Macrophages mediate colon carcinoma cell adhesion in the rat liver after exposure to LPS


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Running title: LPS-induced liver metastases development

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Abstract

Resection of primary colorectal cancer is associated with enhanced risk of liver metastasis development. Moreover, bacterial translocation or anastomotic leakage during surgical resection of primary colorectal tumor was shown to correlate with poor long-term outcome, suggesting that bacterial products may contribute to metastases development. Therefore, the aim of this study was to investigate the role of the bacterial product LPS in liver metastases development. In vivo experiments in rats were performed to investigate the effects of LPS on tumor cell adhesion in the liver. Furthermore, because our previous studies suggested a role for liver macrophages and endothelial damage in metastases formation, we investigated the effects of LPS on endothelial-macrophages co-cultures in vitro. Adhesion of tumor cells in the liver was already increased 45 minutes after LPS injection. Additionally, treatment of macrophages with LPS in endothelial-macrophage co-cultures induced disruption of endothelial monolayer integrity and intercellular gap formation, which was prevented by addition of either the LPS inhibitor polymyxin B or reactive oxygen species (ROS) scavenging enzymes. Depletion of macrophages or treatment with the ROS scavenger Edaravone inhibited in vivo tumor cell adhesion. In conclusion, LPS mediated ROS release by macrophages, which resulted in endothelial damage, allowing tumor cells to adhere in the liver vasculature. Therefore, peri-operative treatment that prevents macrophage activation and hereby inhibits endothelial damage and concomitant tumor cell adhesion may significantly improve patient prognosis.
Introduction

Colorectal cancer (CRC) is the most prevalent malignancy of the digestive tract in men and women in developed countries. Worldwide approximately one million new cases of CRC are diagnosed annually, and over half a million patients die from this disease every year.\(^1\)\(^-\)\(^3\) Surgical removal of primary colorectal carcinoma is the preferred and only treatment that can provide long-term disease-free survival. However, 20-50% of the patients who do not have evidence of metastatic disease at the time of resection will develop liver metastases within 5 years.\(^4\) This indicates that - in spite of removing the bulk of tumor with surgery - these patients still have either undetectable micrometastases or circulating tumor cells, which can grow out into distance metastases.

Previously it was demonstrated that surgery paradoxically contributes to tumor recurrence and liver metastasis development.\(^5\)\(^-\)\(^7\) First, free circulating tumor cells were detected in peripheral blood of patients with CRC prior to surgery.\(^8\) Moreover, the number of circulating tumor cells increased during, or shortly after resection in both peripheral and portal blood, suggesting surgery-induced tumor cell dissemination.\(^8\)\(^,\)\(^9\) Second, surgical trauma was shown to augment metastases outgrowth.\(^7\) And finally, patients with positive bacterial translocation after surgical removal of the tumor had significantly shorter disease-free survival.\(^10\) In line with this it was shown that treatment of mice with bacterial endotoxins enhanced lung and liver metastases development.\(^11\)\(^,\)\(^12\) These data suggest that bacterial spillage during surgery contributes to metastases development.

Bacterial products such as LPS, which is an important component of the outer membrane of Gram-negative bacteria, were detected in post-surgical plasma of patients.\(^13\)\(^,\)\(^14\) LPS concentration in peripheral blood was enhanced 1 hour after surgery and normalized after 24 hours. Elevation of the LPS concentration in blood was accompanied by intestinal permeability, which suggested that the epithelial barrier was impaired after surgery.\(^15\) Moreover, translocation of viable bacteria from the gut lumen to local mesenteric lymph nodes was demonstrated in CRC patients undergoing colectomy.\(^10\)\(^,\)\(^16\)\(^,\)\(^17\)

It was recently demonstrated that LPS induced signaling via TLR4 in human colorectal cancer cells, which led to enhanced tumor cell adhesion.\(^11\) However, LPS additionally activates potent immune responses through TLRs that are expressed by most immune cells, including macrophages.\(^18\) Previously, we demonstrated that macrophages can release reactive oxygen species (ROS) after surgery.\(^19\) Moreover, it was demonstrated that ROS disrupted the endothelial barrier, leading to vascular permeability in tissues, as well as enhanced tumor cell adhesion in the liver.\(^20\)\(^-\)\(^22\) Because macrophages express TLR4, we hypothesize that – in addition to direct effects on tumor cells – macrophages may play a role in LPS-induced metastases development. Therefore, in the current study we investigated the effect of LPS-stimulated macrophages on endothelial integrity in macrophage-endothelial co-cultures. Furthermore, role of LPS in tumor cell adhesion in vivo was determined.
Material and methods

