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

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ABSTRACT

Objective Resection of primary colorectal cancer is associated with enhanced risk of development of liver metastases. It was 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, the effects of ROS on the liver vascular lining and tumour cell adhesion were investigated.

Methods Human endothelial cell monolayers (human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells of the lung (HMEC-1s)) were exposed to ROS production, after which electrical impedance, cellular integrity and tumour cell adhesion were investigated. Furthermore, surgery-induced tumour cell adhesion as well as the role of ROS and liver macrophages (Kupffer cells) in this process were 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 subendothelial 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 the numbers of adhered tumour cells and prevented disruption of expression of tight junction proteins.

Conclusions In this study it is shown that surgery-induced ROS production by macrophages damages the vascular lining by downregulating tight junction proteins. This leads to exposure of ECM, to which circulating tumour cells bind. In light of this, perioperative therapeutic intervention, preventing surgery-induced inflammatory reactions, may reduce the risk of developing liver metastases, thereby improving the clinical outcome of patients with colorectal cancer.

Significance of this study

What is already known about this subject?

- Resection of primary colorectal cancer stimulates metastasis development.
- Surgery increases the amounts of circulating tumour cells by dissemination.
- Surgery initiates inflammatory responses.

What are the new findings?

- Exposure of endothelial monolayers to reactive oxygen species (ROS) elevates tumour cells adhesion.
- ROS-induced tumour cell adhesion is subendothelial extracellular matrix dependent, but does not involve binding to endothelial cells.
- Liver resident macrophages, Kupffer cells, are responsible for enhanced tumour cell adhesion after surgery.
- Surgery-induced tumour cell adhesion in the liver is dependent on ROS production by Kupffer cells.

How might it impact clinical practice in the foreseeable future?

- Prevention of tumour cell adhesion in the liver after resection of the primary colorectal cancer by adjuvant perioperative therapeutic interventions (eg, using an antioxidant with a short half-life) might reduce surgery-induced development of liver metastases, thereby significantly improving patient outcome.

INTRODUCTION

Colorectal carcinoma (CRC) is one of the most prevalent malignancies of the gastrointestinal tract in developed countries. Each year ~1 million new cases of CRC are diagnosed worldwide, and about half a million patients die from this disease every year.[1–3] While resection of primary CRC is the preferred treatment with curative intent, surgery paradoxically contributes to tumour recurrence and metastasis development.[4] Ultimately, 25–50% of patients who had resection of the primary tumour without any sign of metastases before resection will develop liver metastasis.[1 5]

Interestingly, patients who underwent laparoscopy-assisted colectomy showed a longer tumour recurrence-free period, and higher overall survival and cancer-related survival compared with patients who had open colectomy.[6] This strongly suggested that the severity of surgical trauma, which is higher

in patients undergoing open colectomy, is negatively correlated with survival outcome. Notably, in experimental animal models, development of colorectal liver metastases was significantly reduced by minimally invasive operative techniques,[7–9] supporting the idea that reducing trauma favours clinical outcome. Furthermore, free circulating tumour cells were demonstrated in patients with CRC, which increased during surgery, suggesting surgery-induced tumour cell dissemination.[10–12]

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.[13] 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.[13 14] Adhesion of tumour cells to the ECM requires integrins, which are heterodimeric adhesion molecules containing α- and β-subunits. Combination of subunits allows binding to different ECM proteins.[15] Tumour cell adhesion after surgery either to the peritoneal wall or in the liver was abolished by blocking antibodies against integrins β_1 or α_2, respectively,[13 14] 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 development of liver metastases.[14 16] As such, induction of systemic responses after surgery is supported. Previous studies demonstrated release of inflammatory mediators such as cytokines and reactive oxygen species (ROS).[17–19] Enhanced release and activity of the ROS-producing enzyme xanthine oxidase (XO) and its substrates was observed during surgical trauma.[20–22] Moreover, it was demonstrated that ROS damages endothelial barriers, leading to vascular permeability and influx of immune cells into tissues such as the lungs and the brain.[23]

Thus, we hypothesise that ROS, which are released during resection of primary colorectal cancer, affect the vascular lining of the liver, thereby exposing the ECM to which circulating tumour cells bind. Therefore, the effects of ROS production on the 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,[24] and cultured to confluence till passage 5 in medium M199, supplemented with 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 5000 U/ml heparin, 10 μg/ml basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, Connecticut, USA), 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin (Gibco, Irvine, UK) under standard culture conditions.

Human microvascular endothelial cells of the lung (HMEC-1s) were cultured in fibronectin-coated culture flasks with an 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, Virginia, USA) were cultured in Dulbecco's modified Eagle's medium (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%).[25] Cell suspensions were prepared using trypsin–EDTA solution. Viability was assessed by trypan blue exclusion and exceeded 95%.

Human colon carcinoma cells (4.5×10^6 cells/ml) were labelled fluorescently by incubation at 37°C for 20 min in DMEM/10% containing calcein-AM (0.5 μM, Invitrogen), after which cells were washed with Hanks' balanced salt solution containing 0.5% bovine serum albumin (HBSS/BSA).

CC531s cells (5×10^6 cells/ml) were incubated in RPMI/10% containing 5 μg/ml DiI (Sigma-Aldrich, St Louis, Missouri, USA) for 30 min at 37°C and subsequently washed with HBSS/BSA.

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 visualise tumour cell adhesion in the liver, midline laparotomy was performed under anaesthesia and DiI-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 ($2{,}5\times10^6$) was injected. Animals were sacrificed at different time points. Alternatively, Kupffer cell (KC) depletion was accomplished by injecting clodronate (Roche Diagnostics, Mannheim, Germany)—encapsulated in liposomes[26]—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 min prior to surgery and just before closure of the wound. Rats were sacrificed after 1.5 h. Liver samples were frozen for microscopic analyses. Additionally, the 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/group). Because injection of 2.5×10^6 tumour cells (see adhesion experiments) would lead to unacceptably high tumour development, the dose for long-term experiments was lowered to 0.5×10^6 cells. Animals were sacrificed 14 days after surgery and tumour load in livers was scored in a blinded fashion by two independent observers. For transmission electron microscopy (TEM), animals were operated and perfused under anaesthesia as described.[14]

Generation of ROS

ROS were generated using XO (from bovine milk, Sigma Aldrich) and its substrates xanthine and hypoxanthine in a concentration of 1×10^{-6} M.[27] This mixture produces constant levels of superoxide and to a lesser extent hydrogen peroxide and hydroxyl radicals.[28] 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 HUVEC 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 to confluency on collagen- (from calf skin, Sigma Aldrich) coated ECIS Cultureware 8W10E+ (Applied BioPhysics, Troy, New York, USA), washed with M199 and incubated under standard culture conditions. Electrical impedance of cell layers was measured every 10 s at 4000 Hz for 15 min 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 min.

