Chapter 8

General discussion

DISCUSSION

Despite the introduction of new treatment modalities for gastro-intestinal malignancies during the last decades, surgery remains the principal therapy for most gastro-intestinal malignancies, although the recurrence rates after apparently curative surgery are worrisome[1-6] .

For colon cancer, the recurrence rate after surgery with curative intend exceeds 40%, with preferential sites being the liver, lungs and loco-regionally[6-8]. After partial hepatectomy for liver metastases in colon cancer patients even 75% of patients develop tumour recurrence, most commonly within the remnant liver or lungs[9]. As the liver has the unique ability to regenerate to its original size in a relatively short period of time after resection[10,11], it has been the clinical impression for a long time that microscopic residual disease, undetected at the time of operation, might profit from this regeneration. It was hypothesized that the plethora of released growth factors during liver regeneration stimulates the growth of already implanted tumour cells at distant sites.

The first part of this thesis aimed at unravelling the responsible mechanisms of this hypothesis. However, this hypothesis could not be grounded, since Chapter 3 shows that partial liver resection did not result in enhanced growth of the tumour cells. Partial resection of the liver did induce enhanced distant tumour noduli in the lungs. However, this was not specific for partial liver resection, but applied for surgical procedures in general. Because of the short time-period in relation to surgery in which the enhanced tumour take was observed, an influence of surgery on tumour cell implantation seems the most likely explanation. This hypothesis is further supported by data from previous studies investigating the influence of surgery on loco-regional tumour recurrence[12-16]. In these studies, surgical trauma provoked a local inflammatory reaction that is responsible for enhanced loco-regional tumour cell implantation. The question remains if the inflammatory reaction provoked by surgical trauma enhances not only loco-regional tumour recurrences, but recurrences to distant sites as well.

Therefore, the focus of the remaining part of this thesis lay on the influence of surgical trauma on tumour cell implantation in distant organs.

CONSIDERATION OF EXPERIMENTAL MODELS

To investigate whether partial liver resection and surgical procedures in general affect patterns of tumour recurrences, we used *in vivo* rat models. In these models, rats underwent a partial liver resection, a partial ileum resection, a sham operation or no operation and tumour cells were administered at a certain time in relation to surgery. The endpoint was the

development of tumour nodules in the lungs. The different surgical procedures provide insight in the way surgical trauma influences distant tumour recurrence. Furthermore, by varying the time interval between surgery and tumour inoculation, this model differentiates in the contribution of tumour growth and tumour adhesion to enhanced distant tumour recurrence.

In vivo models are essential in investigating the pathology of physiologic processes and animal models permit advances with the use of experimental strategies that would be inappropriate in human studies[17]. However, diversity exists between animal and human physiology and therefore, the limits of these models should be recognized. *In vitro* cell culture studies can provide knowledge using human material. A disadvantage of *in vitro* models is the lack of physiological interactions, although *in vitro* models provide the opportunity to study a specific interaction without disturbance from unknown factors.

To study the interactions between human tumour cell lines and human endothelial cells, reproducible human *in vitro* models were developed. Since tumour cell implantation occurs in the microvasculature, monolayers of microvascular endothelial cells were used. These monolayers were exposed to inflammatory cells and mediators known to play a major role in inflammation and elevated in blood during and after surgery[18-24]. In this way the influence of the inflammatory sequelae, provoked by surgery, on the adhesion of human tumour cell lines to the microvasculature could be investigated without the interference of unknown factors. Next to the lack of unknown factors which may interfere with tumour cell adhesion in this *in vitro* model, mechanical forces like blood flow and shear forces are absent as well. Several studies demonstrated that these forces have an impact on tumour cell adhesion, although more in a quantitative than a qualitative manner[25,26].

Comparable adhesion models were developed to study the adhesion of human tumour cell lines to the extracellular matrix. Each organ is unique in the composition of matrix and the matrix components studied in this thesis constitutes an important part of the matrix surrounding the microvasculature. This model enables to study tumour cell adhesion to each component of the matrix separately, thereby giving insight in the role of each component in adhesion.