Colon carcinoma cell culture
The rat colon carcinoma cell line CC531s is a moderately differentiated and immunogenic cell line. \(^{23}\) 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-tetramethylindocarbocyanine perchlorate (DiI, Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C and 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. \(^{24}\) HUVECs were harvested and cultured to confluence till passage 5 in M199 medium, supplemented with 10% heat-inactivated human serum, 10% heat-inactivated new born calf serum, 5000 U/ml heparin, 10 μg/ml basic fibroblast growth factor (Peprotech Inc, Rocky Hill, CT), 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin (Gibco) at standard culture conditions (hereafter referred to as endothelial growth medium).

Endothelial-macrophage co-cultures
Monocytes were isolated with CD14 MicroBeads (MACS Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions from freshly obtained donor blood. Healthy donors gave informed consent according to the guidelines of the medical ethical committee of the VUmc. Cells were resuspended in DMEM medium containing 10% heat-inactivated human serum, 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin (Gibco referred to as macrophage growth medium). 2x10^5 monocytes were added to transwells (Corning Incorporated, New York) with 0.4μm pore size polyester membrane inserts, which had been turned upside down. Transwells were incubated for three hours to allow optimal adhesion of monocytes to membranes (on bottom side). Transwells were then turned and placed in 24 well plates for 7 days to allow differentiation into macrophages. Hereafter, confluent HUVEC monolayers (5x10^5 cells/well) were cultured on gelatine coating on the upper site of filters.

Cells were incubated with different concentrations of LPS for 30 minutes in M199 with or without the LPS scavenger polymyxin B sulfate (PMB, 50 μg/ml, Applichem,
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Darmstadt, Germany). Alternatively, 5000 U/ml superoxide dismutase (SOD, from bovine erythrocytes, Sigma Aldrich) and catalase (from bovine liver, Sigma Aldrich) were added.

After incubation, cells were fixated with acetone for 10 minutes at room temperature (RT) and air-dried. HUVECs monolayers were stained with Rhodamine-labeled Ulex Europaeus Agglutinin I (Vectors labs, Burlingame, CA) at a concentration of 6 μg/ml for 15 minutes at RT. Cell nuclei were stained with Hoechst (Invitrogen™, Breda, The Netherlands). Membranes were washed, mounted and 5 random pictures were made with a Leica DM6000 microscope (Leica Microsystems B.V., Rijswijk, the Netherlands). Areas of damaged HUVECs monolayers were measured with AnalySIS software (Soft Imaging System GmbH, Münster, Germany).

Scanning and transmission electron microscopy (SEM and TEM)
Freshly isolated human monocytes were allowed to differentiate into macrophages for 7 days in macrophage growth medium in teflon erlenmeyer flasks (Nalgene, Rochester, New York). Macrophages were seeded into 6-well culture plates (Greiner Bio One, Kremsmuenster, Austria) and incubated for different time points with saline or 500 ng/ml LPS. SEM microscopy was performed as described previously. Alternatively, after 1 hour incubation with saline or 500 ng/ml LPS, macrophages were fixated in 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated in ethanol infiltrated with propylene oxide and embedded in Agar 100 Resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with TEM (Philips CM 100 Bio Twin, Philips Eindhoven, the Netherlands).

Animal Models
Male inbred Wag/Rij rats (180-220 g; Charles River, Maastricht, The Netherlands) were housed under standard laboratory conditions and had access to food and water ad libitum. The Committee for Animal Research of the VUmc approved the experiments according to institutional and national guidelines.

Portal veins of rats were 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 10 days. To investigate tumor cell adhesion following an i.p. injection with saline or 10 μg/kg LPS, 2x10<sup>6</sup> Dil-labeled CC531s cells were injected through the catheter and animals were sacrificed after 45 minutes, 1,5 and 3 hours (n= 4 per group). Liver samples were snap frozen. In additional experiments, liver macrophages (Kupffer cells; KCs), were depleted by intravenous injection of clodronate (Roche Diagnostics, Mannheim, Germany) encapsulated in liposomes or saline 2 days prior to tumor cells injection (n= 4 per group). Alternatively, rats received 125 mg/kg of the ROS scavenger Edaravone (3-Methyl-1-Phenyl-2-Pyrazolin-5-one, Calbiochem, Darmstadt, Germany) or the vehicle ethanol (±6%) i.p. 30 minutes prior to and immediately after LPS or saline injection (n= 4 per group). Animals were sacrificed 1.5 hours after inoculation of fluorescently labeled...
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CC531s cells. Liver samples were snap frozen for immunofluorescent analyses.