Colon carcinoma cell adhesion assay

HUVECs were grown to confluency on collagen- or fibronectin- (human, Harbor-Bioproducts, Norwood, Massachusetts, USA) coated flat-bottomed 96-well culture plates (Greiner Bio-One, Frickenhausen, Germany). Cells were washed and incubated with increasing concentrations of XO and substrates for 15 min. Cells were then washed with HBSS/BSA, after which 7×10^4 calcein-labelled human colon carcinoma cells were added and incubated for 1 h at 37°C. Alternatively, calcein-labelled LS180 cells were pretreated with XO for 15 min or incubated for 30 min at room temperature with mouse serum as isotype control or antibodies against integrins α_2, α_5 or β_1 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 microscopy and scanning electron microscopy (SEM)

HUVECs or HMEC-1s 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 h at 37°C. After washing, cells were fixed with 0.5% glutardialdehyde (Brunschwig Chemie, Amsterdam, The Netherlands) for 15 min at room temperature, permeabilised with 0.05% Tween-20 for 2 min and stained with rhodamine–phalloidin (Invitrogen) for 15 min. 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 a fixed exposure time for all photos. The digital image analysis program AnalySIS Pro (Soft Imaging System, Münster, Germany) was used to quantify the number of bound tumour cells.

For SEM experiments, HUVECs were cultured on collagen-coated coverslips and experiments were performed as described above. Cells were fixed with McDowels fixative solution for 10 min at room temperature and 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 a spot size of 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 ethics committee of the VUMC. Cryostat liver tissue sections from human and rat livers were fixed for 10 min in acetone and air-dried. After blocking with 10% normal goat serum for 15 min, slides were incubated for 1 h with primary antibodies against ZO-1, occludin or claudin-5 (Zymed Laboratories, San Fransisco, California, USA) at room temperature. Mouse or rabbit serum was used as isotype control. After washing, visualisation was achieved by incubation with Alexa 488-labelled goat anti-mouse antibody (Molecular Probes, Eugene, Oregon, USA). Nuclei were stained with Hoechst (Molecular Probes). 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 were 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 analysis of variance (ANOVA). Statistical significance was accepted at $p<0.05$. Results are presented as mean±SEM.

RESULTS

Treatment of HUVECs 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 an 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). Exposure of LS180 cells to ROS 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 subendothelial ECM coating, since elevated RKO cell adhesion was only observed when HUVECs were grown on fibronectin. We therefore investigated the influence of 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 (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).

Next, the effect of ROS production on HUVEC monolayers was visualised 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 the volumes and amount of intercellular gaps, thereby exposing the subendothelial collagen coating. Because we previously showed that in vivo tumour cells adhered in both large and smaller vessels in the liver of operated rats,[14] we also tested whether ROS production impairs the integrity of microvascular endothelium (HMEC-1s). Untreated HMEC-1s showed an intact monolayer (figure 2D). Treatment