SURGICAL TRAUMA AND DISTANT TUMOUR RECURRENCE

A major clinical problem is the high tumour recurrence after intentionally curative surgery for most gastro-intestinal malignancies. Since the majority of recurrences occur relatively in a short time period after surgery[27], many clinicians believed that surgery in itself act upon tumour recurrence. During surgery, resection handling of the primary tumour causes an increased detachment of tumour cells resulting in an enhancement of circulating tumour cells[28,29]. The higher detection rate of circulating tumour cells in it self may procure an

increase in haematogenous metastasis, although the development of distant metastases is a highly inefficient process. Creating an environment more beneficial to the tumour cell is essential for enhanced tumour recurrence. In chapter 2 we show that surgical procedures positively modulate the conditions for tumour cells to form metastases, resulting in enhanced distant tumour recurrences. Surgery brings about a range of growth factors[30] that may influence the growth of implanted tumour cells at distant sites. Although enhanced tumour growth may play a role in enhanced tumour recurrence after surgery, we were not able to detect a growth promoting effect in our *in vivo* and *in vitro* experiments.

The step in the metastatic cascade that most likely benefit from the surgical modulation is tumour cell implantation in a distant organ, involving tumour cell – endothelial cell interactions. The question is in which way surgical trauma promotes enhanced tumour cell implantation. Next to the growth factors produced by surgical procedures, these procedures also provoke an inflammatory reaction with the activation of inflammatory cells and release of inflammatory mediators. From previous *in vivo* animal studies we know that the inflammatory reaction enhances loco-regional tumour recurrence and therefore, the inflammatory reaction may enhance distant tumour recurrence as well.

Another factor beneficial for tumour recurrence may be the diminished immune function induced by major surgical trauma. The dysfunction of the immune apparatus during and shortly after surgery may enable tumour cells to survive from host defence mechanisms. In the *in vivo* experiment we utilized the CC531 colon carcinoma cell line, which is weakly immunogenic. Since the cells are only weakly immunogenic, the contribution of diminished immune function to enhanced tumour recurrence may not be more than marginal in our model.

TUMOUR CELL ADHESION

Because tumour growth and immune dysfunction seems not to be the chief responsible mechanism of surgery-induced tumour recurrence, our attention concentrated on the adhesion phase. In the human in vitro adhesion models we investigated the influence of surgery-derived factors on tumour cell – endothelial cell interactions. An important inflammatory mediator abundantly present after surgery is interleukin 6 (IL-6) [23]. In chapter 3, we exposed microvascular endothelial cells to this pro-inflammatory cytokine to investigate if it modulates the adhesion of tumour cells to microvascular endothelial cells. However, no change in adhesion was observed. Two other important pro-inflammatory cytokines of the inflammatory reaction are IL-1β and TNF-α. In low concentrations these cytokines act as tumour promoters, but in higher concentrations they are tumoricidal[31]. So is high dose TNF-α used in isolated limb or liver perfusion as a tumoricidal agent[32,33], but post-operative blood levels of TNF-α do not reach sufficient levels to be lethal for tumour cells.

In lower concentrations these cytokines activate the endothelium, thereby inducing an up-regulation of adhesion molecules on the endothelial cells and in this way more binding places for tumour cells are created. From literature and also from our studies it is known that the binding of both colon and pancreas carcinoma cells to cytokine-activated HUVEC, which are macrovascular endothelial cells, is E-Selectin dependent[34,34-37]. However, we found that the binding of these tumour cells to activated microvascular endothelial cells is not E-Selectin dependent. Other important adhesion molecules on these cells, like ICAM-1 and VCAM-1, are not responsible as well for the tumour cell binding, although the up-regulation of their expression coincides with the adhesion pattern. Another yet unknown adhesion molecule or perhaps a complex of adhesion molecules may be responsible for tumour cell binding to microvascular endothelial cells. The discrepancy between macro- and microvascular endothelial cells necessitates studying interactions using the latter, since the adhesion occurs in the microcirculation. Further experiments therefore were carried out with microvascular endothelial cells.

