Colectomy enhances tumor cell adhesion in the liver and subsequent outgrowth of metastases
Abstract

Surgical resection of the primary tumor provides the best chance of cure for patients with colorectal cancer (CRC). However, we previously demonstrated that laparotomy led to enhanced adhesion of tumor cells in the liver vasculature. Moreover, it was demonstrated that patients with anastomotic leakage after resection of the tumor had poorer survival, supporting that resection of primary CRC paradoxically may have a negative impact on metastases development and long-term patient outcome.

Therefore, the aim of this study was to investigate the influence of bacterial contamination during surgery on liver metastases development. Rats underwent a sham operation (laparotomy, opening and closure of peritoneal cavity), colectomy (resection of part of the colon and anastomosis), or received anesthesia alone, after which tumor cells were injected into the portal circulation. When swaps of the colon wall were taken at the beginning of surgery, no bacterial outgrowth was observed. However, an increased number of bacterial colonies grew out when swaps were taken of the suture, supporting bacterial contamination after subtotal colectomy. Moreover, both tumor cell adhesion and liver metastases development were significantly enhanced in rats that underwent colectomy. Additionally, liver vessel integrity was disrupted, as expression of the tight junction molecules ZO-1 and Claudin-5 were decreased after laparotomy and subtotal colectomy, indicating loss of cell-cell contact after abdominal surgery. This was likely due to increased macrophage activity.

In conclusion, our results support that macrophages were activated by a systemic factor in the plasma of operated rats, which led to impaired liver vasculature and augmented tumor cells adhesion and outgrowth. Exposure to bacterial products after colectomy may contribute to this process, as this will strongly activate macrophages. Understanding the precise mechanisms may aid the rational design of novel strategies to prevent liver metastases development after bacterial contamination, hereby improving prognosis of patients undergoing CRC resection.
Introduction

Colorectal cancer (CRC) is the most frequent tumor of the digestive tract, and the third common solid organ cancer in developed countries. Annually approximately 1.2 million cases of CRC are diagnosed worldwide, and over half a million patients die from this disease each year. Surgical removal of the primary tumor is the cornerstone of the treatment of CRC. Unfortunately, development of metastases is a frequent complication, which is accompanied by high morbidity and mortality. Metastases are found in ~20-25% of patients with CRC at the time of surgical resection of the primary tumor. Moreover, ~10-25% of the patients who do not have evidence of metastatic disease at the time of resection and as such are eligible for curative surgery, will nonetheless develop metastases post-operatively, within a period of five years.

Paradoxically, a growing body of evidence is suggesting that cancer surgery can increase the risk of metastases development, as multiple experimental studies as well as clinical data support the association between surgical trauma and tumor development. For instance, sites of injury were a preferential locus for tumor outgrowth in animal models, and it was previously shown that surgical trauma enhanced loco-regional metastases outgrowth. Moreover, the influence of surgery on tumor development is not confined to local peritoneal sites, as intraperitoneal surgery increased liver metastases outgrowth as well. Thus, surgery can induce changes that facilitate both local and distant metastases development.

We previously demonstrated in a rat model that intraperitoneal surgery led to activation of macrophages in the liver (also referred as Kupffer cells), which produced reactive oxygen species (ROS). This had a destructive effect on the integrity of the liver vasculature, exposing the sub-cellular extracellular matrix (ECM) components to which circulating tumor cells adhered. Because circulating tumor cells were detected in peripheral blood of patients with CRC prior to surgery, alterations in the liver vasculature in patients may contribute to the development of liver metastases as well. The tight junction molecule Claudin-5 was reduced in human liver biopsies that had been taken at the end of surgery, supporting liver vascular damage in patients. Moreover, it was shown that the number of circulating tumor cells increased during, or shortly after resection in both peripheral and portal blood, which suggest that manipulation of the primary CRC may lead to dissemination of tumor cells.

