Role of polymorphonuclear cells in surgery-induced liver metastases development

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CHAPTER 5

Abstract

Surgical resection of primary colorectal tumors is currently the preferred and only treatment that can create long-term disease free survival. However, abdominal surgery caused increased tumor cell adhesion to the peritoneal wall or in the liver that grew out into metastases. Interestingly, depletion of PMNs diminished tumor recurrence, suggesting that PMNs might play a role in surgery-induced tumor development. Therefore, in the current study we investigated the role of PMNs in liver metastases formation. Increased levels of PMNs and tumor cells were observed in the livers of rats that underwent laparotomy, which were even higher in the livers of rats in which colonic resection was performed. As colectomy results in bacterial contamination, we next investigated the role of bacterial products in enhanced PMNs and tumor cell adhesion. Significantly more PMNs and tumor cells were observed in livers of rats that had been treated with the bacterial product lipopolysaccharide (LPS). Moreover, incubation of endothelial monolayers with PMNs and LPS resulted in formation of intercellular gaps. Importantly, administration of an anti-oxidant was sufficient to prevent PMN and tumor cell accumulation in the livers of rats that underwent surgery or rats that had received a LPS injection. Thus, surgery-induced release of ROS by PMNs may be involved in formation of metastases. Therefore, therapies interfering with PMNs activation may prevent liver metastases development and improve patients’ outcome.
Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide.1-3 Approximately 60% of the CRC cases occur in developed countries and accounts for 8% of all cancer deaths, which makes CRC the fourth most common cause of death from cancer. The preferred therapeutic option that creates long-term disease free survival is surgical removal of the primary tumor. However, post-surgical liver metastases development is a frequent complication, that is detected in ~25% patients who did not have evidence of metastatic disease at the time of resection.1 This implicates that the presence of residual disease gave rise to post-surgical metastases development. Previously, it was demonstrated that patients with primary colorectal tumors have circulating tumor cells in their peripheral blood.4-8 Moreover, the numbers of circulating tumor cells were increased significantly during and after surgical resection of the primary tumor, especially in the portal blood.4 This suggested that surgical manipulation of the intestines caused dissemination of tumor cells from the primary tumor. Importantly, liver of rats that underwent abdominal surgery contained significantly more adhered tumor cells, which grew out into metastases compared to control animals.9 Furthermore, enhanced tumor cells adherence to the peritoneal wall was observed in animal studies in which abdominal surgery was performed.10, 11 Moreover, this was accompanied by high numbers of polymorphonuclear cells (PMNs) in abdominal lavage fluids.12 Addition of PMNs that had been incubated with phorbol myristate acetate - which is a potent activator that induces oxidative burst of PMNs - induced damage to mesothelial monolayers.13 Moreover, activated PMNs release chemotactic factors that attract and activate more PMNs and therefore provoke systemic inflammation. It was shown that systemic inflammation stimulates sequestration of activated PMNs in organs such as the liver and the lungs.14 Subsequently, this may lead to local tissue injury in these organs because of release of destructive molecules like reactive oxygen species (ROS) and proteases by PMNs.14 In line with this, it was demonstrated that surgery induced retraction of endothelial cells in the vasculature of liver and the lungs, which caused the exposure of sub-endothelial ECM to which tumor cells preferentially adhered.9, 15 This supported the release of a systemic factor, and in a previous study we demonstrated that increased tumor cells adhesion was a ROS dependent process.16 Surgery-induced tumor cell adherence in the liver was reverted when an anti-oxidant was injected pre-operatively in rats. Additionally, less tumor recurrence was observed in PMN- depleted animals,12 which suggested that PMNs play a role in metastases development after surgery. Therefore, we propose that the oxidative burst of activated PMNs may damage the sinusoidal endothelial lining and therefore mediate tumor cell adhesion. Additionally, previous studies demonstrated that surgical manipulation of the intestines caused transfer of bacterial products, such as lipopolysaccharides (LPS), from the gut lumen into the peripheral blood circulation.17-19 Moreover, patients with bacterial spillage or anastomotic leakage after surgery had poorer survival.20-22
whereas bacterial products augmented metastases outgrowth in the lungs and liver in animal models. This suggested that bacterial products, which are potent activators of PMNs, influences patients’ outcome. Therefore, in the current study we studied the effects of surgery and LPS on PMN activation and the contribution of these cells in surgery-induced metastases development.

