehtgens P. A role for beta(2) integrins (CD11/CD18) in the regulation of cytokine gene expression of polymorphonuclear neutrophils during the inflammatory response. Faseb J 1999;13:1855-65.

36. Andreoli SP, Mallett C, Williams K, McAteer JA, Rothlein R, Doerschuk CM. Mechanisms of polymorphonuclear leukocyte mediated peritoneal mesothelial cell injury. Kidney Int 1994;46:1100-9.
37. Wang P, Chen H, Qin H, Sankarapandi S, Becher MW, Wong PC, Zweier JL. Overexpression of human copper, zinc-superoxide dismutase (SOD1) prevents postischemic injury. Proc Natl Acad Sci U S A 1998;95:4556-60.
38. Shandall AA, Williams GT, Hallett MB, Young HL. Colonic healing: a role for polymorphonuclear leucocytes and oxygen radical production. Br J Surg 1986;73:225-8.
39. van Rossen ME, Sluiter W, Bonthuis F, Jeekel H, Marquet RL, van Eijck CH. Scavenging of reactive oxygen species leads to diminished peritoneal tumor recurrence. Cancer Res 2000;60:5625-9.
40. Breborowicz A, Witowski J, Wieczorowska K, Martis L, Serkes KD, Oreopoulos DG. Toxicity of free radicals to mesothelial cells and peritoneal membrane. Nephron 1993;65:62-6.
41. Stadlmann S, Raffeiner R, Amberger A, Margreiter R, Zeimet AG, Abendstein B, Moser PL, Mikuz G, Klosterhalfen B, Offner FA. Disruption of the integrity of human peritoneal mesothelium by interleukin-1beta and tumor necrosis factor-alpha. Virchows Arch 2003;443:678-85.
42. Oosterling SJ, van der Bij GJ, Bogels M, ten Raa S, Klaverweide JR, Post JA, Jeekel J, Meijer GA, Beelen RH, Meijer S, et al. Anti β1-integrin antibody reduces surgery-induced adhesion of CC531 colon carcinoma cells to traumatized peritoneal surfaces. Submitted.
43. Lohr M, Trautmann B, Gottler M, Peters S, Zauner I, Maier A, Kloppel G, Liebe S, Kreuser ED. Expression and function of receptors for extracellular matrix proteins in human ductal adenocarcinomas of the pancreas. Pancreas 1996;12:248-59.
44. Sawai H, Takeyama H, Yamamoto M, Furuta A, Funahashi H, Okada Y, Sato M, Tanaka M, Manabe T. Enhancement of integrins by interleukin-1alpha, and their relationship with metastatic and invasive behavior of human pancreatic ductal adenocarcinoma cells. J Surg Oncol 2003;82:51-6.
45. Haier J, Nasralla M, Nicolson GL. Different adhesion properties of highly and poorly metastatic HT-29 colon carcinoma cells with extracellular matrix components: role of integrin expression and cytoskeletal components. Br J Cancer 1999;80:1867-74.
46. Laurich C, Wheeler MA, Iida J, Neudauer CL, McCarthy JB, Bullard KM. Hyaluronan mediates adhesion of metastatic colon carcinoma cells. J Surg Res 2004;122:70-4.
47. Ziprin P, Ridgway PF, Pfistermuller KL, Peck DH, Darzi AW. ICAM-1 mediated tumor-mesothelial cell adhesion is modulated by IL-6 and TNF-alpha: a potential mechanism by which surgical trauma increases peritoneal metastases. Cell Commun Adhes 2003;10:141-54.
48. Aalbers AGJ, ten Kate M, van Grevenstein WMU, Hofland LJ, Wiemer EAC, Jeekel J, van Eijck CHJ. Liver surgery enhances implantation of circulating tumour cells, but does not stimulate growth of tumour cells. Thesis: The influence of surgery on the development of distant tumour recurrence 2007:27-44.
49. Ten Kate M, Hofland LJ, van Grevenstein WM, van Koetsveld PV, Jeekel J, van Eijck CH. Influence of proinflammatory cytokines on the adhesion of human colon carcinoma cells to lung microvascular endothelium. Int J Cancer 2004;112:943-50.
50. Ten Kate M, Hofland LJ, van Koetsveld PM, Jeekel J, van Eijck CH. Pro-inflammatory cytokines affect pancreatic carcinoma cell. Endothelial cell interactions. Jop 2006;7:454-64.
51. Ten Kate M, van der Wal JB, Sluiter W, Hofland LJ, Jeekel J, Sonneveld P, van Eijck CH. The role of superoxide anions in the development of distant tumour recurrence. Br J Cancer 2006;95:1497-503.
52. Guvenal T, Cetin A, Ozdemir H, Yanar O, Kaya T. Prevention of postoperative adhesion formation in rat uterine horn model by nimesulide: a selective COX-2 inhibitor. Hum Reprod 2001;16:1732-5.
53. Bernareggi A. Clinical pharmacokinetics of nimesulide. Clin Pharmacokinet 1998;35:247-74.
54. Greene AK, Alwayn IP, Nose V, Flynn E, Sampson D, Zurakowski D, Folkman J, Puder M. Prevention of intra-abdominal adhesions using the antiangiogenic COX-2 inhibitor celecoxib. Ann Surg 2005;242:140-6.

55. Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, Beauchamp RD, DuBois RN. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J Clin Invest 1997;99:2254-9.
56. Williams CS, Watson AJ, Sheng H, Helou R, Shao J, DuBois RN. Celecoxib prevents tumor growth in vivo without toxicity to normal gut: lack of correlation between in vitro and in vivo models. Cancer Res 2000;60:6045-51.
57. Roh JL, Sung MW, Park SW, Heo DS, Lee DW, Kim KH. Celecoxib can prevent tumor growth and distant metastasis in postoperative setting. Cancer Res 2004;64:3230-5.
58. Juni P, Nartey L, Reichenbach S, Sterchi R, Dieppe PA, Egger M. Risk of cardiovascular events and rofecoxib: cumulative meta-analysis. Lancet 2004;364:2021-9.
59. Levesque LE, Brophy JM, Zhang B. The risk for myocardial infarction with cyclooxygenase-2 inhibitors: a population study of elderly adults. Ann Intern Med 2005;142:481-9.
60. Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, et al. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. Jama 2000;284:1247-55.
61. Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 2000;342:1946-52.
62. Zeyneloglu HB, Seli E, Senturk LM, Gutierrez LS, Olive DL, Arici A. The effect of monocyte chemotactic protein 1 in intraperitoneal adhesion formation in a mouse model. Am J Obstet Gynecol 1998;179:438-43.
63. Ar'Rajab A, Dawidson I, Sentementes J, Sikes P, Harris R, Mileski W. Enhancement of peritoneal macrophages reduces postoperative peritoneal adhesion formation. J Surg Res 1995;58:307-12.
64. 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;61:143-6.
65. Vural B, Canturk NZ, Esen N, Solakoglu S, Canturk Z, Kirkali G, Sokmensuer C. The role of neutrophils in the formation of peritoneal adhesions. Hum Reprod 1999;14:49-54.
66. Otterness IG, Chang YH. Comparative study of cyclophosphamide, 6-mercaptopurine, azathiopurine and methotrexate. Relative effects on the humoral and the cellular immune response in the mouse. Clin Exp Immunol 1976;26:346-54.
67. Holmdahl L. The role of fibrinolysis in adhesion formation. Eur J Surg Suppl 1997:24-31.
68. Menzies D, Pascual MH, Walz MK, Duron JJ, Tonelli F, Crowe A, Knight A. Use of icodextrin 4% solution in the prevention of adhesion formation following general surgery: from the multicentre ARIEL Registry. Ann R Coll Surg Engl 2006;88:375-82.
69. Fazio VW, Cohen Z, Fleshman JW, van Goor H, Bauer JJ, Wolff BG, Corman M, Beart RW, Jr., Wexner SD, Becker JM, et al. Reduction in adhesive small-bowel obstruction by Seprafilm adhesion barrier after intestinal resection. Dis Colon Rectum 2006;49:1-11.
70. Vrijland WW, Tseng LN, Eijkman HJ, Hop WC, Jakimowicz JJ, Leguit P, Stassen LP, Swank DJ, Haverlag R, Bonjer HJ, et al. Fewer intraperitoneal adhesions with use of hyaluronic acid-carboxymethylcellulose membrane: a randomized clinical trial. Ann Surg 2002;235:193-9.
71. 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;10:490-4.
72. Reza MM, Blasco JA, Andradas E, Cantero R, Mayol J. Systematic review of laparoscopic versus open surgery for colorectal cancer. Br J Surg 2006;93:921-8.
73. Lacy AM, Garcia-Valdecasas JC, Delgado S, Castells A, Taura P, Pique JM, Visa J. Laparoscopy-assisted colectomy versus open colectomy for treatment of non-metastatic colon cancer: a randomised trial. Lancet 2002;359:2224-9.

