Surgery-induced reactive oxygen species enhance colon carcinoma cell binding by disrupting liver endothelial cell lining

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Abstract

Objective
Resection of primary colorectal cancer is associated with enhanced risk of liver metastases development. We previously demonstrated that surgery initiated an early inflammatory response resulting in elevated tumour cell adhesion in the liver. Because reactive oxygen species (ROS) are shown to be produced and released during surgery, we now investigated the effects of ROS on liver vascular lining and tumour cell adhesion.

Methods
Human endothelial cell monolayers (HUVEC and HMEC-1) were exposed to ROS production after which electrical impedance, cellular integrity and tumour cell adhesion was investigated. Furthermore, surgery-induced tumour cell adhesion as well as the role of ROS and liver macrophages (Kupffer cells) in this process was studied in vivo.

Results
Production of ROS decreased cellular impedance of endothelial monolayers dramatically. Moreover, formation of intercellular gaps in endothelial monolayers was observed, exposing sub-endothelial extracellular matrix (ECM) on which colon carcinoma cells adhered via integrin molecules. Endothelial damage was however prevented in the presence of ROS scavenging enzymes. Additionally, surgery induced downregulation of both rat and human liver tight junction molecules. Treatment of rats with the ROS scavenger Edaravone prevented surgery-induced tumour cell adhesion and downregulation of tight junction proteins in the liver. Interestingly, depletion of Kupffer cells prior to surgery significantly reduced numbers of adhered tumour cells and prevented disruption of tight junction proteins expression.

Conclusions
In the current study we show that surgery-induced ROS production by macrophages damages vascular lining by down regulating tight junction proteins. This leads to exposure of ECM, to which circulating tumour cells bind. As such, peri-operative therapeutic intervention, preventing surgery-induced inflammatory reactions may reduce the risk of developing liver metastases, hereby improving clinical outcome of patients with colorectal cancer.
Introduction

Colorectal carcinoma (CRC) is one of the most prevalent malignancies of the gastrointestinal tract in developed countries. Each year approximately one million new cases of CRC are diagnosed worldwide, and ~half a million patients die from this disease every year. While resection of primary CRC is the preferred treatment with curative intent, surgery paradoxically contributes to tumour recurrence and metastasis development. Ultimately, 25-50% of patients who had resection of the primary tumour without any sign of metastases before resection, will develop liver metastasis.

Interestingly, patients who underwent laparoscopy-assisted colectomy showed a longer tumour recurrence free period, higher overall survival and cancer-related survival compared to patients who had open colectomy. This strongly suggested that severity of surgical trauma, which is higher in open colectomy patients, is negatively correlated with survival outcome. Notably, in experimental animal models, development of colorectal liver metastases was significantly reduced by minimally invasive operative techniques, suggesting that reducing trauma favours clinical outcome. Furthermore, free circulating tumour cells were demonstrated in CRC patients, which increased during surgery, suggesting surgery induced tumour cell dissemination.

We previously demonstrated that abdominal surgery resulted in loss of cell-cell contact between mesothelial cells of the peritoneal wall, inducing intercellular gap formation. This led to exposure of underlying extracellular matrix (ECM) on which tumour cells preferentially adhered. A similar phenomenon was observed in livers of operated rats, as both decreased tight junction molecule expression and increased tumour cell-ECM interaction in the liver were demonstrated after surgery. Adhesion of tumour cells to ECM requires integrins, which are heterodimeric adhesion molecules containing α- and β-subunits. Combination of sub-units allows binding to different ECM proteins. Tumour cell adhesion after surgery to either the peritoneal wall or in the liver was abolished by blocking antibodies against integrins β or α, respectively, indicating that tumour cells adhered to exposed ECM via their integrin molecules.

Development of metastases after surgery is not confined to local sites, as thoracotomy resulted in development of distant tumours in the peritoneal cavity, whereas abdominal surgery led to enhanced liver metastases development. As such, induction of systemic responses after surgery is supported. Previous studies demonstrated release of inflammatory mediators like cytokines and reactive oxygen species (ROS). Enhanced release and activity of the ROS producing enzyme xanthine oxidase (XO) and its substrates was observed during surgical trauma. Moreover, it was demonstrated that ROS damages endothelial barriers, leading to vascular permeability and influx of immune cells into tissues like lungs and the brain. Thus, we hypothesize that ROS, which is released during resection of primary colorectal cancer, affects vascular lining of the liver, hereby exposing
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ECM to which circulating tumour cells bind. Therefore, effects of ROS production on endothelial lining and tumour cell adhesion were investigated in vitro and in vivo.