**Material and methods**

**Colon carcinoma cell culture**
The rat colon carcinoma cell line CC531s is a moderately differentiated and immunogenic cell line. CC531s cells were cultured under standard culture conditions in RPMI 1640 medium (Invitrogen, Breda, the Netherlands) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin (Gibco, Irvine, UK, hereafter referred as complete growth medium). Cell suspensions were prepared by enzymatic detachment using trypsin-EDTA solution (Gibco), and contained single tumor cells as well as small clusters (2–8 cells). Viability was assessed by trypan blue exclusion and always exceeded 95%. For in vivo experiments, CC531s cells (5×10^6 cells/ml) were fluorescently labeled by incubating cells in complete growth medium containing 50 μg/ml 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbo cyanine perchlorate (DiI, Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C and were subsequently washed with HBSS (Gibco) according to the manufacturer’s instructions.

**Endothelial cell cultures**
Human umbilical veins were treated with collagenase (Worthington, Lakewood, UK) for 20 minutes at 37°C according to a standard procedure. Human umbilical vein endothelial cells (HUVECs) were harvested and cultured to confluence till passage 5 in medium M199, supplemented with 10% heat-inactivated human serum, 10% heat-inactivated new born calf serum, 5000 U/ml heparin, 10 mg/ml bFGF (Peprotech Inc, Rocky Hill, CT), 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin (Gibco, Irvine, UK) at standard culture conditions.

**PMN isolation**
PMNs were isolated from blood of healthy human volunteers who gave informed consent, according to the guidelines of the medical ethical committee of the VUmc. The total amount of blood was 1:2 diluted with saline. By performing a density gradient centrifugation step with Lymfoprep (Fresenius Kabi Norge, Oslo, Norway) peripheral blood mononuclear cells were separated from PMNs and red blood cells. To further isolate PMNs, red blood cells were removed from the suspension with shock buffer, containing 155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA. After three washing steps with PBS, the numbers of PMNs were counted. PMNs were labeled fluorescently with Calcein AM (Invitrogen™, Breda, The Netherlands) according to the manufacturer’s protocol at a concentration of 1 μg/ml (2×10^6 cells/ml). Cells were left to rest for two hours in complete growth medium at 37°C. Before
the start of all experiments, PMNs were resuspended in M199 medium.

**HUVECs and PMNs co-culture**

To study the effects of LPS and/or PMNs on HUVEC monolayers, HUVECs were cultured on collagen (from calf skin, Sigma-Aldrich, St. Louis, MO) coated Lab-Tek Chamber Slide™ system 8 well glass slides (Nalge Nunc International, New York, NY). HUVEC monolayers were incubated with 100 ng/ml LPS and/or PMNs (5x10⁵) for 15 minutes at 37°C. After incubation and a gentle washing step with PBS, slides were fixated with 0,5% glutardialdehyde for 10 minutes. HUVEC monolayers were stained with Rhedemine-Phalloidin (Invitrogen) for 15 minutes. Cell nuclei were stained with Hoechst (Invitrogen™, Breda, The Netherlands). Slides were washed, mounted and examined with a Leica DM6000 fluorescence microscope (Leica Microsystems, Heidelberg, Germany). Areas of damaged endothelial monolayers were measured with AnalySIS software (Soft Imaging System GmbH, Münster, Germany).

**Detection of ROS production**

PMNs were washed and resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) medium containing 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM MgSO₄·7H₂O and 1,2 mM K₂HPO₄·3H₂O, supplemented with 1M CaCl₂, 5 g/l bovine serum albumin and 1 g/l glucose. 1x10⁵ cells were added to a 96-wells plate and incubated with 0, 10, 100 or 500 ng/ml LPS (from E. Coli, Sigma Aldrich, St. Louis, MO). A reaction mixture of HorseRadish Peroxidase (HRP) and Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine, Molecular Probes, Breda, The Netherlands) was added. This mixture reacts with H₂O₂ to form highly fluorescent resorufin. Fluorescence was measured every 5 minutes at 37°C in a BMG Fluostar Galaxy (BMG Lab Technologies GmbH, Offenburg, Germany).