Additionally, several studies linked anastomotic leakage after resection of the primary colorectal cancer with increased tumor recurrence and worse survival. The endotoxin lipopolysaccharide (LPS), which is an important component of the outer membrane of Gram-negative bacteria, was furthermore detected in post-surgical plasma of patients. Elevation of the LPS concentration in blood was accompanied by intestinal permeability, which suggested that the epithelial barrier was impaired after surgery. Importantly, patients with positive bacterial
translocation after surgery had significantly shorter disease-free survival, supporting the negative impact of bacterial contamination after surgery on oncological clinical outcome.

Although we could demonstrate in previous studies in rats that laparatomy (open and closure of the abdominal cavity) by itself already resulted in Kupffer cell activation with detrimental consequences for tumor cell adherence, effects of bacterial contamination were not included. We therefore now established a colectomy model in which part of the colon was resected and an anastomosis was made to investigate the influence of per-operative bacterial contamination on liver metastases development.

Materials and Methods

Animals
Male inbred Wag/Rij rats, weighing (200-220g), were obtained from Charles River, Maastricht, The Netherlands. Rats were kept under standard laboratory conditions and had access to food and water ad libitum. The Committee for Animal Research of the VUmc approved all experiments, according to institutional and national guidelines.

Cell culture
The CC531s tumor cell line is a moderately differentiated colonic adenocarcinoma, which is transplantable in Wag/Rij rats, but does not metastasize spontaneously. CC531s tumor cells were cultured under standard incubator conditions in DMEM (Invitrogen, Paisly, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco Irvine, UK), 100 U/ml penicillin, 100m \( \mu \)g/ml streptomycin and 200mM L-glutamine (hereafter referred to as complete DMEM). Cell suspensions were prepared by enzymatic detachment with trypsin-EDTA solution, and contained both single tumor cells and small cell clusters (2-8 cells). Viability was assessed by a tryphan blue exclusion staining and was always > 95%. CC531s tumor cells were fluorescently labeled for short-term experiments, by incubation in complete DMEM containing 10 \( \mu \)l/ml 1,1’dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI, Sigma-Aldrich, St Louis, MO) for 30 minutes at 37ºC. Cells were subsequently washed with Hanks’ balanced salt solution (HBSS), according to the manufacture’s instructions, and suspended in HBSS for further use in experiments.

Animal models
In order to study the adherence of tumor cells and outgrowth of liver metastases in the absence of trauma, or presence of surgical trauma and/ or bacterial products, portal veins of rats were first catheterized as described previously. Briefly, rats received a midline incision under isoflurane anesthesia, after which the portal vein was exposed. A 2-french silicon catheter was secured after insertion in the portal vein with a purse-string suture. The catheter was passed through the muscle wall
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and tunneled subcutaneously towards the subcutaneous pocket between the scapulae of the animal, where an attached mini vascular access port was positioned. The catheter was flushed every other day with glycerol containing heparin (50 IU/ml) to prevent clotting of the catheter.

Animals were allowed a 14-day recovery period, after which they were divided into 3 groups. Group 1 was a control group, which received isofluorane anesthesia only for 45 minutes (= maximum duration of surgery in other groups). Group 2 (laparotomy group) underwent a sham operation, which was defined as midline laparotomy, and transfer of the intestines (covered with moist gauze) out of the abdominal cavity for 45 minutes, after which intestines were replaced in the abdominal cavity and the wound sutured. Group 3 (colectomy group) underwent a subtotal colectomy with an end-to-end anastomosis, in which a segment of the intestine was resected and both ends sutured (surgery lasted 45 minutes in total). Swaps from the colon wall were taken with a cotton swab at the beginning of the operation (pre-operative), during the operation (per-operative) and after the partial resection at the anastomosis (post-operative). Swaps were plated on agar culture plates and placed in an incubator at 37 °C for 24 hours, after which bacterial colonies were counted.

All animals (control-, laparotomy- and colectomy groups; n=6/group) received 2x10⁶ Dil-labeled CC531s tumor cells in 500 µl HBSS through the catheter in short-term experiments. Animals were sacrificed 2 hours post-operatively, after which liver samples were snap frozen for further analysis. Additionally, blood samples were taken via a heart puncture, and centrifuged for 5 minutes at 300 g. Plasma was collected and frozen till further use. Alternatively, for long-term experiments, 2x10⁵ CC531s tumor cells were injected through the catheter. Animals were sacrificed 14 days after tumor cell administration (n=12/group). Tumor nodules were scored blinded macroscopically by two independent investigators.