Materials and methods

Endothelial cell cultures
Human umbilical vein endothelial cells (HUVECs) were isolated according to standard procedures, 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 µg/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.

Human micro vascular endothelial cells of the lung (HMEC-1) were cultured on fibronectin coated culture flasks with EGM-2 MV bullet kit (Lonza, Basel, Switzerland) according to the manufacturer’s instructions.

Colon carcinoma cell cultures
Human colon carcinoma cell lines LS180, WiDR, SW620, HT29, HCT116 and RKO (ATCC, Manassas, VA) were cultured in DMEM (Gibco) containing 10% fetal calf serum (FCS, Gibco), 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin (DMEM/10%). CC531s cells were cultured in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) containing 10% FCS, 2 mM glutamine, 50 U/ml penicillin and streptomycin (RPMI/10%). Cell suspensions were prepared using trypsin-EDTA solution. Viability was assessed by tryphan blue exclusion and exceeded 95%. Human colon carcinoma cells (4.5x10⁶ cells/ml) were labelled fluorescently by incubation at 37°C for 20’ in DMEM/10% containing calcein-AM (0.5 mM, Invitrogen), after which cells were washed with Hanks’ balanced salt solution containing 0.5% bovine serum albumin (HBSS/BSA). CC531s cells (5x10⁶ cells/ml) were incubated in RPMI/10% containing 5 µg/ml Dil (Sigma-Aldrich, St. Louis, MO) for 30’ at 37°C and subsequently washed with HBSS/BSA.

Animal models
Male inbred Wag/Rij rats (200-220g) 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 visualize tumour cell adhesion in the liver, midline laparotomy was performed
under anaesthesia and Dil-labelled CC531s cells were injected in a mesenteric vein. In order to detect a sufficient number of adhered cells in microscopic slides, a high number of cells \((2,5 \times 10^6)\) was injected. Animals were sacrificed at different time points. Alternatively, Kupffer cell (KCs) depletion was accomplished by injecting clodronate (Roche Diagnostics, Mannheim, Germany) -encapsulated in liposomes, intravenously 2 days before surgery.

The ROS scavenger Edaravone (3-Methyl-1-Phenyl-2-Pyrazolin-5-one, Calbiochem, Darmstadt, Germany) was dissolved in 6% ethanol. Animals received 125 mg/kg Edaravone or the vehicle ethanol (±6%) intraperitoneally 30’ prior to surgery and just before closure of the wound. Rats were sacrificed after 1.5 hours. Liver samples were frozen for microscopical analyses. Additionally, effect of Edaravone treatment on tumour outgrowth was studied. Midline laparotomy was performed and all animals received CC531s cells via a mesenteric vein \((n=7/{\text{group}})\). Because injection of \(2,5 \times 10^6\) tumour cells (see adhesion experiments) would lead to unacceptable high tumour development, the dose for long term experiments was lowered to \(0.5 \times 10^6\) cells. Animals were sacrificed 14 days after surgery and tumour load in livers was scored in a blinded fashion by 2 independent observers. For transmission electron microscopy (TEM), animals were operated and perfused under anaesthesia as described.¹⁵

**Generation of ROS**

ROS were generated using XO (from bovine milk, Sigma Aldrich) and its substrates xanthine and hypoxanthine in a concentration of \(1 \times 10^{-6}\) M.²⁸ This mixture produces constant levels of superoxide and to a lesser extent hydrogen peroxide and hydroxyl radicals.²⁹ In additional experiments, ROS scavenging 5000 U/ml superoxide dismutase (SOD) and 5000 U/ml catalase (from bovine liver, Sigma Aldrich) were added. To investigate HUVECs viability, cells were incubated with mixtures of XO and substrates, after which standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assays were performed.