74. Lezoche E, Guerrieri M, De Sanctis A, Campagnacci R, Baldarelli M, Lezoche G, Paganini AM. Long-term results of laparoscopic versus open colorectal resections for cancer in 235 patients with a minimum follow-up of 5 years. Surg Endosc 2006;20:546-53.
75. Backlund MG, Mann JR, Dubois RN. Mechanisms for the prevention of gastrointestinal cancer: the role of prostaglandin E2. Oncology 2005;69 Suppl 1:28-32.
76. Hellebrekers BW, Trimbos-Kemper TC, Trimbos JB, Emeis JJ, Kooistra T. Use of fibrinolytic agents in the prevention of postoperative adhesion formation. Fertil Steril 2000;74:203-12.
77. Kutlay J, Ozer Y, Isik B, Kargici H. Comparative effectiveness of several agents for preventing postoperative adhesions. World J Surg 2004;28:662-5.

Chapter 11

Summary and Conclusions

Samenvatting en Conclusies

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SUMMARY

Peritoneal carcinomatosis and postoperative intra-abdominal adhesion formation still are genuine problems after potentially curative intra-abdominal surgery. After potentially curative surgery for colorectal or pancreatic carcinomas peritoneal recurrence is seen up to 20% or 56% of patients, respectively. In 85 - 95% of patient's postoperative adhesion formation will occur after intra-abdominal surgery. It has been estimated that one third of small bowel obstructions and up to 20% of female infertility is caused by adhesions. The cumulative risk of adhesive small-bowel obstruction after (sub)total colectomy is 11-25% within 1 year, increasing to 30% at 10 years. The clinical implications of peritoneal carcinomatosis and post-operative adhesions are evident. The pathogenesis of the processes responsible for intra-abdominal tumour recurrence and postoperative adhesion formation is largely comparable, but is only partly clarified. To be able to prevent or reduce these surgical "complications" a thorough knowledge of the underlying process is necessary.

Chapter 1 includes the aims and outline of this dissertation.

In **chapter 2** a short review of the literature is presented addressing the clinical implications and the pathogenesis of peritoneal tumour recurrence. Furthermore the hypothesis of the influence of surgical trauma on peritoneal carcinomatosis is explained. Referring to previous studies performed at our laboratory showing the relationship between the amount of surgical trauma and the extent of tumour load. Still for most carcinoma, surgery is the only potentially curative treatment, but should be approached with care. Reducing surgical trauma, by avoiding unnecessary handling, manipulation or clamping of the tumour should continuously have the attention of the surgeon. Unravelling the mechanism, which stimulate postoperative tumour development may lead to new therapeutic strategies.

In **chapter 3** we analysed which process, adhesion and/or proliferation of seeded tumour cells, is mainly stimulated by the postoperative inflammatory reaction. Previous experimental data suggest that wound-healing mediators affect both tumour cell adhesion and growth. However, in most experimental models, effects on tumour cell adhesion and growth of adhered tumour cell clusters might both have contributed to the development of the observed tumour load. The performed experiments showed that the (post) surgical intra-peritoneal microenvironment enhances successful implantation of spilled tumour cells, whereas growth of adhered tumour cell clusters is not affected. Furthermore, this effect on intraperitoneal tumour cell implantation could also be seen after remote surgical trauma, indicating that also (post) surgical systemic factors play a role in the development of peritoneal carcinomatosis.

