INTRODUCTION PART II

ROLE OF MACROPHAGES IN SURGICALLY INDUCED LIVER METASTASES FORMATION

CHAPTER 4

Introduction

With over 700.000 new cases each year, colorectal cancer (CRC) is the second most common malignancy in the western world. Although advances in CRC treatment have increased 5-year relative survival rates from 51% in 1970 to approximately 67% nowadays, CRC still leads to over 300.000 deaths in western society [1], making it the fourth most common cause of death from cancer[1-3]. The basis of curative therapy of CRC is surgical excision of the primary tumour and this is critical for improved survival[2, 4]. Unfortunately colorectal liver metastases (CLM) are detected in approximately 20-25% of CRC patients at time of diagnosis, and with a median survival of 1 year, prognosis of non-treated patients with CLM is extremely poor [5]. Moreover, post-surgical development of metastases is a frequent complication and 25-45 % of patients without detectable CLM at time of diagnosis, will subsequently develop local or distant metastases after removal of the primary tumour within 5 years [3, 6, 7].

Metastasis of CRC often occurs through the portal circulation and therefore favours the development of liver metastases that accounts 70% of colorectal-related deaths [8]. During normal metastases formation, tumour cells undergo a multistep process which enables them to escape the primary tumour and facilitates them towards the portal circulation. Surgery however, can bypass these processes and directly lead to physical dissemination of tumour cells into the portal circulation.[9-11] Therefore an alternative route of surgery induced metastasis of CRC was proposed (figure 1) [12].

Surgery itself therefore seems to act as a double edged sword, on the one hand it is imperative to insure curative therapy, whereas on the other it enables the formation of distant CLM. In this introduction I focus on the role of macrophages in the development of surgery-induced liver metastases and how macrophages can become a therapeutic target against surgery-induced metastases formation.

Surgery-Induced metastases development

Over the years evidence has accumulated, which paradoxically supports that trauma, inflicted by surgical procedures to excise primary tumours, is associated with risk of developing metastases [4, 13, 14]. By imitating surgery procedures in animal models it was shown that surgical trauma enhances loco-regional metastases with preferential outgrowth at injured sites [15, 16]. Furthermore correlated laparoscopy, which induces a milder severity of trauma compared to total laparotomy, to a lower tumour load, suggesting that severity of surgical trauma correlates with the amount of tumour load [17, 18]. Surgical trauma results in systemic alterations which stimulates tumour development in distant organs [19-21]. Thoracotomy (surgery of the chest) for example enhances peritoneal outgrowth of colorectal metastases [22] and increased liver metastases outgrowth was observed as a consequence of abdominal surgery in animal models [12]. Thus, surgery induces both local and systemic changes that facilitate metastases development. Moreover did we previously demonstrate that surgical trauma induced a systemic effect leading to enhanced tumour cell adhesion of circulating tumour cells but not to enhance growth of existing tumour cell clusters [22].

Despite the overwhelming evidence from experimental studies is the notion that live saving surgery itself enhances formation of distant recurrences in patients still a subject of debate. As surgery is mandatory for patient survival it is unethical and impossible to study the effects of surgery in a randomized clinical trial. The limited amount of clinical data on this phenomenon comes from analyses of ancient databases of breast cancer patients. It was demonstrated that patients who did not have surgery showed one peak for death around the fourth and fifth year, whereas in patients who underwent mastectomy, 2 peaks were observed around 3 to 4 years after surgery and one 8 years after surgery [23, 24]. In the greater part of the patient population, surgery elongating life expectancy, whereas surgery led in a smaller subset of patients to shortened survival. Thus, surgery, although greatly reducing tumour mass and is potentially curative, paradoxically can also augment metastases development in some patients.

Tumour cell dissemination

Methods to isolate circulating malignant cells from the blood of CRC patients have been described since 1960-70s [25] and free circulating tumour cells have been detected, in 10-70% of patients with primary colorectal cancer depending on the method of detection [26-28]. Nowadays the presence of free circulating tumour cells has been proposed as an independent prognostic factor for survival of CRC patients [29, 30].