**Endothelial electric cell–substrate impedance sensing (ECIS) measurements**

Endothelial monolayer integrity was investigated by measuring electrical impedance with ECIS. HUVECs were grown confluent on collagen (from calf skin, Sigma Aldrich)-coated ECIS Cultureware™ 8W10E+ (Applied BioPhysics, Troy, NY), washed with M199 and incubated at standard culture conditions. Electrical impedance of cell layers was measured every 10 seconds at 4000 Hz for 15’ to determine baseline. ROS production was initiated by addition of XO and substrates in the presence or absence of ROS scavengers. Electrical impedance was measured for 25’.
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Colon carcinoma cell adhesion assay
HUVECs were grown confluent on collagen or fibronectin (human, Harbour-bioproducts, Massachusetts, NE) coated flat-bottom 96-wells culture plates (Greiner Bio-One, Frickenhausen, Germany). Cells were washed and incubated with increasing concentrations of XO and substrates for 15’. Cells were then washed with HBSS/BSA after which 7x10⁴ calcein-labelled human colon carcinoma cells were added and incubated for 1 hour at 37°. Alternatively, calcein-labelled LS180 cells were pre-treated with XO for 15’ or incubated for 30’ at room temperature (RT) with mouse serum as isotype control or antibodies against integrins α₂, α₅ or β₁ in HBSS/BSA prior to adhesion experiments. After washing, fluorescence was measured (485 nm excitation/520 nm emission filters; Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany).

Fluorescence and scanning electron microscopy (SEM)
HUVECs or HMEC-1 cells were grown on collagen or fibronectin coated glass 8-chamber slides (NUNC, Amsterdam, The Netherlands), and exposed to ROS production by adding XO and substrates, after which calcein-labelled LS180 cells were added and incubated for 1 hour at 37°C. After washing, cells were fixated with 0.5% glutardialdehyde (Brunschwig Chemie, Amsterdam, The Netherlands) for 15’ at RT, permeabilized with 0.05% Tween-20 for 2’ and stained with Rhodamin-phalloidin (Invitrogen) for 15’. Chamber slides were washed, mounted and examined with a Nikon Eclipse E8000 microscope. Five pictures were taken randomly with a digital Nikon DXM1200 camera (Nikon, Lijnden, The Netherlands) with fixed exposure time for all photos. The digital image analysis program AnalySIS Pro (Soft Imaging System, Münster, Germany) was used to quantify number of bound tumour cells.
For SEM experiments, HUVECs were cultured on collagen-coated cover slips and experiments were performed as described above. Cells were fixated with Mc Dowels fixative solution for 10’ at RT, dehydrated in a graded ethanol series and hexamethyldisilazane. Samples were mounted on aluminium, SEM specimen mount stubs and sputter coated with gold, using Balzers Union SCD040. Cells were examined in a Scanning Electron Microscope (Phillips 525, Orion Frame Grabber), operated at 15 KV with spot size 30 nm.
Liver biopsies were taken from patients undergoing liver resection due to colorectal metastases 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’, slides were incubated for 1 hour with primary antibodies against ZO-1, occludin or claudin-5 (Zymed Laboratories, San Fransisco, CA) at RT. Mouse or rabbit serum was used as isotype control.
After washing, visualization was achieved by incubation with Alexa 488-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). Tumour cell numbers at different time points after surgery in rat livers was determined (20 stitched fields per liver sample, 5 liver samples per animal).

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+/SEM.

Results

Treatment of HUVEC with XO leads to enhanced tumour cell adhesion
HUVECs, grown on collagen, were exposed to ROS production by adding increasing XO concentrations. Addition of LS180 cells after ROS production resulted in elevated cell adhesion in a dose dependent manner (Figure 1a). To investigate whether this was a general phenomenon or specific for LS180 cells, experiments were repeated with other human colon carcinoma cell lines. Enhanced tumour cell adhesion after ROS production was observed for WiDr, SW620, HT29 or HCT116 cells in a XO concentration dependent manner (figure 1a). In contrast, no increased RKO cell adhesion was detected (Figure 1a). However, when HUVECs were grown on fibronectin, ROS exposure led to enhanced RKO adhesion (Figure 1b). Addition of SOD and catalase prevented ROS-induced WiDR and RKO adhesion (Figure 1c). ROS exposure of LS180 cells did not affect cell adhesion to collagen in the absence of HUVECs, supporting that ROS-induced tumour cell adhesion was a consequence of alterations to the endothelial monolayer (Figure 1d).