**Fluorescence microscopy**
Cryostat liver tissue sections of 5 μm were fixated for 10 minutes in acetone and air-dried. Nuclei were stained with Hoechst (1:1000, Molecular Probes Inc.). Sections were washed, mounted and examined with a Leica DM6000 fluorescence microscope (Leica Microsystems, Heidelberg, Germany). Numbers of tumor cells at 45 minutes, 1.5 and 3 hours after tumor cell inoculation were determined (20 stitched fields per liver sample, liver samples from 5 different lobules per animal) with AnalySIS software (Soft Imaging System GmbH).

**Statistical analysis**
For comparisons between two groups student T-tests were used. Comparisons between multiple groups (>2) were performed with ANOVA. Statistical significance was accepted at p<0.05. Results are presented as mean +/- standard error of mean.
**Figure 1:** LPS stimulates tumor cell adhesion in the liver by disrupting endothelial layer. 

*a:* tumor cell numbers in the livers of rats which had been treated with saline or LPS and were sacrificed at indicated time points (n=4 per group). Red: Dil labeled CC531s, blue: cell nuclei. The graph represents quantification of the number of tumor cells in different groups.

*b:* schematic overview of *in vitro* co-culture of HUVEC and macrophages.

*c:* HUVEC-macrophages (MΦ) co-cultures were incubated with different concentrations of LPS and/or LPS scavenger polymyxin B (PMB). HUVECs were stained fluorescently and 5 random pictures were taken per condition and damaged endothelial areas were determined. Graph represents quantification of % damaged endothelial area. Red: HUVEC, blue: nuclei. Dotted lines indicate areas without endothelial cells. *p<0.05; **p<0.01; ***p<0.001.
Results

**LPS stimulates tumor cell adhesion in the liver by disrupting endothelial layers**

To investigate the effects of LPS on tumor cell adhesion in the liver, rats were injected with saline or LPS i.p., after which tumor cells were administered into the portal circulation. The number of tumor cells in livers of rats that were treated with LPS significantly increased 45 minutes after injection of tumor cells, compared to the amount of tumor cells in livers of saline treated rats (Figure 1a). Tumor cell adhesion was not further increased 1.5 or 3 hours after LPS injection.

Because our previous studies suggested that macrophages induced enhanced tumor cell adhesion through disrupting sinus endothelial barriers, we next studied the effects of LPS-stimulated macrophages on endothelial cells. Because macrophages and sinus endothelial cells are in close contact in the liver, we developed a novel model allowing us to study endothelial cells and macrophages in intimate contact (Figure 1b). Confluent endothelial cell monolayers were cultured on upper sides of transwell membranes, and macrophages on lower sides. Neither incubation of HUVECs with 100 ng/ml LPS nor co-culture of HUVECs with macrophages without LPS had effect on endothelial monolayers (Figure 1c). However, addition of 10 ng/ml LPS to HUVECs-macrophage co-cultures disrupted endothelial monolayer integrity and induced intercellular gaps between endothelial cells, which were further increased when 100 or 500 ng/ml LPS was added. Addition of the LPS inhibitor PMB prevented endothelial damage significantly.

Since these data supported activation of macrophages by LPS, we used SEM and TEM to visualize the effects of LPS stimulation on macrophages. After 30 minutes incubation with saline, macrophages showed some adherence. However, macrophages that were incubated with LPS were tightly adhered and widely spread, supporting strong activation (Figure 2a). Moreover, LPS-stimulated macrophages formed a monolayer after 2 hours, while saline treated cells demonstrated uncovered areas between cells. Furthermore, saline-treated macrophages contained no intracellular vacuoles and showed barely filopodia (Figure 2b, left panel), supporting low cellular activity. In contrast, LPS-stimulated macrophages contained several intracellular vacuoles and exhibited many filopodia, indicating high cellular activity (Figure 2b, right panel). Thus, incubation of macrophages with LPS caused rapid activation of cells. This is consistent with our in vivo data showing increased tumor cell adhesion in rats that were injected with LPS already within 45 minutes after tumor cell injection (Figure 1a).