In **chapter 4**, we focus our attention on the individual capacity of inflammatory peritoneal cells and soluble factors to ascertain which element is mainly responsible for enhanced tumour recurrence. Peritoneal lavage fluids collected after intra-abdominal surgical trauma were collected and separated in a (neutrophil rich) cellular and supernatant component. By intraperitoneally injecting the separate components in naïve rats together with tumour cells, especially the neutrophil rich cellular component showed to induce tumour load. Secondly, assuming that post-traumatic intra-abdominal influx of neutrophils is possibly responsible for local tumour recurrence, prevention of neutrophil influx might influence this process. ANS was used to block the intraperitoneal influx of neutrophils, which resulted in a significant reduction of the peritoneal tumour load normally seen after surgical trauma. However when an overdose of ANS was used and the systemic immune response was suppressed as well an increase in tumour load was found.

Chapter 5 is a continuation of the studies performed in chapter 3 and 4. Using an *in-vitro* model the influence of the main effectors of neutrophils, reactive oxygen species (ROS), on mesothelial cells and tumour cells is studied. It is demonstrated that ROS stimulate tumour cell adhesion to the peritoneum in a human *in-vitro* model. The mechanism by which ROS are able to enhance tumour cell adhesion is dual. Firstly, a cytotoxic effect of ROS on mesothelial cells is demonstrated, hereby exposing the extra-cellular matrix, which has been shown to be a preferential site for tumour cells to adhere, Secondly, enhanced presentation of adhesion molecules on mesothelial cells is found upon stimulation of the cells with ROS. The latter might explain the enhanced tumour load found at not directly traumatized peritoneal sites.

Another key regulator of the inflammatory process is the enzyme cyclooxygenase-2 (COX-2). COX-2 catalyses the conversion from arachidonic acid to prostaglandins, which are potent inflammatory mediators. In **chapter 6** we demonstrate an impressive reduction in peritoneal tumour load using a high dose of celecoxib, a potent COX-2 inhibitor, peri-operatively. In review of recent publications, showing cardiovascular side effects to chronic COX-2 inhibitor use, the selective COX-2 inhibitor celecoxib might proof an interesting drug for short-term peri-operative use in a select group of patients. The benefits, if reproduced in the clinical setting, should be weighed against the side effects.

In **chapter 7** a short review of the literature is performed addressing the clinical implications and the pathogenesis of post-operative intra-abdominal adhesion formation. The formation of postoperative adhesions is a major cause of morbidity, resulting in multiple complications many of which manifest dozens of years after the initial inciting surgical event and add up to a significant part of health care costs. As treatment of adhesions is associated with an increased morbidity, prevention should be focus of attention. Peritoneal trauma is the basis

of adhesion formation after intra-abdominal surgery, therefore surgical trauma should always be minimized, for instance by the use of laparoscopy. Physical barriers between opposing damaged peritoneal sites are still being developed with varying results.

Chapter 8 focuses on the influence of neutrophils and its products, ROS, on postoperative adhesion formation. In a rat model the influx of neutrophils into the peritoneal cavity is inhibited, resulting in a significant reduction of adhesion formation. Also taking the results of chapter 4 into account, modulating the immune system is a hazardous venue. A more select approach, by locally scavenging its products, ROS, proved as efficient. The hydroxyl radical proved to be of less importance in adhesion formation than the superoxide radical and hydrogenperoxide.

Chapter 9 evaluates the adhesion preventing properties of icodextrin, a glucose polymer solution, and its effect on peritoneal metastasis. In our rat models icodextrin proved to be very effective in reducing postoperative adhesion formation, but no inhibitory or stimulating effect was seen on peritoneal metastasis. To further explore whether hydroflotation is the only mechanism by which icodextrin reduces adhesion formation several *in-vitro* experiments were performed. Icodextrin showed to have anti-oxidant properties, which, as shown in chapter 8, might beneficially influence adhesion formation. However icodextrin negatively influences the fibrinolytic properties of mesothelial cells, although this seems to be of less importance *in-vivo*.