ROS production disrupts endothelial integrity
Previous experiments suggested that tumour cell adhesion depended on the composition of sub-endothelial ECM coating, since elevated RKO cell adhesion was only observed when HUVEC were grown on fibronectin. We therefore investigated the influence ROS on endothelial integrity with ECIS. Replacement of fluid disrupted electron flow temporarily. However, in contrast to 0 and 3 mU/ml, impedance was not restored to basal levels after addition of XO concentrations of 6 mU/ml or higher (Figure 2a), indicating disruption of cellular integrity. Incubation of HUVECs with XO did not affect endothelial cell viability, suggesting that decreased impedance was not caused by detachment of dead endothelial cells (Figure 2b).
Figure 1: Human colon carcinoma cell adhesion to HUVEC monolayers after production of ROS. 

a: HUVEC monolayers were grown on collagen and exposed to ROS production for 15 minutes by adding increasing XO concentrations. Adhesion assays were performed with LS180, WiDR, SW620, HT29, HCT116 and RKO cells. 

b: RKO cell adhesion after ROS production on HUVECs that had been grown on fibronectin. 

c: HUVEC monolayers were grown on collagen or fibronectin after which medium, 25 mU/ml XO, XO+SOD+catalase or SOD+catalase was added. WiDR and RKO cell adhesion was investigated. Adhesion on untreated HUVEC monolayer (XO 0 uM/ml) was set at 100%. Differences between groups were analysed with ANOVA.*p<.05; **p<.01; ***p<.001 compared to XO 0 mU/ml; n=4
Figure 2: Production of ROS results in endothelial cell damage.  

**A**: Cellular impedance of HUVEC monolayers during exposure to increasing XO concentrations. Arrowhead indicates XO addition. 

**B**: HUVEC cell viability after exposure to ROS production by adding increasing XO concentrations. 

**C-D**: Fluorescent microscopy images of HUVEC (c) or HMEC-1 (d) monolayers after ROS production by addition of increasing XO amounts. Intercellular gaps in endothelial monolayers are indicated with asterisks. 

**E**: Left picture: HUVEC monolayer treated with 25 mU/ml XO. Right picture: HUVEC monolayer treatment with 25 mU/ml XO was followed by addition of SOD and catalase. 

**F**: Cellular impedance of HUVEC monolayers after exposure to ROS production in absence or presence of SOD+catalase. First arrowhead indicates addition of SOD+catalase and the second addition of XO. n= 4.
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Next, effect of ROS production on HUVEC monolayers was visualized using fluorescent microscopy. In the absence of XO, tightly adhered HUVEC layers were observed (Figure 2c). Incubation of HUVECs with 6 mU/ml XO resulted in visible loss of cell-cell contact and intercellular gaps. Adding higher XO concentrations increased both volumes and amount of intercellular gaps, hereby exposing the sub-endothelial collagen coating. Because we previously showed that in vivo tumour cells adhered in both large and smaller vessels in the liver of operated rats, we also tested whether ROS production impairs integrity of micro-vascular endothelium (HMEC-1). Untreated HMEC-1 cells showed an intact monolayer (Figure 2d). Treatment of HMEC-1 monolayers with 6 mU/ml XO resulted in formation of gaps between the endothelial cells, which increased after treatment with higher XO concentrations, which was consistent with results obtained with HUVECs.

To investigate whether established damage to endothelial monolayers was reversible, SOD and catalase were added after XO treatment. Again, ROS production resulted in retraction of endothelial cells (Figure 2e). However, when HUVEC treatment with XO was followed by addition of SOD and catalase, endothelial integrity was restored, as spreading of HUVECs was observed (Figure 2e). Moreover, in the presence of SOD and catalase reduction of cellular impedance of HUVEC monolayers after XO treatment was prevented (Figure 2f).

Effects of ROS production on endothelial integrity were further investigated with SEM. Untreated HUVECs showed intercellular contacts (Figure 3a). Exposure of HUVECs to ROS production resulted in blunted endothelial cells without intercellular contacts (Figure 3b). Damage to endothelial layers was reversed by addition of SOD and catalase, restoring intercellular contacts (Figure 3c).