The notion that surgery of mamma carcinomas leads to enhanced dissemination of free tumour cells into the circulation was already hypothesized in the beginning of the last century by William Halsted [31]. Experiments in animal models aptly showed that dissemination of tumour cells into the circulation occurs spontaneously from existing solid tumours, which is enhanced after manipulation of the tumour [32 33]. The augmentation of free circulating tumour cells in the circulation, peritoneal cavity or the liver after and during surgery of the primary tumour has also been extensively described in human patients [34-36]. Thus, loss of cell-cell adhesion and mechanical handling of tumours during surgical removal may permit tumour cells spillage. Furthermore, increased numbers of circulating tumour cells in the portal system have been associated with decreased overall survival [11, 36, 37] and is a strong predictor of CRC recurrence [26, 38].

These studies not only indicate that examination of disseminated tumour cells in blood after surgery can serve as a prognostic tool, but also provide evidence that surgical resection results in an increase of exfoliated tumour cells, which can develop into metastases. However, implantation of circulating tumour cells is a highly inefficient process and most circulating tumour cells are rapidly removed by the immune system before they can initiate in metastases formation. Therefore, spillage of tumour cells during operation cannot fully explain the heightened incidence of metastases development after surgery. Surgery unavoidably leads to tissue injury, which initiates a systemic stress response that encompasses a wide range of endocrinologic, immunologic, and hematologic effects. It is now clear that the systemic inflammatory responses after surgery also contribute to heightened tumour development via several mechanisms:

Post-surgical immune suppression

After an initial pro-inflammatory acute phase response directly after surgery, characterized by increased IL1β, TNFα, IL6 and CRP secretion [39-41], compensatory anti-inflammatory mediators are released [42]. An unbalanced systemic compensatory response to acute phase responses may result in immune suppression, which can last for several days [42, 43] and renders the patient susceptible for post-operative infections and hampered anti-tumour immunity [42, 44-47]. Moreover, the severity of trauma, blood transfusion, anaesthetics and psychological stress were demonstrated to play a role in the magnitude and duration of immune suppression [18, 40, 42].

An imbalance in pro- and anti-inflammatory immune responses after surgery may hamper antitumor cytotoxicity and is thought to facilitate metastases outgrowth.

Enhanced tumour growth

An increased proliferation rate of tumour cells is another suggested mechanism explaining the rapid tumour recurrence after CRC resection. Factors secreted by the primary tumour may hamper growth of dormant metastases [48] and after resection of the primary tumour distant metastases show reduced apoptosis and increased proliferation [49, 50]. Thus, surgery may induce growth of earlier undetected dormant metastases due to the loss of the suppressive capacity of the primary tumour.

Tumour cell adhesion through reactive oxygen species mediated cell damage

The enhanced acute phase response during and immediately after surgery initiates a systemic inflammation leading to rapid activation of innate immune cells and subsequently to increased IL1β, TNFα and IL6 production. These pro-inflammatory cytokines can, by enhancing expression of adhesion molecules like ICAM-1, VCAM-1 and E-selectin on endothelial cells, increase adhesion of tumour cells *in vitro* and *in vivo* [51-54]. In the liver this enhanced tumour cell adhesion to endothelial cells was shown to be dependent on activation of macrophages. Subsequently led macrophage depletion to a decreased adhesion molecule expression and lessened tumour cell adhesion [52]. Although incubation with pro-inflammatory cytokines eventually leads to enhanced tumour cell adhesion to endothelial cells, this effect only occurs after 2-4 hours of cytokine incubation [51, 52]. Maximal adhesion of tumour cells to activated endothelial cells is observed 12 hours after pro-inflammatory