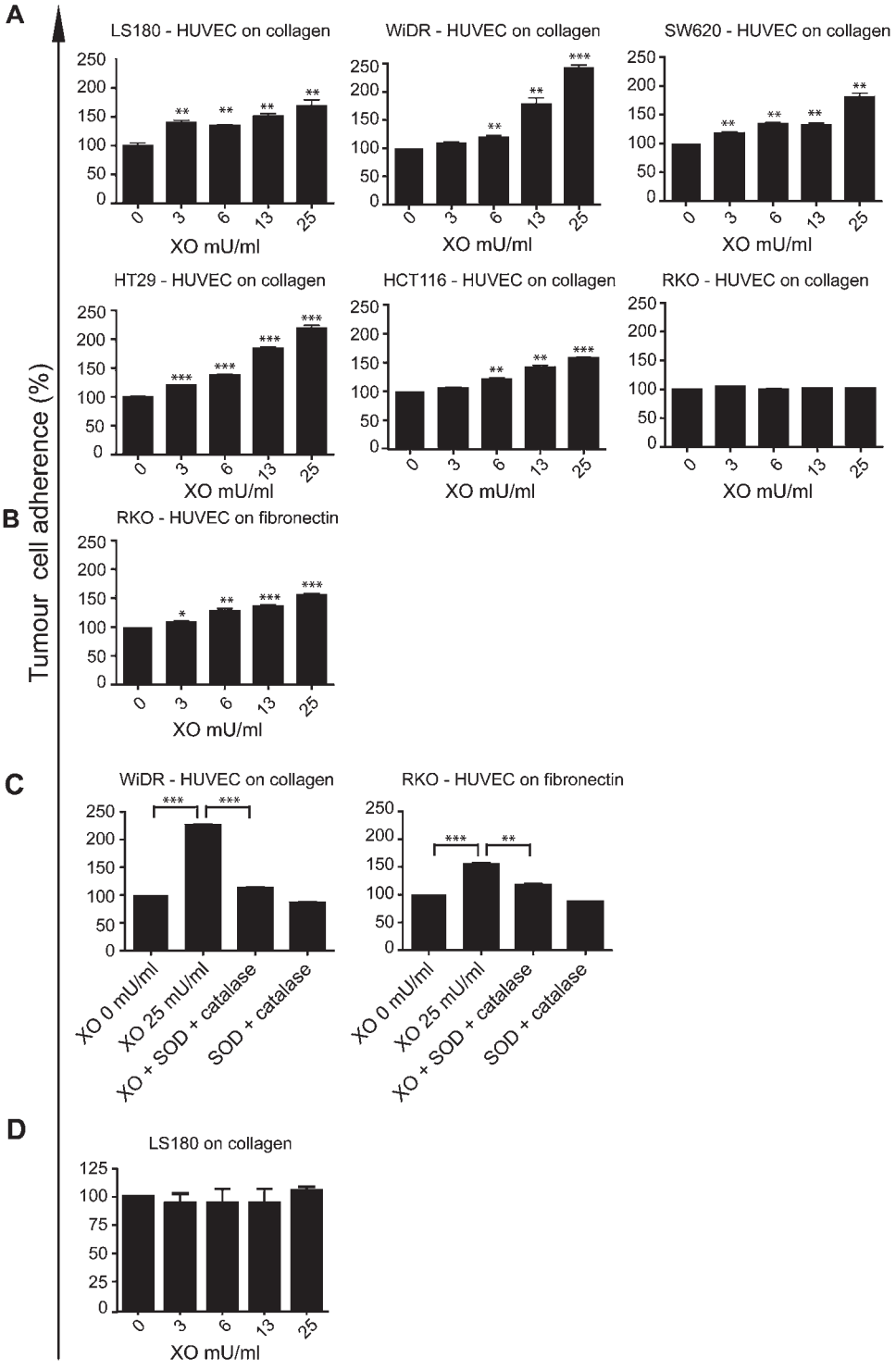

Figure 1 Human colon carcinoma cell adhesion to human umbilical vein endothelial cell (HUVEC) monolayers after production of reactive oxygen species (ROS). (A) HUVEC monolayers were grown on collagen and exposed to ROS production for 15 min by adding increasing xanthine oxidase (XO) concentrations. Adhesion 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 +superoxide dismutase (SOD) +catalase or SOD+catalase was added. WiDR and RKO cell adhesion was investigated. Adhesion on untreated HUVEC monolayers (XO 0 mU/ml) was set at 100%. (D) LS180 cells were treated with increasing concentrations of XO for 15 min and tumour cell adhesion on the collagen coating was assessed. Adhesion of untreated cells (XO 0 mU/ml) was set at 100%. Differences between groups were analysed with analysis of variance (ANOVA).*$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with XO 0 mU/ml; n=4

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 the 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).