**Animal models**

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

To investigate the effects of abdominal surgery on tumor cells adhesion and PMNs dynamics in the liver, midline laparotomy was performed under anesthesia and 2,5x10⁶ Dil-labeled CC531s cells were injected in a mesenteric vein. Rats were sacrificed at different time points (n= 3 per group). Alternatively, effect of the bacterial product LPS on tumor cell adherence and PMNs dynamics in the liver was studied by injection of PBS or 10 µg/kg LPS (from E. Coli, Sigma Aldrich). Portal veins of rats were first catheterized as described previously. Catheters were flushed every 3 days with glycerol (Merck, Darmstadt, Germany) containing heparin (50 IU/mL) to prevent obstruction of catheters by blood clots. All animals were allowed to recover for 10 days. 2x10⁶ Dil-labeled CC531s cells were injected through the catheter and animals were sacrificed after 45 minutes, 1,5 or 3 hours (n= 4 per group). The role of ROS in this process was studied by using the ROS scavenger Edaravone (3-Methyl-
1-Phenyl-2-Pyrazolin-5-one, Calbiochem, Darmstadt, Germany), which was dissolved in 6% ethanol. Animals received 125 mg/kg Edaravone or the vehicle ethanol (±6%) intra peritonealy 30’ before and immediately after surgery or LPS injection and were sacrificed after 1.5 hours.

**Fluorescence microscopy**
Liver samples from animal experiments were snap frozen for immunohistochemical staining. Furthermore, liver biopsies were taken from patients undergoing resection of liver metastases from colorectal cancers at the start and end of surgery. All patients gave informed consent according to the guidelines of the medical ethical committee of the VUmc. Cryostat liver tissue sections from human and rat livers were fixed for 10’ 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 His48 (mouse-anti-rat granulocytes, Serotec, Planegg, Germany) or CD66b (rabbit-anti-human granulocytes, BD Pharmingen, Franklin Lakes, NJ) at room temperature. Mouse or rabbit serum was used as isotype control. After washing, visualization was achieved by incubation with Alexa 488 or 594-labelled goat-anti-mouse antibody (Molecular Probes Inc). Nuclei were stained with Hoechst (Molecular Probes Inc). Sections were washed, mounted and examined with a Leica DM6000 fluorescence microscope (Leica Microsystems, Heidelberg, Germany). Representative pictures of livers were taken (3 per liver sample, 5 liver samples from different lobules per animal). The numbers of PMNs and tumor cells were determined blinded by two independent investigators with AnalySIS software (Soft Imaging System GmbH).

**Statistical analysis**
For comparisons between two groups Student T-tests were performed. Comparisons between multiple groups (>2) were analyzed with ANOVA. Statistical significance was accepted at p<0.05.

**Results**

**Laparotomy leads to enhanced numbers of PMNs and tumor cells in the liver**
The effect of abdominal surgery on PMN and tumor cell adherence in the liver was studied in a rat model. Rats underwent laparotomy and were sacrificed at different time points. The numbers of PMNs and tumor cells were significantly increased in the livers of rats that underwent surgery (Figure 1a). The amount of PMNs reached a maximum 6 hours after surgery and was decreased to basal levels in the livers of rats that were sacrificed 24 and 48 hours after surgery. However, the numbers of tumor cells reached a maximum 1.5 hours after surgery, after which the numbers of CC531s declined in time in the livers of rats (Figure 1a, black bars). Furthermore, liver biopsies were taken from patients that underwent surgery for the resection of colorectal liver metastases. Importantly, compared to the amount of PMNs in liver samples that were taken at the start of surgery, enhanced PMN numbers were observed at the end of the surgical procedure (Figure 1b).
The bacterial product LPS mediates PMN and tumor cell adhesion in the liver