cytokine incubation [51]. A significant increase in tumour cell accumulation in liver vasculature is observed already 45 minutes after surgery [12]. This implicates that surgical trauma leads to a different mechanism of tumour cell adhesion to liver vasculature. In earlier animal studies we showed that surgery of the abdominal cavity not only led to damage of mesothelial monolayers of the peritoneal wall but also to impairment of liver vasculature [12, 16]. A hallmark of an acute inflammatory response, as induced during surgery, is the rapid release of reactive oxygen species (ROS) by innate immune cells like neutrophils and macrophages [55, 56]. High local concentrations of ROS during acute inflammation in the liver can lead to hepatocyte cell death [55] and incubation with ROS enhanced expression of adhesion molecules ICAM-1 and VCAM-1 on mesothelial cells [57]. Exposing endothelial cell layers to high local ROS doses leads to detachment en subtraction of endothelial cells and subsequently to enhanced adhesion of tumour cells to exposed underlying extracellular matrix proteins in *in vitro* assays [58]. By imitating surgical procedures in animal models it was demonstrated that surgical trauma also *in vivo* results in retraction and detachment of mesothelial cells or sinusoidal endothelial lining [12, 16]. Retraction of endothelial or mesothelial cells results in the formation of intercellular gaps and subsequent exposure of the extracellular matrix (ECM) proteins which serve as preferable adhesion sites for tumour cells [16, 58].

During tumour development even small tumours can release large numbers of malignant cells into the circulation [59, 60] which however do not survive and are quickly cleared from the circulation. Free floating tumour cells are subjected to intense mechanical stress by shear forces through contact between intact endothelial lining and tumour cells in narrow capillaries. These shear stress forces and tumour cell shape-deformations are lethal to the majority of malignant cells [61, 62]. During and after surgery however, precisely these shear stress forces enable disseminated tumour cells to come in close contact to the now available sub-endothelial ECM proteins in the liver vasculature [63]. Because tumour cells express a wide array of adhesion molecules of which integrins are especially important during haematological metastasising [64-66], the availability of sub-endothelial ECM in the liver vasculature after surgery enhances the adhesion of disseminated malignant cells [12, 16, 22].

Cancer cell intravasation into the circulation normally involves cleavage of cell adhesion molecules like E-cadherin and β-catenin, degradation of surrounding ECM and migration through vessel walls [67]. Trauma inflicted during operation therefore seems to propagate a novel model of surgery-induced metastasis. During surgery, handling of the primary tumour enhances tumour cell spillage, which overcomes the need of complex cellular changes tumour cells must go through to escape the primary tumour mass. Surgery creates permissive circumstances for tumour cells to adhere in target organs and thereby increase chances of metastases development.

Macrophages and metastasis

Macrophages are a structural component of the tumour microenvironment and play a pivotal role in the initiation of metastases formation. Macrophages produce different proteases, which enable tumour cells to extravasate from the solid tumour and macrophages are actually seen to actively guide migratory tumour cells towards the circulation [67-69]. As macrophages predispose certain sites in vessel walls for tumour cell intravasation they can also play a role in the pre-dispositioning of distant organs, making vasculature more susceptible for tumour