CONCLUSIONS

- Surgery induces enhanced adhesion of disseminated tumour cells, but does not increase growth of already implanted tumour cell clusters
- The systemic inflammatory response influences intra-peritoneal tumour cell adhesion
- The postoperative peritoneal influx of neutrophils plays an important role in post-operative adhesion formation and peritoneal carcinomatosis
- Inhibiting the peritoneal influx of neutrophils without compromising the systemic immune response reduces post-surgical adhesions and peritoneal carcinomatosis
- Reactive oxygen species stimulate tumour cell adhesion to mesothelial cells by enhancing the expression of adhesion molecules
- Orally administered high dose celecoxib dramatically reduces intra-abdominal tumour cell adhesion and growth
- The fibrinolytic properties of mesothelial cells are compromised by reactive oxygen species
- Scavenging the products of neutrophils, reactive oxygen species, with anti-oxidants reduces postoperative intra-abdominal adhesion formation
- Icodextrin reduces post-operative intra-abdominal adhesions mainly through hydroflotation.

SAMENVATTING

Het locoregionaal recidief, peritoneale metastasering en postoperatieve intra-abdominale adhesievorming zijn nog altijd serieuze problemen in de in opzet curatieve intra-abdominale chirurgie. In de follow-up van in opzet curatieve operaties voor colorectale of pancreas tumoren worden bijvoorbeeld in respectievelijk 20% en 56% van de patienten peritoneale metastasen gevonden. Daarnaast worden postoperatieve adhesies gezien in 85 - 95% van de patienten na een intra-abdominale ingreep. In ongeveer 30% van de dunne darm obstructies en in 20% van de infertiele vrouwen ligt de oorzaak in postoperatieve intra-abdominale adhesievorming. De klinische consequenties van het locoregionaal recidief, peritoneale metastasen en postoperatieve adhesies is duidelijk. Het mechanisme achter het ontstaan van het postoperatieve recidief en van de postoperatieve adhesievorming is nog niet geheel duidelijk, maar er lijkt een duidelijke overlap te bestaan in de pathogenese. Bij het zoeken naar een oplossing voor deze postoperatieve “complicaties” is het essentieel om de onderliggende pathogenese te bestuderen.

Hoofdstuk 1 zet de doelstelling van deze thesis uiteen.

In **hoofdstuk 2** wordt een korte review gegeven van de literatuur, met betrekking tot de pathogenese en de klinische consequenties van peritoneale tumor recidivering. Daarnaast wordt de hypothese met betrekking tot de invloed van chirurgisch trauma op het ontstaan van het periteneale recidief uiteengezet. Hierbij wordt gerefereerd naar eerdere studies verricht op ons laboratorium, waarbij een duidelijke relatie werd gevonden tussen de mate van intra-abdominaal chirurgisch trauma en de uitgebreidheid van de tumor. Echter is chirurgie vaak de enige manier om een patient curatief te behandelen. De operatie moet dan ook zo min mogelijk traumatiserend voor het weefsel verlopen. Bijvoorbeeld door onnodige manipulatie van de omringende structuren en de tumor te voorkomen tijdens de operatie. Uiteindelijk wordt geconcludeerd dat door het verder analyseren van het mechanisme waarmee chirurgie de postoperatieve recidivering stimuleert, er mogelijk nieuwe strategieen kunnen worden ontwikkeld om dit mechanisme te doorbreken.

In **hoofdstuk 3** wordt bestudeerd of door chirurgisch trauma met name de adhesie van tumorcellen aan het peritoneum wordt gestimuleerd of dat juist groei van reeds geimplanteerde tumor cellen wordt gestimuleerd. In de literatuur wordt ervan uitgegaan dat de stoffen welke vrijkomen tijdens de wondgenezing zowel tumorcel aanhechting als groei stimuleren. Echter de opzet van deze experimenten maakt het niet mogelijk om onderscheidt te maken tussen deze twee processen. Hier is specifiek rekening mee gehouden in het experiment beschreven in hoofdstuk 2 en het blijkt dat met name adhesie van vrije tumorcellen wordt gestimuleerd. Er wordt geen groei stimulerend effect gezien op reeds

geimplanteerde tumorcellen. Dit effect wordt niet alleen gezien bij intra-abdominaal chirurgisch trauma, maar adhesie wordt ook gestimuleerd wanneer het chirurgisch trauma wordt verricht buiten de peritoneaal holte. Ook systemische factoren beinvloeden dus de adhesie van tumorcellen aan het peritoneum. Het immuunsuppressieve effect van een operatie, waardoor meer tumorcellen overleven en dus de mogelijkheid krijgen om aan te hechten, zou hier een rol bij kunnen spelen.