Strikingly, tumour cells adhered preferentially in intercellular gaps, but did not bind to endothelial cells (Figure 4a and b). Since we previously demonstrated that metastasis development in liver or abdominal cavity was integrin α2 or β1 dependent, we next investigated tumour cell adhesion after blocking integrins α2, β1, or α5 (as control). HUVEC monolayers cultured on collagen were exposed to ROS production. Incubation of LS180 cells with anti-integrin α2 antibody reduced tumour cells adhesion significantly, while integrin β1 blockade resulted in a small but significant inhibition (Figure 4c and d). Blocking α5 did not prevent cell adhesion to collagen.
**Figure 3**: ROS production leads to intercellular gap formation in endothelial lining. *a*: Untreated HUVEC monolayer. *b*: HUVEC exposed to ROS production. *c*: Incubation of HUVEC monolayer with XO was followed by addition of SOD+catalase. 372x magnification.
Figure 4: Effects of ROS generation on endothelial monolayers and tumour cell adherence. a: Production of ROS results in formation of intercellular gaps. b: SEM pictures of tumour cell adhesion on HUVEC monolayer treated with 0 and 6 mU/ml XO. TC: tumour cell, EC: endothelial cell. c-d: Adhesion of LS180 cells after incubation with blocking antibody against integrin α2, β1 or α5. Adhesion in presence of isotype control antibody was set at 100%. Red: HUVEC; green: LS180 cells. Differences between groups were analysed with ANOVA. *p<.05, ***p<.001, compared to isotype control. n=4
**Figure 5:** Surgery-induced tumour cell adhesion is mediated by production of ROS. 

a: The number of CC531s cell adhesion in the liver at different time points after surgery. n=4 per time point.

b: CC531s cell adhesion in livers of rats after vehicle or Edaravone treatment. n=4 vehicle group, n=5 Edaravone group.

c: Tight junction molecules expression in non-operated rats vs. vehicle or Edaravone treated rats. Green: ZO-1 (upper panels) or occludin (lower panels), blue: nuclei.

d: Liver metastases development in vehicle and Edaravone treated rats. Arrowheads indicate tumour nodules. n=7 per group. Differences between groups (in a, right panel) were analysed with ANOVA, *p<.05; **p<.01; ***p<.001. The difference between the vehicle or EDA groups was determined with the Student T-test (in b, right panel). **p<.01, compared to vehicle.
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**Treatment with ROS scavenger Edaravone prevented tumour cell adhesion**

Previously, we demonstrated that surgery stimulates tumour cell adhesion. Because rats do not spontaneously develop metastasizing colon carcinoma, presence of tumour cells in the portal circulation was introduced by injecting tumour cells in a mesenteric vein. First, laparotomy was performed, followed by injection of fluorescently labelled CC531s, after which rats were sacrificed at different time points. After 45 minutes tumour cells had adhered in the liver, which further increased 1.5 hours after surgery, after which CC531s numbers declined (Figure 5a). To study whether ROS production underlies surgery-induced tumour cell adhesion, rats were treated with Edaravone, which is a hydroxyl and peroxyl scavenger that inhibits oxidative endothelial damage and is used for clinical applications. Rats were treated 30 minutes prior to surgery, after which tumour cells were injected in the mesenteric vein. Rats received a second dose of Edaravone just before closure of the wound. Edaravone treated rats contained significantly less tumour cells in livers compared to vehicle treated rats (Figure 5b). Incubation with Edaravone neither affected direct cell adhesion nor viability of tumour cells in vitro (data not shown). We therefore next investigated whether surgery would affect liver vascular integrity, which might be inhibited by Edaravone, by staining for tight junction proteins ZO-1 and occludin in rat livers. Both expression of ZO-1 and occludin was decreased in operated rats compared to non-operated control rats (Figure 5c). Interestingly, Edaravone treatment prevented decrease in expression of tight junction molecules in rat livers, supporting that ROS were responsible for disruption of endothelial cell integrity. Because of successful inhibition of tumour cell adhesion after surgery, we also investigated whether Edaravone prevented liver metastases development. Unfortunately, Edaravone treatment did not prevent outgrowth, but enhanced liver metastases development as a trend towards higher tumour load was observed (Figure 5d).