Surgery-Induced metastases development

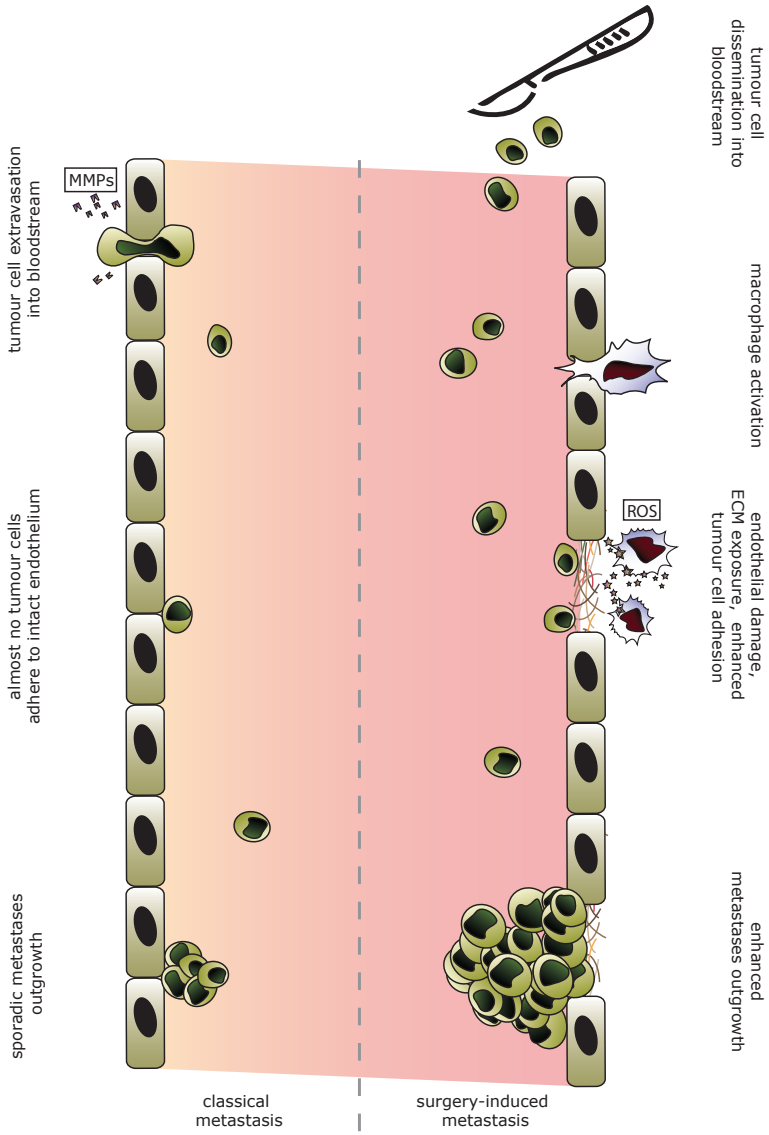

Figure 1 Classical vs surgery-induced liver metastases.
Left panel: To escape the primary tumour, tumour cells must down regulate of adhesion molecules, degrade surrounding basal membranes by producing different MMPs and invade the surrounding tissue. Detached tumour cells extravasate, with the help of MMPs and other matrix degrading enzymes, into the bloodstream and arrive in the liver vasculature. Here some tumour cells can adhere to the intact endothelial lining where they potentially grow out in distant metastases. **Right panel**: because of mechanical handling during surgery, tumour cells disseminate from the primary tumor without the need of down regulation of adhesion molecules directly into the bloodstream. Furthermore, surgery leads to systemic activation of macrophages, which subsequently will locally produce reactive oxygen species (ROS). ROS leads to endothelial cell damage, retraction and subsequent exposure of sub-endothelial extra cellular matrix (ECM). Disseminated tumor cells more easily adhere to the exposed ECM e.g. through integrin a2 subsequently leading to enhanced liver metastases development.

cell implantation [69]. A connection between surgical trauma, macrophage activation and metastasis predisposition at points of injury has therefore been suggested [70-73]. Accordingly was tumour cell adhesion to liver endothelium diminished in mice, in which functional liver macrophages were removed prior to tumour cell injection [58].

During surgery or trauma, peritoneal and blood monocytes, which are the source of tissue-fixed macrophages, are activated and shown to release high levels of cytokines TNF-a or IL-6 [74, 75]. Furthermore, activated macrophages are an important source of ROS [58]. In the liver the Kupffer cells (KCs), which form the major portion (80-90%) of tissue-fixed macrophages in the body, are adhered to sinusoidal endothelial lining and reside in the luminal site of the sinusoids. During operation bacterial components and other unknown factors are released into the circulation. KCs recognize these bacterial products by Toll like receptors (TLRs) and KCs become activated during surgery. Because the KCs are in close proximity of sinusoidal endothelial lining, inflammatory mediators and ROS that are released by KCs may lead to damage and retraction of the hepatic endothelial cells. Eventually, this could facilitate tumour cell adhesion and augment metastases outgrowth.