Tijdens de postoperatieve ontstekingsreactie worden verschillende ontstekingscellen naar de peritoneaal holte getrokken en worden vele cytokinen, chemokinen en groeifactoren geproduceerd. In **hoofdstuk 4** wordt gekeken naar welk van deze onderdelen met name verantwoordelijk is voor het tumor stimulerende effect. Hiervoor werden buikspoelingen verricht bij intra-abdominaal geopereerde ratten. De spoelvloeistof werd vervolgens gescheiden in een cellulaire fractie en een supernatant (cytokinen etc.). Door de gescheiden componenten vervolgens tesamen met tumorcellen intra-peritoneaal toe te dienen bij niet geopereerde ratten kon het effect van beide componenten worden bestudeerd. Met name de neutrofiel rijke cellulaire fractie stimuleerde de tumor. De neutrofiel lijkt dus een belangrijke rol te spelen. In het vervolg experiment werd de postoperatieve influx van neutrofielen geremd met anti-neutrofielen serum (ANS). Wanneer de influx van de neutrofiel naar de peritoneaalholte selectief werd geblokkeerd werd een significante reductie gezien in tumorvolume, echter wanneer te veel ANS werd toegedient trad ook een systemisch suppressie op van alle afweercellen, met als gevolg juist een stimulatie van het tumorvolume.

Hoofdstuk 5 is een vervolgstudie op hoofdstuk 3 en 4, alleen wordt nu een humaan model gebruikt. Het effect van zuurstofradicalen (het belangrijkste product van de neutrofiele granulocyt) op humane mesotheelcellen en de pancreas tumorcellijn Panc-1 wordt geanalyseerd. In een *in-vitro* tumorcel adhesie model kan de aanhechting van Panc-1 worden gestimuleerd door de mesotheelcellen bloot te stellen aan zuurstofradicalen. Het mechanisme waarmee zuurstofradicalen deze stimulatie kunnen bewerkstelligen is tweeledig. Allereest bestaat er een direct cytotoxisch effect op de mesotheelcel, hierdoor komt de extra-cellulaire matrix bloot te liggen, wat voor tumorcellen een voorkeursplaats is om aan te hechten. Ten tweede wordt gedemonstreerd dat er een verhoogde expressie van adhesiemolekulen plaatsvindt wanneer de mesotheelcellen in aanraking komen met zuurstofradicalen. Dit laatste mechanisme zou een verklaring kunnen zijn voor het feit dat ook op onbeschadigd peritoneum een verhoogde tumorvolume wordt gezien na chirurgisch trauma.

Een op dit moment omstreden groep medicijnen zijn de hoog selectieve cyclo-oxygenase-2 (COX-2) remmers. COX-2 speelt een belangrijke rol in de initiatie van de post-operatieve

ontstekingsreactie. Daarnaast is aangetoond dat COX-2 in verhoogde mate voorkomt in kankerweefsel. In **hoofdstuk 6** wordt een indrukwekkende resultaat gedemonstreerd, waarbij nauwelijks tumoren gezien worden in de met hoge orale dosering COX-2 remmer behandelde groep. De ophef rond deze groep middelen heeft met name betrekking op de cardiovasculaire bijwerking bij chronisch gebruik. Echter dergelijke effecten zijn niet beschreven bij gebruik voor een beperkte perioden en zal alleen geindiceerd zijn voor een selecte populatie. De voordelen, mits de experimentele resultaten in de klinische setting kunnen worden gereproduceerd, zouden heel goed kunnen opwegen tegen de bijwerkingen.