The 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,B). Since we

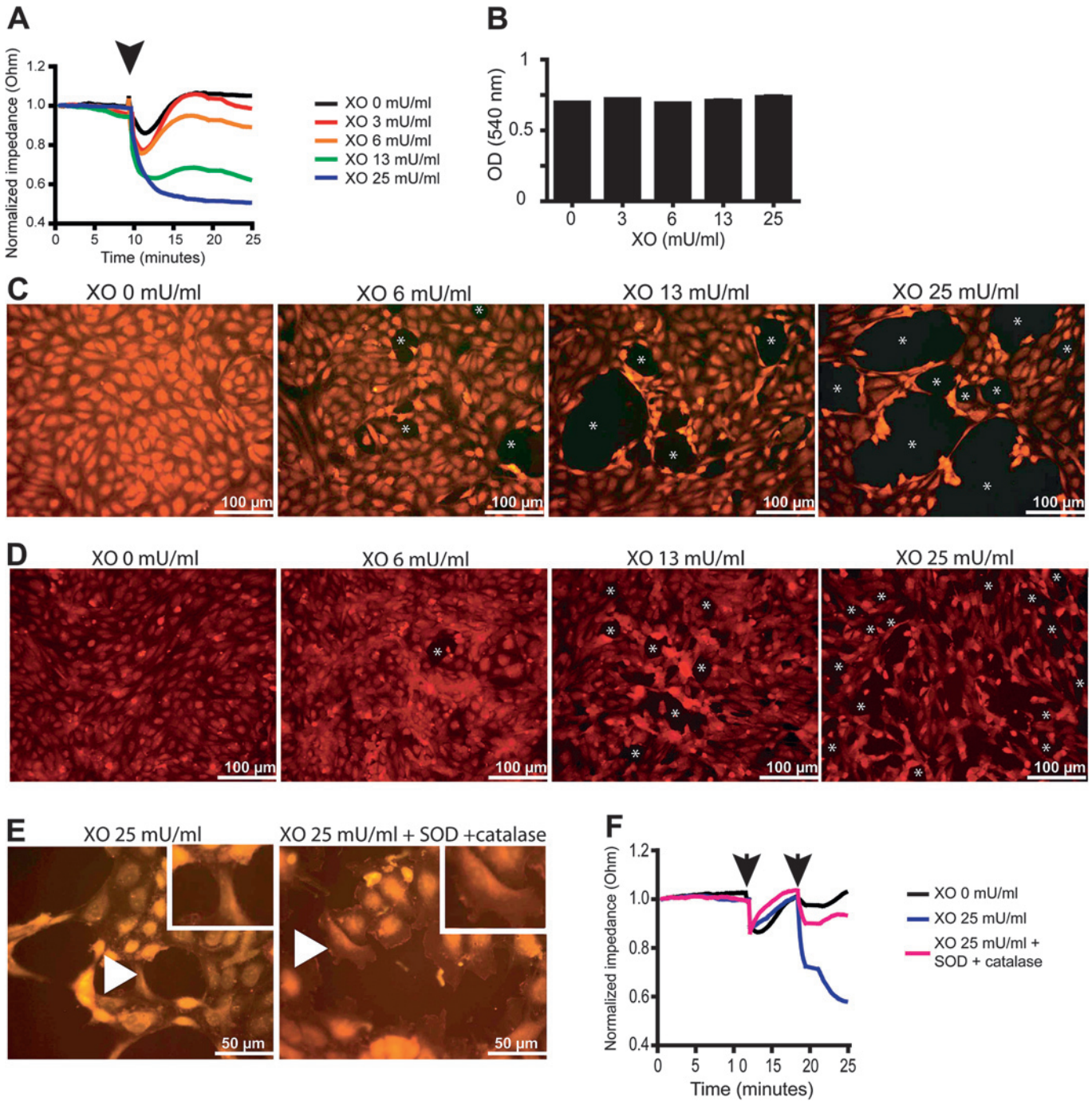

Figure 2 Production of reactive oxygen species (ROS) results in endothelial cell damage. (A) Cellular impedance of human umbilical vein endothelial cell (HUVEC) monolayers during exposure to increasing xanthine oxidase (XO) concentrations. The arrowhead indicates XO addition. (B) HUVEC viability after exposure to ROS production by adding increasing XO concentrations. (C and D) Fluorescence microscopy images of HUVEC (C) or HMEC-1 (human microvascular endothelial cell of the lung; D) monolayers after ROS production by addition of increasing amounts of XO. Intercellular gaps in endothelial monolayers are indicated with asterisks. (E) Left picture: a HUVEC monolayer treated with 25 mU/ml XO. Right picture: a HUVEC monolayer treated with 25 mU/ml XO followed by addition of superoxide dismutase (SOD) and catalase. (F) Cellular impedance of HUVEC monolayers after exposure to ROS production in the absence or presence of SOD+catalase. The first arrowhead indicates addition of SOD+catalase and the second addition of XO. n=4.

previously demonstrated that metastasis development in the liver or abdominal cavity was integrin α_2 or β_1 dependent,[13 14] we next investigated tumour cell adhesion after blocking integrins α_2, β_1 or α_5 (as control). HUVEC monolayers cultured on collagen were exposed to ROS production. Incubation of LS180 cells with anti-integrin α_2 antibody reduced tumour cell adhesion significantly, while integrin β_1 blockade resulted in a small but significant inhibition (figure 4C,D). Blocking α_5 did not prevent cell adhesion to collagen.

Treatment with the ROS scavenger edaravone prevented tumour cell adhesion

Previously we demonstrated that surgery stimulates tumour cell adhesion.[14] Because rats do not spontaneously develop

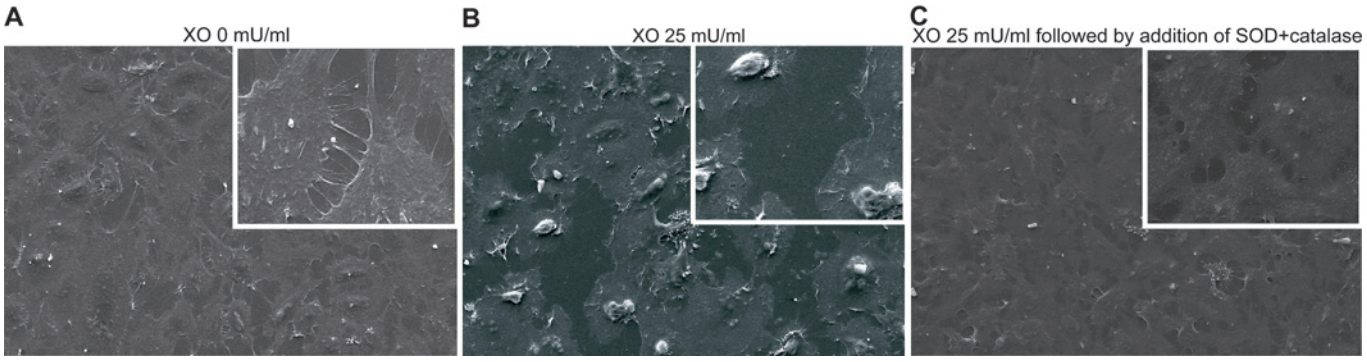

Figure 3 Reactive oxygen species (ROS) production leads to intercellular gap formation in the endothelial lining. (A) An untreated human umbilical vein endothelial cell (HUVEC) monolayer. (B) HUVECs exposed to ROS production. (C) Incubation of a HUVEC monolayer with xanthine oxidase (XO) was followed by addition of superoxide dismutase (SOD)+catalase. ×372 magnification.

metastasising colon carcinoma, the 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 cells, after which rats were sacrificed at different time points. After 45 min tumour cells had adhered in the liver, which further increased 1.5 h after surgery, after which CC531s cell 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.[29 30] Rats were treated 30 min prior to surgery, after which tumour cells were injected into the mesenteric vein. Rats received a second dose of edaravone just before closure of the wound. Edaravone-treated rats were found to have significantly fewer tumour cells in their livers compared with vehicle-treated rats (figure 5B). Incubation with edaravone affected neither 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 the tight junction proteins ZO-1 and occludin in rat livers. The expression of both ZO-1 and occludin was decreased in operated rats compared with non-operated control rats (figure 5C). Interestingly, edaravone treatment prevented the 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 development of liver metastases. Unfortunately, edaravone treatment did not prevent outgrowth, but enhanced development of liver metastases, as a trend towards higher tumour load was observed. (figure 5D).

Surgery induced downregulation 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 with 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. Comparably with the human situation, claudin-5 expression was decreased in rat livers that were obtained at the end of the surgery (figure 6B).

KCs are involved in surgery-induced tumour cell adhesion

Treatment with edaravone decreased tumour cell adhesion in the liver, but was not able to prevent outgrowth of metastases. We therefore hypothesised 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.[31 32] In light of this, we next investigated the role of KCs in surgery-induced tumour cell adhesion by depleting these cells. The absence of KCs or newly recruited monocytes was confirmed by ED2 (a marker for KCs) or ED1 (a marker for monocytes) staining (data not shown). Animals in which KCs were depleted prior to surgery had significantly lower numbers of tumour cells in their liver compared with control rats (figure 7A). Moreover, livers of KC-depleted operated rats had higher expression of occludin compared with control rats after surgery (figure 7B), indicating that endothelial integrity was still intact, supporting the role of KCs in ROS-mediated endothelial damage. Activation of macrophages is, among other factors, characterised 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 7C). KCs of vehicle-treated operated rats had many pseudopodia and contained several vacuoles, supporting that the cells were highly activated. In contrast, when ROS production in KCs was inhibited by edaravone treatment, KCs showed fewer pseudopodia and contained hardly any vacuoles.