Although the exact mechanisms of surgery-induced tumour cell adhesion have not yet been completely elucidated, it was shown that production of reactive oxygen species (ROS) leads to damage of the liver vasculature and subsequent enhanced tumour cell adhesion [58]. The peri-operative period therefore remains an attractive window of opportunity in which expunging the remaining free disseminated tumour cells may reduce formation of liver metastases and improve overall patient survival [4, 13, 14].

Therapeutic options for prevention of metastases development

Blocking tumour cell adhesion

Adhesion molecules mediate tumour cell arrest in the target organs and as such play an important role in metastases formation. In animal models, development of metastases was significantly diminished when tumour cells were incubated with antibodies directed against adhesion molecules such as carcinoembryonic antigen (CEA) before injection in animals [76]. This suggested that inhibiting tumour cell adhesion might be a promising approach to prevent surgery-induced liver metastases. One of the most important adhesion factors on tumour cells, which play an important role during metastasising are the integrins [64-66]. Integrins are a family of cell adhesion molecules consisting of an a and a b subunits heterodimer [77] and are important in binding different ECM proteins, such as collagens and fibronectin. Interaction between integrin heterodimers and ECM proteins of the target organs facilitates tumour cell attachment and eventually formation of metastases. Following abdominal surgery, liver microvasculature integrity is impaired [58] leading to exposure of ECM proteins. Blocking either the a2 or β1 integrin subunit on tumour cells inhibited surgically induced metastases formation of colorectal cells to liver vasculature or peritoneal surfaces in a rat model [12, 16]. This suggested that blockade of integrins on tumour cells would potentially prevent recurrence of metastatic disease in patients undergoing operation of primary colorectal carcinoma. However, surgery is inevitably associated with tissue injury and immediately after surgery wound healing processes are initiated, in which the interaction between integrins and ECM proteins play an essential role [78]. Moreover, non-malignant cells like keratinocytes, which are involved in wound healing processes, also express integrins. A previous study demonstrated that interference with integrin b1 affected correct wound healing [79] resulting in abnormalities of epithelial structure. More importantly, lack of integrin b1 prolonged the inflammatory response after injury. So although blocking integrins on circulating tumour cells diminishes tumour cell adhesion and subsequent may decreasse metastases outgrowth after surgery, it can also lead to severe side effect in patients which countermand any beneficial effects of the therapy. Therefore, caution should be taken in attempting to treat patients with integrin blocking agents to improve patients' survival.

Reactive oxygen species scavengers

Surgical injury-induced immune responses result in activation of immune cells which can produce high amounts of ROS [80-82]. Surgical trauma is shown to enhance production of ROS [83-86] which can lead to damage of endothelial monolayers and subsequently to exposure of sub-endothelial ECM proteins, which are favourable adhesion sites for tumour cells [58, 87]. Accordingly, enhanced release and activation of the ROS producing enzyme xanthine oxidase (XO) and its substrate were observed during surgical trauma [84-86, 88-90]. Besides enhanced ROS production by immune cells, colon carcinoma tumour cells themselves can also generate the ROS superoxide by expressing NADPH oxidases (NOX) [91]. Members of Nox family are activated by inflammatory mediators such as cytokines that are released directly after surgery [74, 75]. Therefore, it was suggested that tumour cells potentially regulate their own adhesion and grow out in local or distant metastases [92].

Prevention of tumour cell adherence by treatment with ROS scavengers therefore may be an elegant option to prevent surgery-induced metastases outgrowth. Correspondingly, pre-

operative injection with a ROS scavenger led to significantly reduced tumour cell adhesion in a rat model for surgically induced colorectal liver metastases formation [58]. Whereas an operation in the peritoneal cavity led to decreased expression of tight junction proteins between endothelial cells of the liver vasculature, suggesting an impaired and damaged endothelial barrier, pre-operative injection with this anti-oxidant abrogated these effects of surgical trauma [58].