To investigate the role of macrophages in tumor cell adherence in the livers of rats after LPS injection, we used control rats and rats in which KCs were depleted by liposomes containing clodronate. Absence of liver macrophages or newly recruited monocytes after treatment with clodronate liposomes was confirmed by ED2 (marker for resident macrophages) or ED1 (monocyte marker) staining (data not shown).
Figure 2: LPS stimulates rapid macrophage activation. Human macrophages were incubated either with saline or LPS and cells were analyzed with SEM or TEM. Representative SEM (a) or TEM (b) pictures.
shown). All animals were treated with LPS and tumor cells were injected into portal circulation. The number of tumor cells in livers of KCs-depleted rats that had been treated with LPS was drastically decreased compared to tumor cell numbers in livers of control rats after LPS treatment (Figure 3). Thus, macrophages played an essential role in adherence of tumor cells in the livers of LPS treated animals.

**LPS stimulation of macrophages leads to damaged endothelial layer by ROS production**

It was previously shown that exposure to ROS induce endothelial damage.\(^{19}\) Therefore, we investigated whether the damage to endothelial cells in HUVEC-macrophage co-cultures that was initiated by LPS is ROS mediated. Incubation of endothelial cell-macrophage co-cultures with 100 ng/ml LPS resulted in endothelial damage (Figure 4). Addition of the ROS scavenging enzymes SOD or catalase reduced endothelial damage. Moreover, endothelial damage was decreased to basal levels when a combination of SOD and catalase was added. Next, we investigated the role of ROS in LPS induced tumor cell adhesion in vivo by treating rats with the anti-oxidant Edaravone, which is clinically used to treat ischemic stroke.\(^{26}\) Animals were treated with saline, Edaravone, LPS or LPS in combination with Edaravone. The liver of rats that were treated with LPS contained significantly higher numbers of tumor cells than the livers of saline or Edaravone treated rats (Figure 5). Importantly, significantly lower numbers of tumor cells were observed in livers of animals receiving LPS in combination with Edaravone, compared to rats receiving only LPS. Thus, this suggested that LPS induced tumor cell adhesion in the liver was ROS dependent.

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**Figure 3:** LPS-induced tumor cell adhesion is Kupffer cell (KCs) dependent.

Number of tumor cells in control or KCs depleted rats that were treated with LPS. Animals were sacrificed 1.5 hour after tumor cell inoculation and liver samples were taken from 5 different liver lobes. Number of tumor cells was determined in liver sections. Graph represents the numbers of tumor cells in both groups (n=4 per group). Red: Dil-labeled CC531s, blue: cell nuclei, ***p<0.001.
**Figure 4:** LPS-induced endothelial damage is mediated by ROS. HUVECs or co-culture of HUVECs with macrophages (MΦ) were incubated with 100 ng/ml LPS in the presence or absence of catalase and/or SOD. HUVECs were stained fluorescently and 5 random pictures were taken per condition and damaged endothelial areas were determined. The graph represents quantification of % damaged endothelial area. Red: HUVEC, blue: nuclei. Dotted lines indicate areas without endothelial cells. *p<0.05; **p<0.01; ***p<0.001.