DISCUSSION

Despite surgery with curative intent, ~25–50% of patients with CRC without detectable metastases at the time of resection of the primary tumour will develop metastases within 2 years, with the liver as the major site.[1 5] Surgical trauma was shown to induce early inflammatory systemic responses.[14 33] 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 the ROS scavenger edaravone.

Previously, it was demonstrated that human tumour cell adhesion was increased 12 h after exposure of endothelial cells to

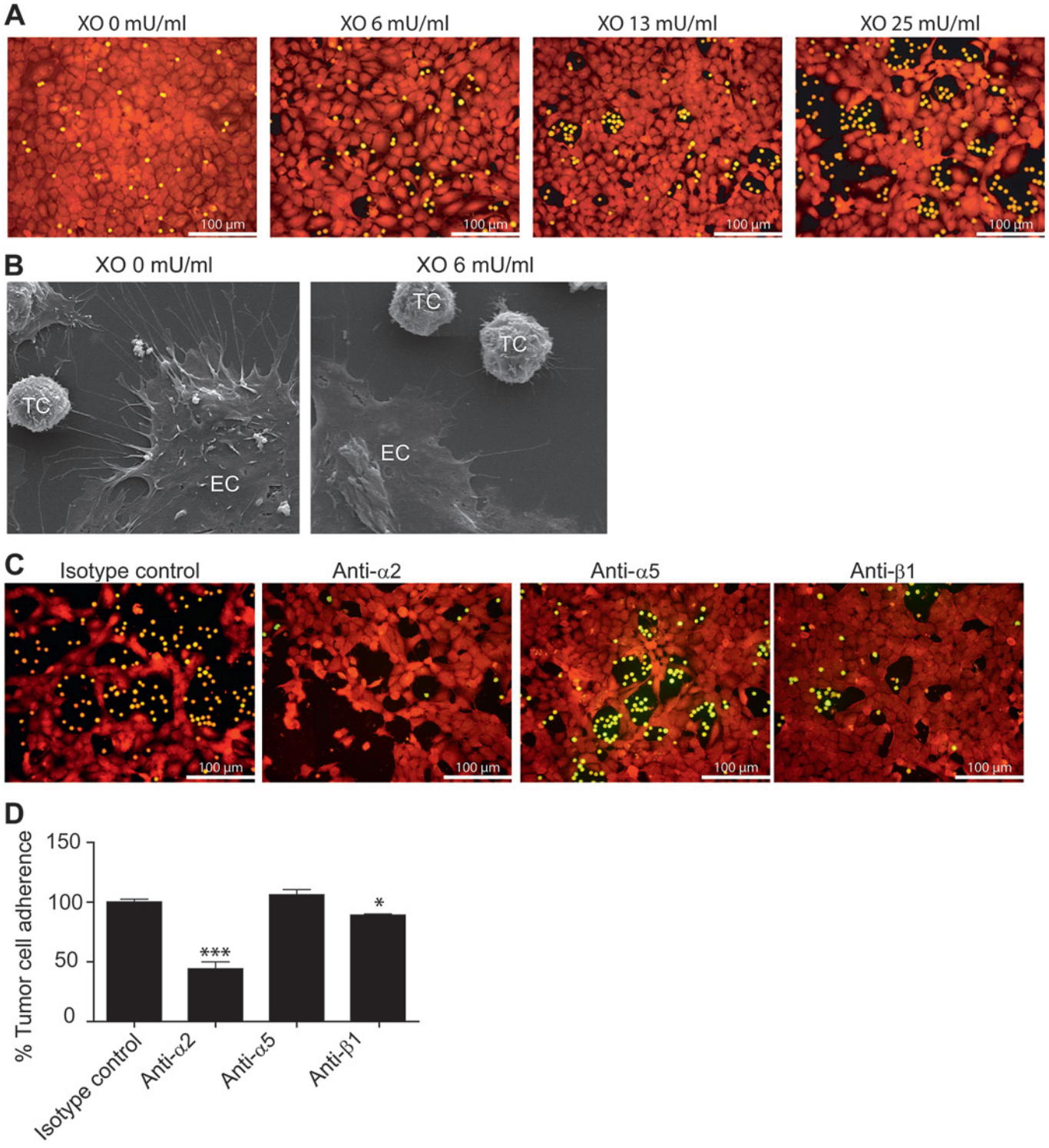

Figure 4 Effects of reactive oxygen species (ROS) generation on endothelial monolayers and tumour cell adherence. (A) Production of ROS results in formation of intercellular gaps. (B) Scanning electron microscopy pictures of tumour cell adhesion on a human umbilical vein endothelial cell (HUVEC) monolayer treated with 0 and 6 mU/ml xanthine oxidase (XO). TC, tumour cell; EC, endothelial cell. (C and D) Adhesion of LS180 cells after incubation with blocking antibody against integrin α_2, β_1 or α_5. Adhesion in the presence of isotype control antibody was set at 100%. Red, HUVECs; green, LS180 cells. Differences between groups were analysed with analysis of variance (ANOVA). *$p<0.05$, ***$p<0.001$, compared with isotype control. n=4.

ROS in vitro, which was accompanied by upregulation of adhesion molecules by endothelial cells including endothelial-selectin, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1.[27] 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 min. We demonstrate that ROS production had a transient destructive effect on electrical impedance and initiated formation of intercellular gaps, exposing subcellular 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 liver of rats, leading to retraction of sinusoidal endothelial cells and thereby facilitating tumour cell