**Surgery induced down-regulation of the tight junction molecule claudin-5 in human livers.**

To investigate whether the results obtained in our rat experiments translate to the human situation, we collected liver biopsies from patients undergoing liver resection due to colorectal metastases. Biopsies were taken at the start and end of the surgery. Because a high background was observed when livers were stained for either ZO-1 or occludin, obscuring expression of tight junctions (data not shown), livers were additionally stained for the tight junction molecule claudin-5. Expression of claudin-5 in human livers was significantly reduced at the end of the surgery, compared to expression in biopsies, which had been taken at the beginning (Figure 6a). For comparison, we also stained rat livers that had been collected at the beginning and end of surgery for claudin-5 expression. Comparable to the human situation, claudin-5 expression was decreased in rat livers that were obtained at the end of the surgery (Figure 6b).
Kupffer cells are involved in surgery-induced tumour cell adhesion
Treatment with Edaravone decreased tumour cell adhesion in the liver, but was not able to prevent metastases outgrowth. We therefore hypothesized that treatment with Edaravone interfered with the ability of macrophages to generate ROS, since we previously demonstrated that prevention of tumour development requires proper macrophage functioning, and ROS production is essential for macrophage-mediated killing of tumour cells. As such, we next investigated the role of KCs in surgery-induced tumour cell adhesion by depleting these cells. Absence of KCs or newly recruited monocytes was confirmed by ED2 (marker for KCs) or ED1 (marker for monocytes) staining (data not shown). Animals, in which KCs were depleted prior to surgery, contained significantly lower numbers of tumour cells in their liver compared to control rats (Figure 6a). Moreover, livers of KC-depleted operated rats had higher expression of occludin compared to control rats after surgery (Figure 6b), indicating that endothelial integrity was still intact; supporting the role of KCs in ROS mediated endothelial damage. Activation of macrophages is amongst others characterized by the presence of pseudopodia and intracellular vacuoles. To investigate whether treatment with Edaravone influenced macrophage activity, we determined KC morphology of vehicle or Edaravone treated rats with TEM (Figure 6c). KCs of vehicle treated operated rats had many pseudopodia and contained several vacuoles, supporting highly activated cells. By contrast, when ROS production in KCs was inhibited by Edaravone treatment, KCs showed less pseudopodia and contained hardly any vacuoles.

Discussion
Despite of surgery with curative intent, approximately 25-50% of CRC patients without detectable metastases at the time of resection of the primary tumour will develop metastases within 2 years, with the liver as major site. Surgical trauma was shown to induce early inflammatory systemic responses. In the current study, we show that surgery-induced ROS formation is responsible for increased tumour cell adhesion in the liver of rats, which is prevented by treatment with ROS scavenger Edaravone.

Previously, it was demonstrated that human tumour cell adhesion was increased 12 hours after exposure of endothelial cells to ROS in vitro, which was accompanied by up-regulation of adhesion molecules by endothelial cells including endothelial-selectin, inter-cellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. This suggested that tumour cells adhered to endothelial cells. However, in the current study, exposure of HUVEC monolayers to ROS production enhanced human tumour cell adhesion already after 15 minutes. We demonstrate that ROS production had a transient destructive effect on electrical impedance and initiated formation of intercellular gaps, exposing sub-cellular ECM to which tumour cells adhered. This is supported by our previous and current findings in which we demonstrated surgery-induced loss of expression of tight junction proteins in the
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liver of rats, leading to retraction of sinusoidal endothelial cells hereby facilitating tumour cell adhesion to exposed ECM.\textsuperscript{15} Furthermore, \textit{in vivo} circulating rat colon carcinoma cells adhered already 45 minutes after surgery on exposed ECM in the liver. Scavenging ROS prevented down-regulation of tight junction molecules, supporting that surgery-induced ROS production damages liver vascular lining. Because we observed similar surgery-induced down-regulation of the tight junction molecule claudin-5 in both rat and human livers, we hypothesize that surgery-induced endothelial damage also contributes to adhesion of circulating tumour cells in patients that undergo resection of colorectal cancer.

Previously, an imbalance in ROS production and the ROS scavenging system was shown during laparotomy, which resulted in intercellular gap formation between intestinal epithelial cells.\textsuperscript{36} When ROS was scavenged, intercellular gaps formation was prevented in animal models.\textsuperscript{21, 36} Thus, surgery-induced ROS production similarly may lead to initial loss of endothelial cell-cell contact, hereby exposing sub-endothelial ECM to which tumour cells preferentially bind. In a later stage up-regulation of adhesion molecules on endothelial cells may furthermore contribute to tumour cell adhesion.