Previous studies demonstrated that surgery caused bacterial translocation.\textsuperscript{17-19} Moreover, patients with positive bacterial translocation had poor survival,\textsuperscript{22} which suggested that bacterial products, which are potent activators of PMNs, might influence the patients’ outcome. To investigate whether bacterial products induce accumulation of PMNs or tumor cells in the liver, rats received an intraperitoneal injection of PBS or 10 µg/kg LPS and were sacrificed at different time points. The numbers of PMNs in the livers of rats that were treated with LPS were already significantly increased after 45 minutes compared to PBS treatment (Figure 2), which further increased after 1.5 hours compared to 45 minutes. Tumor cell adhesion was increased 45 minutes after LPS injection as well, but this did not further increase over time. Thus, surgery and LPS cause enhanced tumor cell adhesion in the liver. It was demonstrated that endothelial or mesothelial integrity was disrupted either by direct incubation with ROS or by activated PMNs.\textsuperscript{13, 16} In the liver microvasculature, ECM proteins in the space of Disse are covered by a lining of endothelial cells.\textsuperscript{28} Damage to the endothelial lining may cause the exposure of ECM proteins, which are preferable adhesion sites for circulating tumor cells.\textsuperscript{9, 15} Therefore, we next investigated the effects of LPS stimulation of PMNs on endothelial integrity. Incubation of HUVEC monolayers with 100 ng/ml LPS did not affect endothelial monolayers (Figure 3a). When PMNs were added, minimal damage to the monolayer integrity was observed, which may be due to some activation of PMNs after isolation. However, incubation of HUVEC monolayers with PMNs in the presence of LPS introduced severe retraction and detachment of endothelial cells. Since we previously demonstrated that endothelial damage was caused by ROS,\textsuperscript{16} we next investigated whether LPS treatment induced ROS production by PMNs. Incubation of PMNs with varying concentrations of LPS resulted in production of high levels of $\text{H}_2\text{O}_2$ in a LPS concentration dependent manner (Figure 3b).

Therefore, we investigated the role of ROS in enhanced adhesion of PMNs and tumor cells after laparotomy or LPS injection in our animal model. Pre-surgical treatment of rats with the anti-oxidant Edaravone decreased the adherence of PMNs and tumor cells compared to livers of rats that had been treated with the vehicle alone (Figure 4a). Similarly, livers of rats that were treated with Edaravone before they received a LPS injection contained significantly less PMNs and tumor cells than animals that were injected with LPS alone (Figure 4b).
**Figure 1:** Surgery stimulates adhesion of PMNs and tumour cells in the liver. 

- **A:** numbers of PMNs and tumor cells in the livers of rats undergoing laparotomy that were sacrificed at the indicated time points after surgery. Green: His48+ cells (PMN), blue: nucleus.
- **B:** numbers of PMNs in liver samples of patients that were taken at the begin and end of surgery. Red: CD66+ cells, blue: cell nuclei, scale bar is 100 µm. fov= field of view.

*p<0.05, **p<0.01, ***p<0.001.

**Figure 2:** LPS induces PMN and tumor cell adherence in the liver of rats. *p<0.05, **p<0.01, ***p<0.001.
Figure 3: Addition of LPS and PMNs to endothelial monolayers leads to severe endothelial damage. a: Incubation of HUVEC monolayers with PMNs in the absence or presence of LPS. Insets are higher magnifications of areas indicated by *. Arrow heads point to intercellular gaps. Red: Rhodamine-Phalloidin staining of HUVECs. Scale bar is 100 μm. b: Quantification of damaged endothelial areas. c: Effect of LPS on ROS production by PMNs. ***p<0.001.