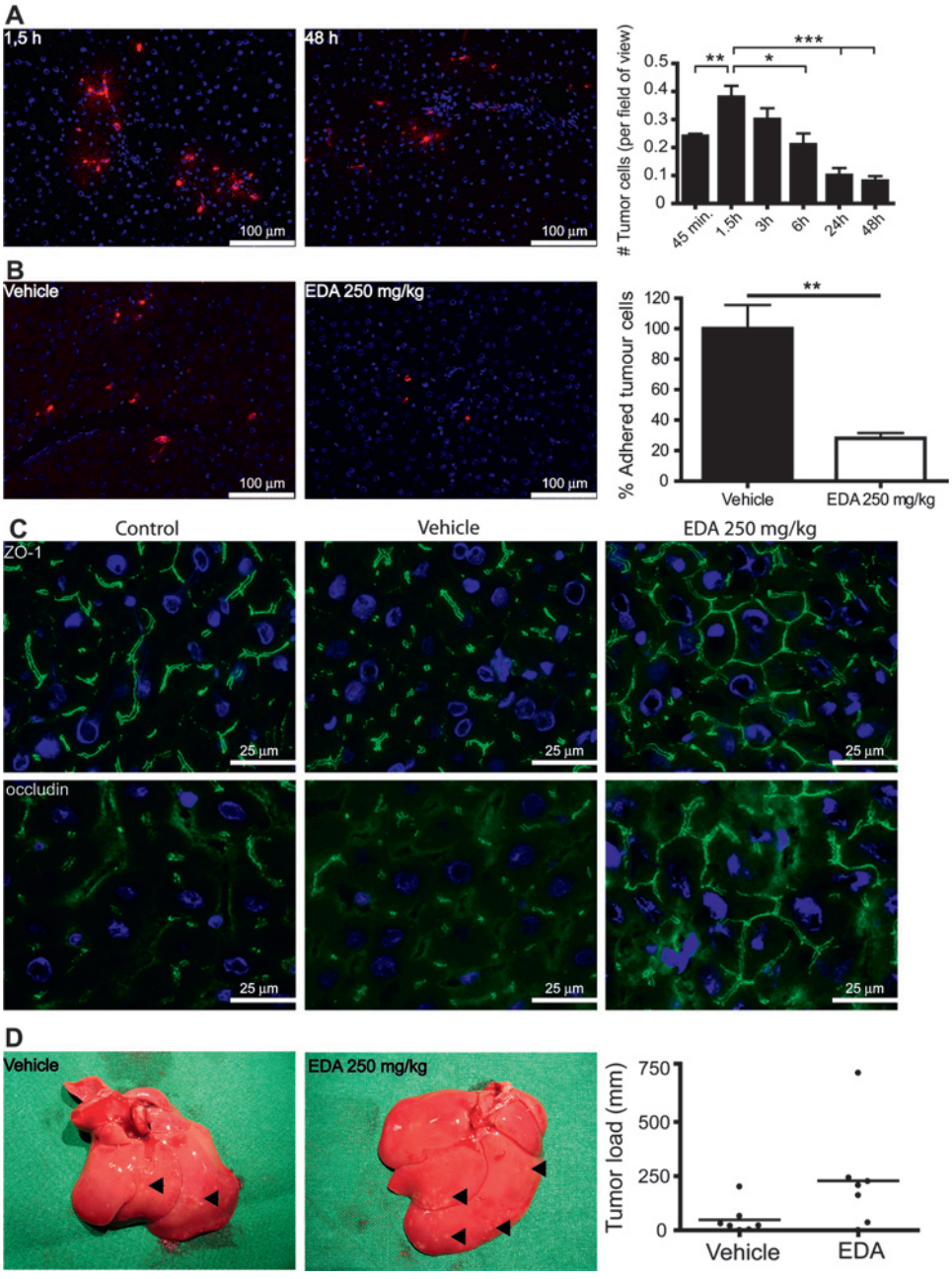

Figure 5 Surgery-induced tumour cell adhesion is mediated by production of reactive oxygen species (ROS). (A) The number of CC531s cells adhering in the liver at different time points after surgery. n=4 per time point. (B) CC531s cell adhesion in livers of rats after vehicle or edaravone (EDA) treatment. n=4 vehicle group, n=5 EDA group. (C) Expression of tight junction molecules in non-operated rats versus vehicle- or EDA-treated rats. Green, ZO-1 (upper panels) or occludin (lower panels); blue, nuclei, (D) Development of liver metastases in vehicle- and EDA-treated rats. Arrowheads indicate tumour nodules. n=7 per group. Differences between groups (in A, right panel) were analysed with analysis of variance (ANOVA). *$p<0.05$; **$p<0.01$; ***$p<0.001$. The difference between the vehicle or EDA groups was determined with the Student t test (in B, right panel). **$p<0.01$, compared with vehicle.

adhesion to exposed ECM.[14] Furthermore, in vivo circulating rat colon carcinoma cells already adhered 45 min after surgery on exposed ECM in the liver. Scavenging ROS prevented downregulation of tight junction molecules, supporting that surgery-induced ROS production damages the liver vascular lining. Because we observed similar surgery-induced downregulation of the tight junction molecule claudin-5 in both rat and human livers, we hypothesise 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.[34] When ROS were scavenged, intercellular gap formation was prevented in animal models.[20 34] Thus, surgery-induced ROS production similarly may lead to initial loss of endothelial cell–cell contact, thereby exposing subendothelial ECM to which tumour cells preferentially bind. In a later stage, upregulation of adhesion molecules on endothelial cells may contribute further to tumour cell adhesion.

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[35] 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 neutralises H_2O_2, was decreased in patients with tumours in the rectum, stomach, pancreas or intestine.[36] Furthermore, catalase activity in leucocytes and the liver was depressed in tumour-bearing mice.[37] Therefore, initiation of ROS production by surgery in cancer patients with an already imbalanced ROS neutralising system can result in damaged endothelial lining.

Although treatment of rats with the ROS scavenger edaravone successfully prevented downregulation of tight junction proteins