**Figure 5:** LPS-induced tumor cells adhesion in the liver is prevented by treatment with Edaravone. Numbers of tumor cells in rats that were treated with saline, Edaravone, LPS or LPS in combination with Edaravone and animals were sacrificed 1.5 hour after tumor cells inoculation (n=4 per group). Graph represents the numbers of tumor cells in different groups. Red: DiI labeled CC531s cells, blue: cell nuclei. Arrowheads point to tumor cells. **p<0.01; ***p<0.001.
Surgical resection of the primary tumor is the preferred therapy that can provide long-term disease free survival of patients with CRC. However, surgical trauma is paradoxically correlated with liver metastases development. Additionally, it was demonstrated that LPS led to enhanced lung and liver metastases. Moreover, LPS was detected in portal and peripheral blood of patients after colectomy, and patients with positive bacterial translocation had significantly shorter disease-free survival, supporting the negative effect of bacterial product LPS exposure on long-term clinical outcome. In the current study, we demonstrate that LPS treatment elevated tumor cell adhesion in the livers of rats already 45 minutes after tumor cells injection. It was recently shown that LPS could directly increase colon carcinoma cell adhesion through signaling via TLR4. This led to functional activation of β1 integrin, which is an important adhesion molecule for ECM. However, whereas β1 integrin was essential for adhesion of CC531s colon carcinoma cells to damaged peritoneal surfaces, it was not required for adherence in liver vasculature, which supports that our data can not be explained by direct effect of LPS on CC531s cells. LPS is however also a potent activator of immune responses by triggering TLR4, which is expressed on immune cells. In the liver macrophages are in intimate contact with sinusoidal endothelial cells and capable of ROS release upon LPS stimulation. We now demonstrate that LPS led to activation of macrophages, which resulted in damaged endothelial monolayers in vitro. This may cause the exposure of sub-endothelial extracellular matrix (ECM) in vivo as well. Because we previously showed that exposure of sub-endothelial ECM in the liver vasculature leads to increased tumor cell adhesion, we propose that LPS treatment caused endothelial damage in vivo as well, allowing free circulating tumor cells to adhere on exposed ECM. This hypothesis is supported by a previous study in which it was demonstrated that increased binding of polymorphonuclear cells (PMNs) in the liver microvasculature after LPS treatment was due to altered interaction with ECM. As we observed significant accumulation of PMNs in livers of rats after LPS administration as well (data not shown), it is likely that both PMNs and tumor cells use similar mechanisms to adhere in the liver because of altered ECM exposure. Importantly, downregulation of endothelial tight junction molecules (indicating endothelial cell stress) in post-surgical liver samples of CRC patients has been reported, which supports exposure of sub-endothelial ECM after surgery in patients as well. Furthermore, enhanced numbers of circulating tumor cells during surgery are detected in CRC patients, LPS-induced endothelial damage may contribute to poor patient outcome, by allowing tumor cell adherence in the liver.

Damage to endothelial monolayers that was mediated by LPS was prevented by addition of ROS scavenging enzymes, indicating an essential role for ROS. Both PMNs and macrophages can be activated by LPS and are potent ROS producers. Therefore these cells may contribute to endothelial damage in vivo. Depletion
of liver macrophages or treatment with the antioxidant Edaravone decreased tumor cell adherence in the liver drastically, demonstrating that ROS production by macrophages played an essential role in tumor cell adherence. Additionally, impairment of ROS scavenging systems has been observed both in cancer patients and tumor bearing mice. Catalase activity, which neutralizes $H_2O_2$, was decreased in patients with tumors in rectum, stomach, pancreas or intestines and in leukocytes and liver of tumor bearing mice. Thus, initiation of ROS production initiated by bacterial translocation due to surgery, in cancer patients with an already imbalanced ROS neutralizing system can result in damaged endothelial lining in the liver.

Because we previously demonstrated that enhanced tumor cell adherence resulted in increased liver metastases development, we also investigated tumor development after LPS injection. However, in spite of increased tumor cell adhesion, rats that received LPS had less liver metastases compared to control rats (data not shown). In contrast, previous studies demonstrated that LPS injection led to tumor outgrowth from HT29 or 4T1 cells. However, HT29 cells were grown in immunocompromised mice, and 4T1 cells are poorly immunogenic, whereas CC531s cells have been reported to induce immune responses. Since TLR ligands can induce anti-tumor immunity, we stained tumor samples for influx of CD8+ and CD4+ T cells and NK cells (data not shown). Tumors from the livers of rats that were treated with LPS contained significantly higher numbers of these immune cells, suggesting that LPS stimulated immune responses against immunogenic CC531s cells resulting in elimination of liver metastases development. Alternatively, it is possible that in the study with 4T1 mammary carcinoma cells endotoxin-tolerance was induced, which results in unresponsiveness of the immune system, since a five times higher concentration of LPS was used.

In conclusion, LPS exposure can have opposing effects on tumor cell adherence and tumor development. First, LPS triggers TLR signaling in macrophages, which produce ROS resulting in endothelial damage and exposure of sub-endothelial ECM to which circulating tumor cells adhere. Second, LPS can stimulate clearance of immunogenic tumor cells by long-term initiation of adaptive immune responses. However, lack of specific tumor-associated antigens is a major problem in most clinically manifested tumors. Therefore, it is likely that many patients may not benefit from LPS-induced anti-tumor immune responses because tumor antigens are poorly recognized by immune cells. Additionally, exposure to high LPS concentration – as in the case of anastomotic leakage - may induce endotoxin tolerance, explaining the poorer oncological outcome of these patients. Increased understanding of short-term and long-term activation of immune cells after surgery may help to identify patients at risk for development of post-surgical liver metastases. Designing suitable peri-operative therapies to reduce this risk may ultimately greatly improve patient outcome.
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