In **hoofdstuk 7** wordt een korte review gegeven van de literatuur, met betrekking tot de pathogenese en de klinische consequenties van postoperatieve intra-abdominale adhesievorming. Het ontstaan van postoperatieve adhesies brengt aanzienlijke morbiditeit met zich mee. Tot lange tijd na de initiele operatie kunnen complicaties optreden van adhesies en adhesies veroorzaken dan ook voor significante kosten voor de gezondheidszorg. Voorkomen is beter dan genezen, aangezien het behandelen van complicaties van adhesies aanzienlijke morbiditeit met zich meebrengt. Beschadiging van het peritoneum staat ten grondslag aan de vorming van adhesies en de mate van peritoneale schade zal dan ook zo minimaal mogelijk gehouden moeten worden, bijvoorbeeld door gebruik te maken van laparoscopische chirurgie waar mogelijk. Vele middelen zijn ontwikkeld met wisselend en met name inconsistente resultaten.

Hoofdstuk 8 richt zich op de invloed van de postoperatieve influx van de neutrofiel en zijn effector, zuurstofradicalen, op de ontwikkeling van adhesies. In navolging op het model beschreven in hoofdstuk 4 wordt in een rat model de postoperatieve influx van neutrofielen geremd met ANS, met als gevolg een significante reductie in de vorming van adhesies. Mede gezien het nadelige effect dat moduleren van ontstekingscellen kan hebben, zoals gedemonstreerd in hoofdstuk 4, wordt vervolgens gekeken naar het effect van scavengers op adhesievorming. Hierbij wordt gezien dat toediening van superoxide dismutase en catalase ook een significante reductie in adhesie vorming kon bewerkstelligen. Het hydroxyl radicaal bleek minder van invloed te zijn.

Lokale barriers hebben als nadeel dat de chirurg moet voorspellen waar de meeste adhesie zullen ontstaan. Icodextrin is een glucose polymeer oplossing welke als vloeistof zich geheel door de buikholte verspreid. In **hoofdstuk 9** wordt gekeken naar de anti-adhesieve werking van icodextrin en of icodextin invloed heeft op peritoneale metastasen. In ons rattenmodel bleek icodextrin zeer efficient in het voorkomen van adhesies en werd geen effect gezien op peritoneale metastasering. Het meest waarschijnlijke mechanisme waarmee icodextrin werkt is “hydroflotatie”. Mogelijke andere invloeden van icodextrin op het peritoneum werden *in-vitro* bestudeerd. Het blijkt dat icodextrin milde scavenger eigenschappen bezit, welke

mogelijk bij zullen dragen aan de anti-adhesieve eigenschap (in navolging op de resultaten gezien in hoofdstuk 8). Echter bleek icodextrin een negatieve invloed te hebben op de fibrinolytische eigenschappen van de mesotheel cel, *in-vivo* lijkt dit van ondergeschikt belang.

CONCLUSIES

- Chirurgie beinvloedt met name de adhesie van vrij tumorcellen en heeft geen invloed op de groei van reeds geimplanteerde tumorcellen.
- De systemische ontstekingsreactie veroorzaakt door chirurgie beinvloedt intra-peritoneale tumorcel adhesie.
- De postoperatieve peritoneale influx van neutrofielen speelt een belangrijke rol in postoperatieve adhesie vorming en peritoneale metastasering
- Het selectief remmen van de influx van neutrofielen naar de peritoneaal holte zonder de systemisch immuniteit aan te tasten geeft een reductie in postoperatieve peritoneale metastasen en adhesie vorming.
- Zuurstofradicalen stimuleren de adhesie van tumorcellen aan het peritoneum door de expressie van adhesiemolekulen op mesotheelcellen te stimuleren.
- Een bijna volledige remming van het ontstaan van postoperatieve peritoneale metastasen wordt gezien bij orale hoge doses celecoxib rondom de operatie.
- De fibrinolytische eigenschappen van mesotheelcellen worden gecompromitteerd door zuurstofradicalen.
- Het scavengen van zuurstofradicalen geeft een reductie in de vorming van postoperatieve adhesies.
- Icodextrin verminderd postoperatieve adhesievorming met name door hydroflotatie.

Appendices

Dankwoord

List of Publications

Curriculum Vitae Auctoris

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Lieve Saskia, mijn antwoord op 12 en 19 mei 2007 staat vast. Vele reizen reeds samen gemaakt, maar deze reis zal de mooiste en langste zijn.