\textbf{A} Human \hspace{1cm} begin surgery \hspace{2cm} end surgery

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{image1}
\caption{Surgery induced down-regulation of the tight junction molecule claudin-5 in \textit{a}: human and \textit{b}: rat livers}
\end{figure}

\textbf{B} Rat \hspace{1cm} begin surgery \hspace{2cm} end surgery

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{image2}
\end{figure}
KCs are involved in initiating enhanced tumour cell adhesion, since depletion of these cells in rats decreased the amount of adhered tumour cells after surgery. Additionally, we observed accumulation of polymorphonuclear cells in rat livers after surgery (data not shown), which are potent ROS producers and as such may also contribute to endothelial damage. In addition to enhanced ROS production, impairment of ROS scavenging systems was found in tumour bearing humans and mice. Catalase activity, which neutralizes $\text{H}_2\text{O}_2$, was decreased in patients with tumours in rectum, stomach, pancreas or intestines. Furthermore, catalase activity in leucocytes and liver was depressed in tumour bearing mice. Therefore, initiation of ROS production by surgery in cancer patients with an already imbalanced ROS neutralizing system can result in damaged endothelial lining.

**Figure 7**: KCs are involved by surgery-induced tumour adhesion. a: Tumour cell adhesion in operated control or KCs depleted rats. n=4 per group. b: occludin expression in livers of operated control vs KCs depleted rats. Green: occludin, blue: nuclei. c: Morphology of KCs in vehicle or Edaravone treated rats. Arrows indicate pseudopodia and vacuoles. Ps: pseudopodia, Va: vacuoles. The difference between the control or KC- depleted groups was determined with the Student T-test (in a, right panel). ***p<.001 compared to control.
Although treatment of rats with the ROS scavenger Edaravone successfully prevented downregulation of tight junction proteins and decreased tumour cell adhesion in the liver, it was not able to prevent outgrowth of liver metastases. This is most likely due to the fact that tumour cell killing by KCs is ROS dependent.\textsuperscript{40} We observed that the number of tumour cells decreased after 1.5 hour following surgery, which supports elimination by KCs, as these were previously shown to play an essential role in preventing liver metastases development.\textsuperscript{32, 33} Thus, ROS scavenging by Edaravone during surgery likely acts as a double-edged sword. First, Edaravone prevents short-term surgery-induced endothelial damage by neutralizing ROS production by KCs, hereby decreasing tumour cell adhesion. Second, it unfortunately promotes tumour cell survival through inhibition of cytotoxic activity of macrophages. Because KCs induced endothelial damage occurs within minutes, whereas killing of macrophages takes several hours (Figure 5A, and data not shown), we hypothesize that designing an antioxidant with a short half-life might prevent increased tumour cell adhesion without interfering with the killing capacity of KCs. Edaravone is currently used in the clinic for treatment of diseases involving oxidative stress like stroke,\textsuperscript{41} but has a half-life of 5.6 hours.\textsuperscript{42} As such, it is likely not suitable for peri-operative intervention, because it may interrupt tumour cell killing by macrophages. We speculate that developing novel antioxidants with short half life may interrupt early ROS production, hereby leading to less damaged vessels, while preserving long-term macrophage function. However, this requires a delicate balance, extensive research is required to exclude long-acting effects of ROS scavenging on KCs or other cell populations.

Alternatively, blocking integrins on circulating tumour cells may represent an attractive therapeutic strategy. We previously demonstrated that integrin $\alpha_2$ and $\beta_1$ are the main adhesion molecules for collagen binding, whereas $\alpha_5$ is involved in binding to fibronectin. Furthermore, in our rat model we demonstrated that blocking integrin $\beta_1$ or $\alpha_2$ abolished tumour cell adhesion to the peritoneal wall or in liver vessels, respectively.\textsuperscript{14, 15} Thus, adhesion of tumour cells and metastases development in different organs may depend on integrin expression by tumour cells and ECM expression in that specific organ. As such, the integrin expression profile of the primary tumour might potentially be used as a diagnostic tool for prediction of metastases development.

In conclusion, surgery results in production of ROS by KCs, which disrupts endothelial cell integrity in the liver. This leads to exposed ECM to which tumour cells preferentially adhere. Outcome in patients may be improved by perfecting surgical techniques, since resection of primary colon cancer involving less trauma can result in reduced liver metastases development.\textsuperscript{7} However, inflicting a limited amount of surgical trauma will be unavoidable, warranting the need for novel adjuvant peri-operative therapies. Preventing surgery-induced tumour cell adhesion by inhibiting harmful inflammatory responses may represent such a promising strategy for reducing metastasis development, hereby improving clinical outcome.
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