The first leukocytes triggered by inflammation are the polymorphonuclear leukocytes (PMN)[38]. By the production of reactive oxygen species, these PMN are found to affect local tumour recurrence after surgical trauma[12]. Also in distant tumour recurrence these PMN may play a role, since we found that they enhance the adhesion of both colon and pancreas carcinoma cells to the microvasculature (chapter 4). The production of reactive oxygen species by PMN is the main mechanism by which these leukocytes enhance tumour cell adhesion, since the addition of anti-oxidant enzymes largely abrogate the enhanced tumour adhesion. Although the amount of added anti-oxidant enzymes was sufficient to dismutate the produced reactive oxygen species, the level of tumour cell adhesion did not reach basal levels following the addition of these enzymes. Next to the production of cytokines and proteases by activated PMN that can interfere with adhesion, another possibility is the dismutation of superoxide anion and hydrogen peroxide into other reactive oxygen radicals like the hydroxyl radical that are not scavenged by the used anti-oxidants.

Comparable to the cytokines, reactive oxygen species induce an up-regulation of adhesion molecules corresponding to the pattern of tumour cell adhesion. Exposure to the cytokines and reactive oxygen species lead to apoptosis among other things in endothelial cells. Apoptotic cells are in a highly activated state with an increase in adhesion molecule expression[37,39,40], which may be the mechanism in enhanced tumour adhesion in inflammation.

Modulations of the tumour cells by inflammation were not detected. The range of most promising adhesion molecules on the tumour cells is not up-regulated by inflammatory mediators and exposure of the cells to these mediators did not affect the adhesion to endothelial cells. It is thought that tumour cells do have a high intrinsic activity state and therefore are not influenced by additional activators[41].

The inflammatory sequelae promote tumour cell adhesion to the endothelium, causing the endothelial cells to retract and so enable the tumour cells to invade this layer. Underneath the endothelial cell layer lays the extracellular matrix. We demonstrated that the tested tumour cells have a high affinity for components of this matrix. Because the affinity of the tumour cells for extra cellular matrix components exceeds the affinity for the endothelium, an invasion gradient is created into the organ.

FUTURE DIRECTIONS

Progress is being made in the unravelling of mechanisms by which surgical trauma promotes distant tumour recurrences. However, several aspects of the tumour promoting effect remain underexposed.

In vivo, we found that surgery enhances distant tumour recurrence. Preceding studies demonstrated a loco-regional tumour promoting effect by surgery in which reactive oxygen species are the most potent perpetrators. At this moment, a model will be developed in which the effect of anti-oxidant enzymes on the development of distant tumour recurrence after surgery will be evaluated. Contrary to the previous *in vivo* models in which rats are used together with the rat CC531 colon carcinoma cell line, this new model exploits nude mouses in which the human tumour cell lines, used in our *in vitro* assays, can be evaluated. Furthermore, it will be interesting to investigate whether the addition of functional antibodies against the pro-inflammatory cytokines IL-1β and TNF-α diminish the enhanced distant tumour recurrence after surgery in this *in vivo* nude mouse model. In literature, promising data are published from *in vivo* studies using anti-TNF-α antibodies[42].

Another point of interest is the effect of tumour cell binding to the endothelium and subsequently to the extracellular matrix, since by this binding a range of processes will be activated. Interesting is if tumour cell binding activates the production of the matrix metalloproteinases (MMP). The MMP are pivotal in successful tumour cell implantation in a distant organ, since these enzymes are necessary in the breakdown of the extracellular matrix enabling tumour cells to invade this layer and next settle in the tissue and grow out to form a metastasis[43,44]. *In vitro* assays looking at the MMP production by tumour cell adhesion to endothelial cells and the effect of inflammatory mediators on this production may provide information regarding the step in the metastatic cascade subsequent to the adhesion step.

CLINICAL APPLICATION

Treatment modalities like chemotherapy, radiotherapy, radiofrequency ablation and photodynamic therapy caused progress in the management of malignancies affecting the gastro-intestinal system, with longer survival or disease free interval[45]. Most of these treatment modalities are aimed at abrogating manifest tumour foci locally or at distant sites. However, further improvements are required demanding to focus on other steps in the metastatic cascade. For haematogenous metastasis, an interesting step in the metastatic cascade to focus on is the implantation of circulating tumour cells in distant organs.

Enhanced distant tumour recurrence is the result of the up-regulation of adhesion molecules on the endothelium. Preventing adhesion of circulating tumour cells with the use of monoclonal antibodies might prove difficult, since the profile of adhesion molecules on tumour cells shows heterogeneity among different tumours and further is the adhesion in such a way complex involving a number of adhesion molecules that the use of a single antibody will not prohibit adhesion. Binding to components of the extracellular matrix is accomplished by integrins. Many of the integrins share an affinity toward the RGD recognition sequence in their matrix ligands. Peptides containing this sequence interfere with integrin function and proved to block experimental metastasis[46].