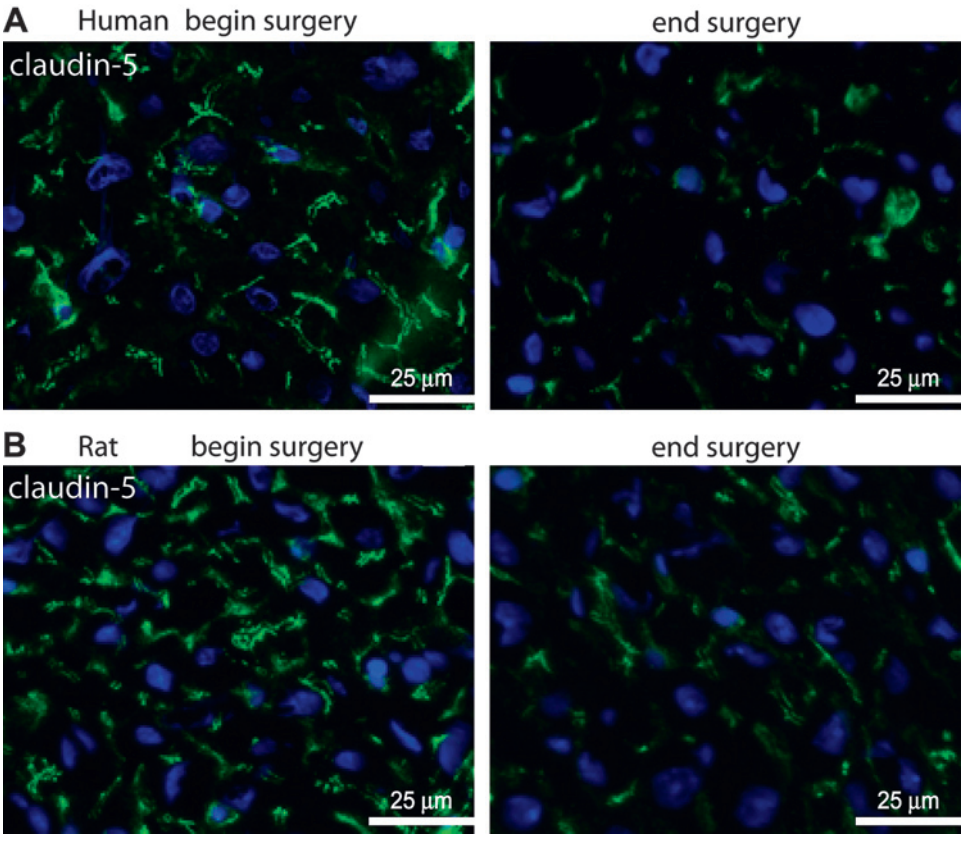

Figure 6 Surgery-induced downregulation of the tight junction molecule claudin-5 in (A) human and (B) rat livers.

and decreased tumour cell adhesion in the liver, it was not able to prevent outgrowth of liver metastases. This is most probably due to the fact that tumour cell killing by KCs is ROS dependent.[38] We observed that the number of tumour cells decreased after 1.5 h following surgery, which supports elimination by KCs, as these were previously shown to play an essential role in preventing development of liver metastases.[31 32] Thus, ROS scavenging by edaravone during surgery probably acts as a double-edged sword. First, edaravone prevents short-term surgery-induced endothelial damage by neutralising ROS

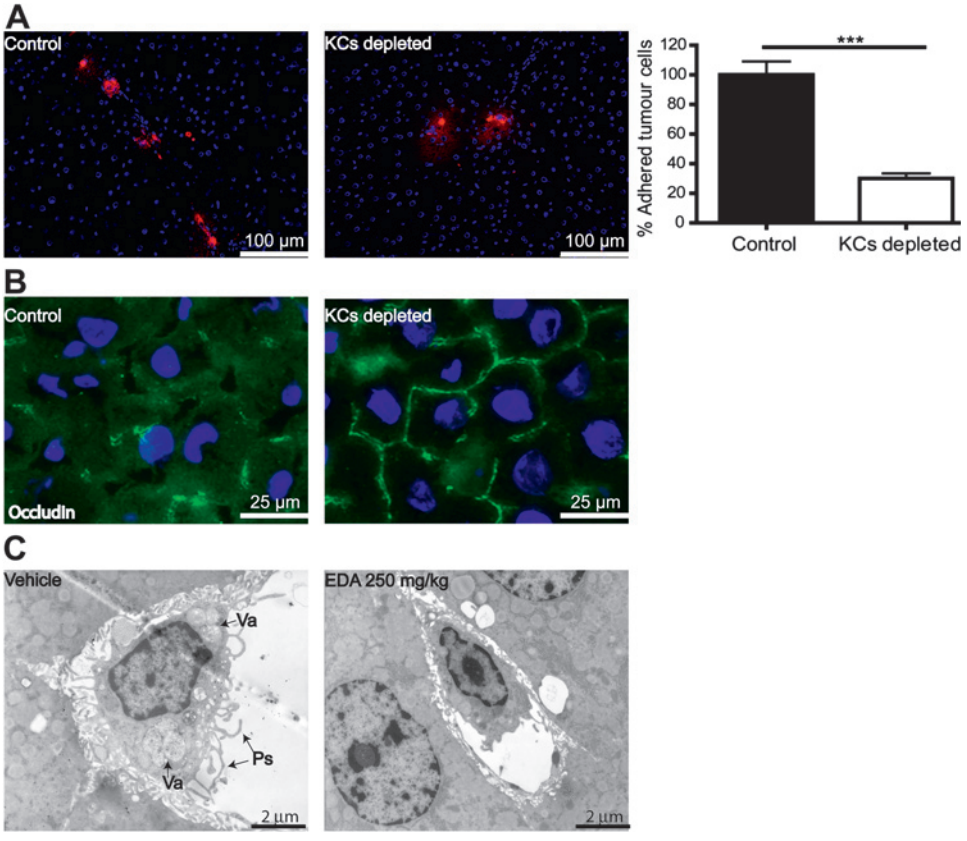

Figure 7 Kupffer cells (KCs) are involved by surgery-induced tumour adhesion. (A) Tumour cell adhesion in operated control or KC-depleted rats. n=4 per group. (B) Occludin expression in livers of operated control versus KC-depleted rats. Green, occludin; blue, nuclei. (C) Morphology of KCs in vehicle- or edaravone (EDA)-treated rats. Arrows indicate pseudopodia (Ps) and vacuoles (Va). The difference between the control or KC-depleted groups was determined with the Student t test (in A, right panel). ***$p<0.001$ compared with control.

production by KCs, thereby decreasing tumour cell adhesion. Secondly, it unfortunately promotes tumour cell survival through inhibition of the cytotoxic activity of macrophages. Because KC-induced endothelial damage occurs within minutes, whereas killing of macrophages takes several hours (figure 5A, and data not shown), we hypothesise 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 such as stroke,[39] but has a half-life of 5.6 h.[40] As such, it is probably not suitable for perioperative intervention, because it may interrupt tumour cell killing by macrophages. We speculate that developing novel antioxidants with a short half-life may interrupt early ROS production, thereby leading to fewer damaged vessels, while preserving long-term macrophage function. However, as 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 α_2 and β_1 are the main adhesion molecules for collagen binding, whereas α_5 is involved in binding to fibronectin. Furthermore, in our rat model we demonstrated that blocking integrin β_1 or α_2 abolished tumour cell adhesion to the peritoneal wall or in liver vessels, respectively.[13 14] Thus, adhesion of tumour cells and development of metastases in different organs may depend on integrin expression by tumour cells and ECM expression in that specific organ. In this regard, the integrin expression profile of the primary tumour might potentially be used as a diagnostic tool for prediction of development of metastases.