Bone marrow-derived macrophages
Bone marrow was harvested from freshly isolated femur, tibia and humerus from Wag/Rij rats. After removal of connective tissues and muscles, bone marrow was flushed and single cell suspensions were made by passing bone marrow through sterile 70 µm filters (BD Falcon, Bedford, MA). Macrophages differentiation was induced by incubating bone marrow cells for 7 days with complete DMEM supplemented with 15% L929 conditioned medium (containing macrophage-colony stimulating factor, hereafter referred to as Mø medium). Macrophages were harvested by a 15 minutes incubation with trypsin-EDTA and subsequent scraping with a cell scraper. Macrophages were seeded in 96-well plates (10⁵/well) for experiments.

MTT assay
Macrophages were incubated with rat plasma for 24 hours, after which (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml)
was added for 1.5 hours at 37°C. Viable cells convert MTT into insoluble formazan. Plates were washed with PBS to remove excess MTT, after which formazan was dissolved in 85% DMSO/14mM glycine. Purple color was measured at 450 nm in a microplate reader (BioRad, Hercules, CA).

**Fluorescence microscopy**
Cryostat liver sections (5 µm) were fixed for 10 minutes in acetone and air-dried. After blocking with 10% normal goat serum for 15 minutes, slides were incubated for 1 hour with primary antibodies against ED1 (Serotec, Oxford, UK), His 48 (BD Pharmingen, Germany), Claudin-5, Occludin or zona occludens 1 (ZO-1, Zymed Laboratories, San Fransisco, California,) at room temperature in a humidified tissue chamber. After washing, visualization was achieved by incubating with Alexa 488- or 555- labeled secondary goat anti- mouse antibodies (1:400, Molecular Probes, Eugene, Oregon). Nuclei were stained with Hoechst (10 µg/ml, Molecular Probes). Sections were washed, mounted and examined with a Leica DM6000 fluorescent microscope (Leica Microsystems B.V., Rijswijk, the Netherlands). The numbers of Dil-labeled tumor cells, ED1+ or His48+cells were quantified in 20 stitched fields per liver sample with the digital image analysis program AnalySIS (Soft Imaging System GmbH, Munster, Germany). Through a constant predefined threshold for color components, expression of ZO-1, Claudin-5 and Occludin tight junction molecules was examined.

**Statistical analysis**
ANOVA tests were used for comparison between three groups (control-, laparotomy- and colectomy operation). Statistical significance was accepted at p<0.05.

**Results and Discussion**

**Bacterial translocation during subtotal colectomy**
Resection of colorectal carcinoma is a necessary procedure, and provides the best chance of cure for patients. Unfortunately, in a sub-population of patients, who do not have evidence of metastatic disease at the time of resection, liver metastases will develop post-operatively.², ²¹ This supports that these patients have either undetectable micro-metastases or circulating tumor cells, which can grow out into distant metastases after successful removal of the primary tumor. Scientific evidence, which supports that surgery can increase the risk of liver metastases development has been accumulating in the last years.⁵, ⁶, ²¹ Previously, we demonstrated that laparotomy (opening and closure of the peritoneal cavity) enhanced tumor cell adhesion in a rat colon carcinoma model, with subsequent concomitant outgrowth of liver metastases.⁴, ⁶ This suggested the release of a systemic inflammatory factor(s). Furthermore, patients with anastomotic leakage or positive bacterial translocation after a (sub)total colectomy had significantly shorter disease-free survival,²⁰ indicating that bacterial contamination had a nega-
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tive impact on long term patient outcome as well. To determine the influence of exposure to bacterial products after surgery, in addition to release of inflammatory mediators, we therefore investigated liver metastases after laparotomy (release of systemic factors) or colectomy (release of systemic inflammatory factors and bacterial contamination).