From previous studies as well as from this thesis, we know that the inflammatory reaction caused by surgery promotes the implantation of circulating tumour cells in distant organs. The inflammation activates inflammatory cells like the PMN and monocytes with the production of inflammatory mediators. Patients undergoing surgery need these cells in the defense against pathogens and to recover from surgical trauma, and therefore, depletion of these cells as well as the produced cytokines will impede normal wound healing and disturb the balance in inflammatory mediators inducing unknown side effects. TNF-α antagonists are already used in clinical studies for several inflammatory conditions and side effects include injection site and intravenous reactions as well as an increased risk of infection[47]. Long term side effects are unknown at the moment. Other products redundantly present after surgery are the reactive oxygen species. The body has an elaborate defense system to protect itself from the deleterious effects of oxidative stress. This includes catalytic antioxidants (such as superoxide dismutases (SODs), glutathione peroxidases, and catalase) and low molecular weight antioxidants such as atocopherol, ascorbate, and glutathione. However, these defenses are often overwhelmed in many pathophysiological states, like in surgical trauma. Administration of exogenous anti-oxidants as therapeutic agent is therefore promising. Anti-oxidants in preservation fluids have already earned credits in diminishing ischaemia-reperfusion injury. Clinical studies investigating the use of orally vitamin C and E in ischemia-reperfusion injury are currently under way[48] as well as in vivo animal studies investigating the use of several anti-oxidants systemically[49].

Other ways of interfering with the inflammatory reaction will be promising. The ubiquitous transcription factor NF-kappaB is a central regulator in the expression of a number of pro-inflammatory genes and adhesion molecules. Targeting NF-kappaB will form a promising selective therapeutic intervention and is currently studied in several studies[50].

Another promising target may be the COX-2 receptor, constitutively expressed in many human cancers including colon, gastric and pancreatic cancer and up-regulated in stromal and inflammatory cells by cytokines and other mediators[51]. There are nowadays indications that COX-2 inhibitors not only reduce the risk of many human cancers but diminish tumour recurrence as well. These observations are based on both animal studies as well as clinical studies[52-54].

Another therapeutic option is to interfere with the invasion of tumour cells through the extracellular matrix. This step is necessary for tumour cells leaving the primary tumour and again if the tumour cell has to invade into a distant organ. The matrix metalloproteinases play a crucial role in the invasion and therefore, MMP-inhibitors may be a powerful tool in preventing these steps[55]. Indeed, down-regulation of the expression of tissue inhibitors of metalloproteinases (TIMP) is associated with increased invasiveness while overexpression leads to reduced tumour growth and metastasis[56,57]. Moreover, a phase II study using a synthetic MMP inhibitor in advanced pancreatic cancer and a phase III gastric cancer study are underway[58,59]

Prevention of tumour cells to enter the circulation during surgery will be ideal to decrease haematogenous metastases. In this light the 'no touch isolation technique' was developed[60]. This technique implies the vascular ligation before manipulation of the tumour during the surgical resection. Although tumour cell dissemination can be reduced in this way, the benefit in terms of improved patient survival remains unproven[61,62].

Finally, operative techniques itself may influence the recurrence rates. The extent of surgical trauma correlates with the degree of the inflammatory reaction that is provoked[18]. Studies clearly demonstrate that the inflammatory reaction provoked by laparoscopic procedures by far do not reach the level of the reaction provoked by laparotomy[22]. In severe inflammation more inflammatory mediators are present compared to mild inflammation. Therefore, major surgical trauma exceeds minor surgical trauma in the promotion of distant tumour recurrence[63] and in this way less traumatic operative procedures will be beneficial to the oncologic patient. Currently, this advantageous aspect of laparoscopy was not revealed in clinical studies comparing laparoscopic and open (hemi)colectomy for colon cancer, since recurrence rates for distant metastases did not differ significantly[64,65]. However, a trend was observed for a lower local recurrence rate after laparoscopy compared to laparotomy[66].

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