Figure 4: Role of ROS in accumulation of PMNs and tumor cells in the livers. a: PMNs and tumor cells in post-operative livers of rats, which were treated with the vehicle EtOH or EDA. b: rats were treated with PBS, EDA, LPS or LPS+EDA and sacrificed after 1.5 hours. Green: His48+ cells (PMN), blue: nucleus. Scale bar is 100 μm. *p<0.05, **p<0.01, ***p<0.001.
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Discussion

Resection is the preferred treatment for colorectal tumors. However, abdominal surgery was shown to induce enhanced tumor cell adhesion to the peritoneal wall. Additionally, high numbers of PMNs were recruited into the abdominal lavage fluids. Importantly, it was demonstrated that depletion of PMNs was effective in prevention of local tumor recurrence, which suggested that PMNs might play a role in tumor development. In the current study we demonstrate that laparotomy (opening and closure of the peritoneal cavity) led to accumulation of both PMNs and tumor cells in the liver, suggesting that PMNs may be involved in tumor cell adhesion in the liver, as well.

Previous studies furthermore demonstrated that surgery resulted in spillage of bacterial products like LPS, which are potent activators of PMNs. Interestingly, when we performed colectomy in rats (partial resection of the colon with anastomosis), significantly enhanced PMN and tumor cell adherence was observed compared to laparotomy alone (data not shown). This resulted in augmented liver metastases development, as an increased number of tumor nodules was observed in rats that underwent colectomy. When swaps of the anastomosis were plated, we observed prominent bacterial outgrowth (data not shown), supporting that resection of colorectal cancer leads to bacterial contamination. Moreover, stimulation of PMNs by LPS resulted in severe damage to endothelial monolayers, which was likely caused by ROS release. We observed that treatment of rats with an anti-oxidant either before surgery or LPS injection prevented attraction of PMNs and tumor cells to the liver. This suggested that exposure of PMNs to LPS in vivo also resulted in ROS production that damaged the endothelial lining of liver sinusoids, allowing tumor cell adherence. This is supported by previous studies, which showed that surgery-induced retraction of pulmonary and liver endothelial lining and promoted adherence of tumor cells to exposed sub-endothelial ECM, which was likely due to ROS production. McDonald et al. showed that LPS injection in mice led to modification of the ECM component hyaluronic acid in the liver, resulting in enhanced PMN accumulation. The authors speculated that LPS injection induced ROS production stimulating PMN adherence to modified ECM.

Alternatively, it was demonstrated that acute inflammation led to endothelial ICAM-1 dependent extravasation of PMNs into the liver paranchyme. Moreover, a previous study reported enhanced expression of ICAM-1 in livers 3 hours after surgery. In contrast, in the current study we found increased numbers of PMNs already 45 minutes after surgery. This suggests that surgery- or LPS-induced PMNs accumulation may be ICAM-1 independent, but depends on interaction with ECM, as it was suggested that extensive endothelial damage caused by ROS facilitate PMN extravasation without the need of adhesion molecules on endothelial cells. This is supported by a previous study, which showed that preventing interaction between integrins and ICAM-1 did not inhibit PMN extravasation during inflammation.
Thus, surgery-induced inflammatory mediators and/or bacterial products that enter the blood circulation due to bacterial spillage during colectomy, induce a systemic inflammation that leads to activation of PMNs. Subsequently, PMNs produced ROS, which damage the sinusoidal endothelial lining hereby allowing adhesion of tumor cells. Since circulating tumor cells can be found in blood of patients undergoing resection for primary colorectal cancer we hypothesize that these tumor cells may adhere in the liver after surgery and grow out into metastases. Therefore, peri-operative therapies interfering with bacterial spillage and/or PMN activation might prevent this process, which will greatly improve patient outcome.
Figure 5: simplified schematic overview of liver microvasculature before and after surgery. In pre-operative colorectal cancer patients, polymorphonuclear cells (PMNs) and cancer cells circulate through the microvasculature of the liver (left). Surgical trauma results in translocation of bacterial products such as LPS. These molecules are recognized by the immune cells of the liver, including circulating PMNs. Activation of PMNs by LPS stimulates the release of inflammatory mediators like reactive oxygen species (ROS). Integrity of sinusoidal endothelial lining becomes disrupted by ROS, exposing the sub-endothelial extracellular matrix (ECM) to which circulating PMNs and/or tumor cells adhere (right).
Reference list