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. The outcome in patients may be improved by perfecting surgical techniques, since resection of primary colon cancer involving less trauma can result in reduced development of liver metastases.[6] However, inflicting a limited amount of surgical trauma will be unavoidable, warranting the need for novel adjuvant perioperative treatments. Preventing surgery-induced tumour cell adhesion by inhibiting harmful inflammatory responses may represent such a promising strategy for reducing metastasis development, thereby improving clinical outcome.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the ethics commission of VUMC.

Provenance and peer review Not commissioned; externally peer reviewed.

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Editor's quiz: GI snapshot

An obscure mass in the head of the pancreas of an adolescent

CLINICAL PRESENTATION

An 18-year-old male student presented with epigastric pain of 10 days duration. The symptoms were moderate and continuous but without radiation. The pain was aggravated by food and lying on his back. The patient denied a history of exposure to tuberculosis and any other medical or surgical history. There had been no weight loss or fever prior to the presentation. Physical examination was normal. Chest and abdominal x-ray showed no evidence of abnormality. Both abdominal CT and MRI confirmed a 3.5 cm round mass with enhancement in the pancreatic uncinate process, compressing the inferior vena cava medially and anteriorly constricting the mid part of the inferior vena cava (figure 1). Both the common bile duct and main pancreatic duct were mildly dilated. The patient was not willing to accept any invasive examinations such as fine needle aspiration (FNA) cytology under the guidance of endoscopic ultrasound and endoscopic retrograde cholangiopancreatography (ERCP). A FDG-PET (fluorodeoxyglucose-positron emission tomography) scan showed an intense accumulation of FDG in the head of the pancreas (mean standardised uptake value (SUV)=7.2) (figure 2). Liver function tests and the glucose level were normal. The Mendel—Mantoux test was weakly positive. The white blood cell count was 6.74×10^9/l, C-reactive protein 9.3 ng/l, erythrocyte sedimentation rate 13 mm/h and the HIV serological test was negative. No data suggest immunodeficiency.

QUESTIONS

Is the mass from the pancreas? What is the property of this mass?

See page 1138 for answers

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Competing interests None.

Ethics approval This study was conducted with the approval of the Ethics Committee of West China Hospital, Sichuan University.

Patient consent Obtained.

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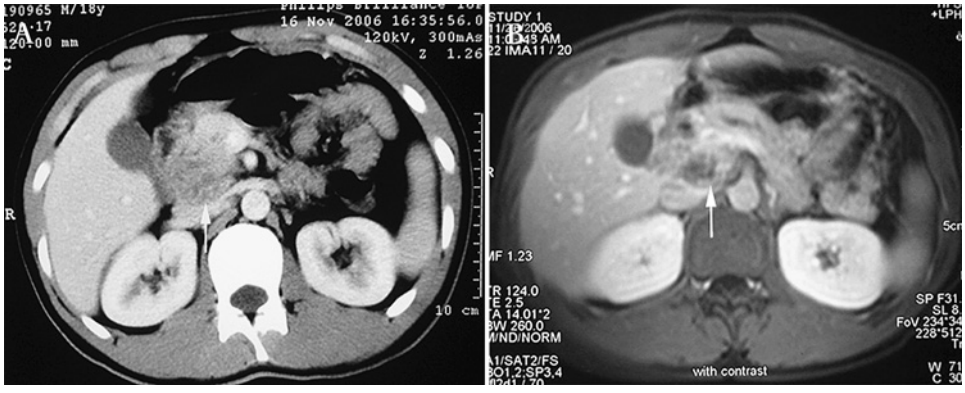

Figure 1 Contrast-enhanced axial CT and MRI showed a round enhanced mass (arrow) probably located in the pancreatic uncinate process.

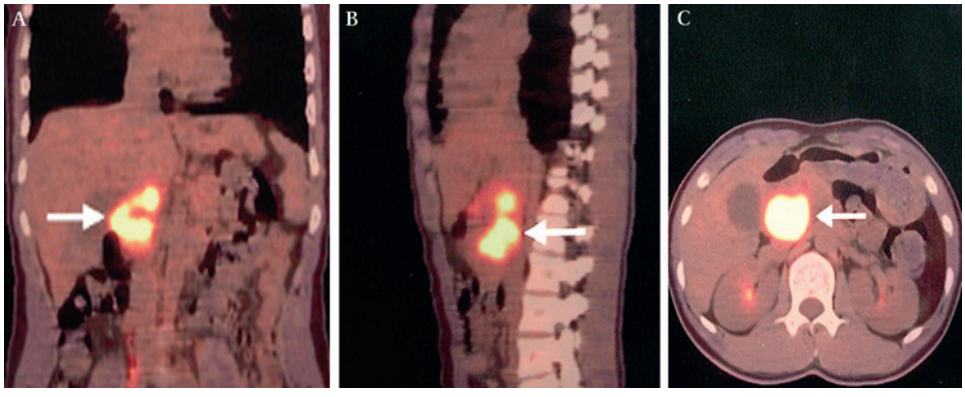

Figure 2 Hybrid 3D PET/CT images showed intense FDH accumulation (arrow) in the pancreatic head (mean standard uptake value(SUV)=7.2.

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

Nuray Gül, Marijn Bögels, Simran Grewal, et al.

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