LIST OF PUBLICATIONS

S. ten Raa, M.R.H.M. van Sambeek, T. Hagenaars, H. van Urk. Management of aortic graft infection. *J Cardiovasc Surg* 2002; 43: 209-215.

S. ten Raa, M.P. van den Tol, J. Jeekel. Intra-abdominal adhesions in the experimental setting. *Adhesions: news and views* 2003; 2: 22-25.

S. ten Raa, D.J. Swank, J. Jeekel. Treatment of Complications of Abdominal Adhesions: Laparoscopy or Laparotomy? *Adhesions: news and views* 2004; 5: 12-15.

M.P. van den Tol, **S. ten Raa**, W.M.U. van Grevenstein, R. Marquet, J. Jeekel. Icodextrin reduces postoperative adhesion formation in rats without affecting peritoneal metastasis. *Surgery* 2005; 137(3): 348-354.

H.C. van der Wal, **S. ten Raa**, J. Jeekel. The impact of irrigants on the peritoneal cavity. *Adhesions: news and views* 2005; 8: 17-19.

S. ten Raa, S.J. Oosterling, N.P. van der Kaaij, M.P. van den Tol, R.H. Beelen, S. Meijer, C.H.J. van Eijck, J.R.M. van der Sijp, M. van Egmond, J. Jeekel. Surgery promotes implantation of disseminated tumor cells, but does not increase growth of tumor cell clusters. *J Surg Oncol.* 2005; 92(2): 124-129.

J.W. Burger, J.A. Halm, A.R. Wijsmuller, **S. ten Raa**, J. Jeekel. Evaluation of new prosthetic meshes for ventral hernia repair. *Surg Endosc.* 2006; 20(8): 1320-1325.

S. ten Raa, M.P. van den Tol, W. Sluiter, L.J. Hofland, C.H.J. van Eijck, J. Jeekel. The role of neutrophils and oxygen free radicals in post-operative adhesions. *J Surg Res.* 2006; 136(1): 45-52.

M.P. van den Tol, **S. ten Raa**, W.M.U. van Grevenstein, M.E.E. van Rossen, J. Jeekel, C.H.J. van Eijck. The post-surgical inflammatory response provokes enhanced tumour recurrence; a crucial role for neutrophils. *Accepted Dig Surg.*

W.M.U. van Grevenstein, A.G.J. Aalbers, **S. ten Raa**, W. Sluiter, L.J. Hofland, J. Jeekel, C.H.J. van Eijck. Surgery-derived reactive oxygen species produced by polymorphonuclear leucocytes promote tumour recurrence: studies in a in-vitro model. *J Surg Res.* epub dec 2006.

S. ten Raa, W.M.U. van Grevenstein, M. ten Kate, G. van ‘t Hof, K.M. Mangundap, L.J. Hofland, J.Jeekel, W. Sluiter, C.H.J. van Eijck. The influence of reactive oxygen species on the adhesion of pancreatic carcinoma cells to the peritoneum. *Submitted.*

S. ten Raa, S.J. Oosterling, G.J. van der Bij, H.C. van der Wal, J.A. Halm, R.H.J. Beelen, S. Meijer, M. van Egmond, J. Jeekel. Peri-operative celecoxib treatment prevents tumor outgrowth in an intra-abdominal surgical model. *Submitted.*

CURRICULUM VITAE AUCTORIS

Sander ten Raa was born on January 25th 1976 in Amersfoort. In 1994 he completed secondary school at the Farel College in Amersfoort. In the same year he started medical school at the medical faculty of the Erasmus University Rotterdam. Clinical rotations were started in September 1998 and he passed his medical qualifying exams in March 2001. Subsequently, he worked as a surgical resident at the department of General Surgery of the University Hospital Dijkzigt, Rotterdam (Prof. dr. J. Jeekel). In the meanwhile he applied for a research position at the Laboratory of Experimental Surgery in Rotterdam. In March 2002 he started working on this thesis under the supervision of prof. dr. J. Jeekel and dr. C.H.J. van Eijck. He started his surgical training at the “Medisch Centrum Rijnmond Zuid” in August 2004 (dr. E. van der Harst).