Although savaging ROS initially leads to decreased tumour cell adhesion, it does not inhibit liver metastases outgrowth [58]. ROS however, also play an important role in tumour cell killing by macrophages [93]. So although a high local ROS production during surgery damages endothelial linings, long term scavenging of ROS leads to diminished tumour cell killing by macrophages and subsequently to enhanced outgrowth of remaining adhered tumour cells [94].

Conclusively, caution should be taken in using ROS scavengers for pre-operative intervention in patients. Therefore, developing novel anti-oxidants with short half life, which may interrupt early ROS production during surgery, hereby leading to less damaged liver vasculature, while preserving long-term macrophage function may have therapeutic potential.

Antibody therapy

Instead of limiting immune cell activation during surgery, another promising therapeutic approach to eliminate the remaining disseminated circulating tumor cells after surgery is to enhance the host immune response against tumour cells with the use of monoclonal antibodies (mAb) [14]. mAb can have direct effects on tumour cells, like the induction of apoptosis or inhibition of proliferation [95, 96]. Furthermore, mAb can activate the complement pathway, which leads to complement-dependent cytotoxicity (CDC), and mAb can recruit immune cells for antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis (ADPh) [95]. Therapeutic mAbs have been developed to specifically target proteins that are (over-) expressed on tumour cells and have become one of the largest classes of new therapeutic agents approved for use in oncology [97]. The Fc tail of monoclonal antibodies is specifically important in the destruction of antibody-coated tumour cells. Binding of complement factors to the Fc region of the antibody can lead to direct CDC of the tumour cells and binding of FC region to specific Fc-receptors (FcR) on immune effector cells like neutrophils and macrophages induces ADCC and ADPh of antibody coated tumour cells. Of the different antibody isotypes, IgG1 is most effective in inducing CDC, ADCC and ADPh [98-101]. Most monoclonal antibodies currently FDA approved for clinical use are therefore human(ised) IgG1 or chimeric human/murine IgG1 antibodies, which are successfully used in treating different haematological malignancies like lymphomas and leukaemias [97]. However, in contrast to treatment of haematological cancers [102], treatment of established solid tumours with mAbs has been disappointing [103]. A protein that drew the attention as a potential target for mAb therapy against solid tumours is the epithelial growth factor receptor (EGFR). This protein is over-expressed in 80% of colorectal carcinomas [104]. Anti-EGFR mAb are currently used to treat (metastatic) colorectal cancer and squamous cell carcinoma of the head and neck (SCCHN) in patients [105, 106]. Binding of its specific ligands (e.g. EGF and TGFα) to EGFR on the surface of cells sends a signal down a pathway, including K-RAS and B-RAF, which

signals cells to divide. mAb against EGFR were developed as inhibitors of EGFR turning of this signal. Response to monotherapy with anti-hEGFR mAb of patients with existing metastatic colorectal cancer is however limited to ≈ 10% of patients and this is enhanced to ≈ 20% when anti-hEGFR therapy is used in combination with chemotherapy [105]. This disappointing response rate is due to the fact that the majority of tumours of colorectal cancer patients contain mutations in the K-RAS and B-RAF proteins leading to an exuberant activation of the pathways downstream of EGFR, which are independent on binding of EGFR by its natural ligands [96, 107, 108]. If mutated, KRAS continuously sends a signal to divide uncontrollably, even if EGFR has been blocked by mAbs.