First, we determined bacterial outgrowth after partial colectomy in rats. Swaps were taken from the exterior of the colon wall at the beginning of the operation (pre-operative), during the operation (per-operative) and after the colon segment had been resected and an anastomosis was made (post-operative). Swaps were plated and bacterial outgrowth was allowed overnight. No bacterial colonies were seen after plating pre-operative swaps (Figure 1). However, we observed a significant increase of outgrowth of bacterial colonies from cultures of per-operative swaps that were taken after the first incision in the colon was made. Furthermore, the highest number of bacterial colonies was observed when swaps were taken of the suture, after closure of the anastomosis. This demonstrated that resection of a small part of the colon led to bacterial contamination of the peritoneal cavity. Moreover, this may lead to dissemination of bacteria and bacterial products such as endotoxins into the blood stream, as LPS was detected in portal and peripheral blood of patients after colectomy.17, 18

Figure 1: bacterial contamination during surgery. Bacterial outgrowth from swaps that were taken from the exterior of the colon wall at the beginning of the operation (pre-operative), during the operation (per-operative) and after the colon segment had been resected and an anastomosis was made (post-operative). **p<0.01

Colorectal cancer surgery augmented adhesion of tumor cells
It was recently shown that exposure to LPS enhanced colon carcinoma cell adhesion.22 We therefore next investigated whether colectomy influenced tumor cell adherence in the liver. We observed an increase in tumor cell adhesion after laparotomy, which was even augmented after a subtotal colectomy compared to laparotomy surgery (Figure 2a). Thus release of bacterial products during a subtotal colectomy facilitated adhesion of tumor cells. It was demonstrated that LPS activated Toll-like receptor 4 (TLR4) on colon carcinoma cells led to functional activation of adhesion molecule β1 integrin, which mediated cell adhesion to ECM
proteins. Although β1 integrin was required for CC531s tumor cells adhesion to damaged peritoneal surfaces, it was not involved in binding to the liver vasculature. As such, increased adherence in the liver is likely not explained by direct effects of LPS on CC531s tumor cells.

**Post-operative serum induced macrophage activation**

TLR4 is however also expressed on a multitude of immune cells. For instance, Kupffer cells, which are the macrophages of the liver were reported to release reactive oxygen species (ROS) upon LPS stimulation. We previously demonstrated that ROS production after laparotomy was detrimental to the liver vasculature, allowing adherence of circulating tumor cells. Release of ROS was likely due to macrophage activation, as depletion of Kupffer cells abrogated surgery-induced tumor cell adhesion. We first investigated whether surgery enhances the recruitment of monocytes/macrophages. However, we did not observe any significant difference in the number of monocytes/macrophages between the three groups (Figure 2b).

![Figure 2](image-url)

**Figure 2**: Colorectal cancer surgery augments tumor cell adhesion. a: Tumor cell numbers in the livers of rats after anesthesia alone, laparotomy or subtotal colectomy. (n=6 per group). Red: Dil-labeled CC531s cells, blue: cell nuclei. b: the amount of ED1+ (monocytes/macrophages) cells in liver samples from the control rats or rats that underwent either laparotomy or colectomy. Green: ED1+ cells, blue: cell nuclei. Scale bar is 100 µm. c: macrophage activity after incubation with plasma of rats taken at different time points after surgery. fov= field of view ***p<0.01, **p<0.001.
To investigate whether systemic factors in plasma of operated rats increased activation of macrophages, we next incubated bone marrow-derived macrophages in the presence of plasma that had been collected at different time points after surgery. Macrophages showed higher activity when they had been incubated with rat plasma that was obtained 45 minutes after surgery (Figure 2c). Activity further increased when cells were incubated with plasma that was obtained either 1.5 or 3 hours after surgery. However, systemic activating factors were absent in plasma that had been collected 6 and 24 hours after surgery. This correlated with previous in vivo experiments, in which we demonstrated that adhesion of circulating tumor cells was increased 45 minutes and 1.5 hours after surgery.4