In contrast to the disappointing effect of mAb treatment on existing solid tumours did animal studies demonstrate that mAbs could successfully prevent the formation of surgery-induced liver metastases that originated from circulating tumour cells [109, 110]. In rat and mouse models for surgically induced colorectal liver metastasising peri- or post-operative administration of syngeneic anti-tumour mAb resulted in binding of free accessible circulating tumour cells with mAbs, enabling the recognition of tumour cells by immune cells as malignant. Then, recognition of the Fc tail of mAb by immune cells with phagocytic capacity like the Kupffer Cells (KCs) of the liver, led to clearance of tumour cell via antibody dependent phagocytosis (ADPh). Moreover, it was demonstrated that when KCs were depleted, mAb treatment did not prevent tumour formation, suggesting that KCs play important role mAb-mediated anti-tumoural actions [109, 110]. Clinical effects of mAb therapy in patients to prevent new distant recurrences of disease are however sparse [111]. One single study showed increased 7 year survival and reduced overall mortality in patients in whom the primary colorectal tumour was microscopically completely resected and were postoperatively treated with mAb targeting the human Ep-CAM on colon carcinoma cells [112]. Treatment with this murine IgG2a mAb directed against human Ep-CAM on colon carcinoma cells had no effect on local recurrence of the primary tumour. However, the occurrence of distant metastases was reduced, albeit only in ~30% of the treated patients [112]. This may be due to the use of murine anti-EpCam antibodies, but the until now unprecedented promising outcome of this trial warrants further investigation as recent randomized clinical trials did not confirm the previous promising results [113-115]. As human and mouse differ in both expression pattern and in binding abilities of their FcR for different mAb isotypes [116], using syngeneic human mAb in patients may potentially enhance results in these clinical trials.

Thus, whether mAb that target Ep-CAM will provide clinical benefits is under intense debate. An alternative target for mAbs may be represented by epithelial growth factor receptor (EGFR) that is up-regulated in 80% of colorectal tumors[105]. Pre- or peri-operative treatment of colorectal patients without any evidence of distant metastases (e.g. stage I/II cancer) with less immunogenic human(ised) IgG1 antibodies that have extended serum half-lives and harness efficient human effector mechanisms may be an elegant way to reduce surgery-induced distant metastases formation. As such, we investigated whether the fully human anti-EGFR mAb Zalutumumab represents a suitable candidate for peri-operative treatment of patients undergoing resection for primary CRC. We found that killing of human colorectal tumour cells by macrophages via antibody dependent phagocytosis in

the presence of fully human anti-EGFR mAb Zalutumumab was independent on KRAS or BRAF mutational status of tumour cells. As such, we propose a novel mAb-based therapeutic strategy that may potentially benefit a significant population of cancer patients.
As such, we propose that patients undergoing resection for primary colorectal cancer may greatly benefit from peri-operative mAb immunotherapy, as this will lead to elimination of any remaining circulating tumour cells by the myeloid mononuclear network in the liver.

In conclusion, we experimentally demonstrated in animals that an operation in the peritoneal cavity to excise the primary tumour mass leads to systemic changes and induces the outgrowth of metastases. Surgical trauma, inevitably inflicted by operating animals or patients, is shown to enhance the adhesion of free circulating tumour cells to liver vasculature. Furthermore is has now become clear that this surgical trauma leads to activation of immune cells and subsequent production of damaging amounts of ROS. Locally produced ROS lead to changes in tight junction proteins between endothelial cells and subsequently, to damaged endothelial layers, which can be prevented by scavenging the produced ROS. Damage and retraction of endothelial cells leads to exposure of extracellular matrix proteins and subsequently, to enhanced adhesion of circulating tumour cells. Scavenging ROS or inhibiting adhesion molecules like integrins on tumour cells decreases the enhanced tumour cell adhesion, which however comes at a price. Inhibiting ROS cripples the ability of macrophages to kill tumour cells, subsequently enhancing the growth of remaining tumour cells, whereas blocking integrins decreases normal tissue repair and enhances the acute inflammatory phase after surgery. The best option in reducing surgery induced liver metastases formation therefore seems mAb therapy. Peri-operative treatment with specific mAb targeting membrane expressed tumour antigens labels free accessible circulating tumour cells as foreign enabling the recognition of tumour cells by immune cells as malignant. The myeloid mononuclear network in the liver, which is comprised mostly of Kupffer Cells can eliminate all adhered tumour cells and abrogate metastases formation.

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