Liver vessel integrity is impaired after abdominal surgery
Previously, it was demonstrated that tumor cells adhered to sub-endothelial ECM in the liver vasculature, due to damage of endothelial cells as a result of exposure to ROS.4 Incubation of endothelial cell- macrophage co-cultures with LPS resulted in significant damage to endothelial monolayers, which was prevented by addition of ROS scavenger. (N. Gül et al, unpublished data) Since Kupffer cells are in vivo in close contact with sinusoidal endothelial cells we hypothesized that exposure of Kupffer cells to LPS may result into damage of the liver vessel integrity. Liver vascu-
lature continuity was investigated by analysis of expression levels of the tight junction proteins ZO-1, Claudin-5 and Occludin. No obvious difference in Occludin expression was observed between rats that received anesthesia alone, laparotomy, or colectomy (data not shown). However, both ZO-1 and Claudin-5 expression was decreased after laparotomy and subtotal colectomy, indicating loss of cell-cell contact (Figure 3a and b). This supports that release of inflammatory mediators and/or contamination with bacterial products after abdominal surgery induces endothelial cell stress, which may result in exposure of sub-endothelial ECM. It was previously reported that LPS injection manipulated the composition of the liver ECM, resulting in enhanced granulocyte accumulation.\textsuperscript{25} We therefore studied the number of granulocytes after surgery, and observed a significant increase after surgery, especially in rats that underwent a subtotal colectomy (Figure 3c). This supports that exposure to bacterial components likely led to sequestration of granulocytes in the liver. Furthermore, when endothelial monolayers were incubated with granulocytes and LPS, formation of intercellular gaps were observed, which was due to ROS production by granulocytes. (N. Gül \textit{et al.}, unpublished data) As such, granulocytes may contribute to the induction of damage to the liver vasculature after surgery and subsequent facilitation of tumor cell adhesion.

\textbf{Colectomy enhances liver metastases development}

Importantly, long term experiments showed that laparotomy increased the development of liver metastases (Figure 4). This is in agreement with earlier studies that demonstrated that surgery has a negative impact on oncological outcome.\textsuperscript{6, 26-28} However, this is further aggravated by post-surgical exposure to bacterial products as the highest number of metastases was observed in the colectomy group. No metastases were apparent in other organs at the time of sacrifice in all groups.

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{figure4.png}
\caption{Colectomy enhances liver metastases development. Number of tumor nodules in the livers of rats from control, laparotomy or colectomy groups. (n=12/group). *p<0.05, ***p<0.01.}
\end{figure}
Thus, our data suggest that surgery-induced inflammation and/or bacterial products that are spilled during surgery cause the activation of Kupffer cells and/or neutrophils in the liver. It was previously demonstrated that activation of these cells results in release of ROS that damages the sinusoidal endothelial lining and disrupts liver microvasculature. (ref 4 and data not shown) Subsequently, this leads to exposure of sub-endothelial ECM proteins, allowing adherence of circulating tumor cells, which grow out into metastases. Unfortunately, experimental therapy using an anti-oxidant to prevent metastases formation proved unsuccessful.4 This may be due to interference with ROS dependent tumor cell killing by macrophages. A more successful approach may be the prevention of undesired immune cell activation by inflammatory mediators and/or bacterial components. For instance, the interaction between LPS and TLR4 on immune cells may be blocked by treatment with either LPS scavengers or TLR4 antagonists. Alternatively, it has been demonstrated that selective decontamination of the digestive tract with antibiotics prior to resection of colorectal carcinoma reduced post-surgical infectious complications and anastomotic leakage.29, 30 As such, it is likely that the bacterial load as well as blood endotoxin concentrations were reduced in these patients, which may lead to a decreased risk of developing liver metastases.

In conclusion, we demonstrate that laparotomy and especially (sub)total colectomy facilitates the process of liver metastases development, as it increases tumor cell adhesion. Understanding the precise mechanisms will help to design optimal per-operative therapeutic strategies, which will ultimately greatly improve patient outcome.
CHAPTER 4

Reference list