Chapter *6*

The role of superoxide anions in the development of distant tumour recurrence

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ABSTRACT

We hypothesize that reactive oxygen species released from activated polymorphonuclear leukocytes during surgery play a crucial role in enhanced tumour recurrence seen after surgery. Therefore, the effect of reactive oxygen species on adhesion of tumour cells to microvascular endothelium in a reproducible human *in vitro* model was studied.

Pre-incubation of microvascular endothelial cells with the superoxide anion producing xanthine - xanthine-oxidase complex significantly increased adhesion of the human colon carcinoma cells HT29 (167% *vs.* control, $p<0.01$), Caco2 (164% *vs.* control, $p<0.01$) and of the pancreas carcinoma cells PanC1 (180% *vs.* control, $p<0.01$). Addition of the antioxidant enzymes superoxide dismutase or catalase significantly decreased tumour cell adhesion ($p<0.01$).

Exposure of endothelial cells to superoxide anions increased the apoptotic rate to 7.9 times the normal rate. Additionally, exposure increased expression of the endothelial adhesion molecules E-Selectin, ICAM-1 and VCAM-1 of maximally 170% *vs.* control ($p<0.01$).

In conclusion, this study shows that superoxide anions promote the adherence of tumour cells to the microvasculature by inducing endothelial apoptosis that subsequently induces the expression of various adhesion molecules for tumour cells. This indicates that by tackling the production of reactive oxygen species preventing tumour recurrence at distant sites might be feasible.

INTRODUCTION

Despite the introduction of new treatment modalities for gastro-intestinal malignancies during the last decades surgery remains the principal therapy for most gastro-intestinal malignancies, although the recurrence rates after intentionally curative surgery are high [1-4]. Operative trauma in itself may favour development of tumour recurrence. This relation between abdominal surgery and locoregional tumour recurrence was investigated in previous *in vivo* and *in vitro* experiments. These studies illustrated that surgical trauma enhanced locoregional tumour recurrence and that this phenomenon involved a dose-response relation, i.e. severe trauma was associated with a higher locoregional tumour recurrence rate compared to mild trauma [5-9]. Further experiments demonstrated that abdominal surgical trauma provoked a local inflammatory reaction with influx of mainly polymorphonuclear cells (PMN). These activated PMN produced reactive oxygen species (ROS) which are found to play an important role in the observed enhanced locoregional tumour recurrence in which binding of the tumour cells to the mesothelium is an essential step [8,9].

The inflammatory reaction caused by abdominal surgical trauma is not confined to the abdominal cavity, but spreads out systemically [10-16]. So is it found that during and shortly after major surgery, the peripheral blood level of elastase, which is an indicator of PMN activity, is elevated [17-21]. Furthermore, major abdominal surgery results in an elevated PMN concentration at distant sites, for example in the lung leading to a distant inflammatory reaction [22]. Therefore, surgical trauma may not only promote local tumour recurrence, but also tumour recurrence at distant sites.

Cancer dissemination is frequently accomplished via the blood stream. While many circulating tumour cells fail to survive this phase of the metastatic cascade, the establishment of metastases depends upon the arrest of surviving cells and their exit from the circulation, which involves adhering to and crossing the barriers imposed by the microvascular endothelium and extracellular matrix [22,23].

Based on the previous studies that demonstrate an important role for ROS in locoregional tumour recurrence after surgical trauma, combined with the systemic inflammatory process after surgical trauma, we hypothesize that ROS enhance distant tumour recurrence by increased tumour cell adhesion to the endothelium.

In this study therefore, we investigate the influence of ROS on tumour cell-endothelial cell interactions. The underlying mechanism of the enhanced adhesion by PMN-derived ROS will be further elucidated. Two tumour cell types were used, namely colon and pancreas carcinoma cells to assess the effect of superoxide anions on tumour cell-endothelial cell

interactions with focus on the expression of a variety of cellular adhesion molecules and the occurrence of apoptosis of both the tumour and microvascular endothelial cells.

MATERIALS & METHODS

Cells

Human microvascular endothelial cells of the lung (MEC) were purchased from Cambrex (Verviers, Belgium) at passage 4 and maintained in EGM-2-MV Bullet kit according to the manufacturer's instructions at 37°C, 95% relative humidity and 5 % CO_2. Confluent monolayers were passaged by 0.025% trypsin / 0.01% ethylenediaminetetraacetic acid (EDTA) and cells were used up to passage 8.

The human colon carcinoma cell lines HT29 and Caco2 and the human pancreas carcinoma cell line PanC1 were grown in EGM-2-MV Bullet kit as well in order to create similar conditions and maintained by serial passage after trypsinization using 0.05% trypsin / 0.02% EDTA (Gibco, Breda, the Netherlands).

Before the adhesion assay, tumour cells were trypsinized and maintained in suspension for 2 hours to regenerate cell-surface proteins.

ROS and scavengers

In this study the xanthine (X) – xanthine oxidase (XO) complex was used in a concentration of 100 *μ*M and 30 mU/ml respectively (Sigma-Aldrich, Zwijndrecht, the Netherlands), to produce superoxide anions.

Superoxide anions were inactivated by the addition of 400 U/ml superoxide dismutase (SOD) (Roche Applied Science, Almere, the Netherlands) that converts superoxide anions into molecular oxygen and hydrogen peroxide. Since hydrogen peroxide may itself affect tumour cell adhesion, 400 U/ml catalase (Sigma-Aldrich, Zwijndrecht, the Netherlands) was added to the *in vitro* model alone or in combination with SOD to decompose any hydrogen peroxide.

Ferricytochrome c reduction assay

To assess production of superoxide anions generated by the combination of X and XO in our model we used the ferricytochrome *c* reduction assay [24]. This assay was performed in phenol red-free and phosphate buffered Hank's Balanced Salt Solution (Invitrogen, Breda,

the Netherlands) with 5% fetal calf serum, since phenol red and pH changes effect the assay. After addition of 75 μM cytochrome *c* (Roche Applied Science, Almere, The Netherlands) the change in absorbance at 550 nm and 540 nm (reference) was continuously recorded by the thermostatted Versamax microplate reader (Molecular Devices) for 125 minutes at 37°C.

Adhesion assay

To quantify tumour cell adhesion to MEC, a standardised cell adhesion assay was developed as described before [25]. Briefly, endothelial monolayers were established in 96 well microtiter plates (Perkin Elmer, Groningen, the Netherlands). To do this, confluent cells were trypsinized and 2×10^{4} endothelial cells were added to each well.

The plates were incubated at 37°C, 95% relative humidity, 5% CO_2 and medium was daily replaced by fresh medium. MEC reached confluence in 3 to 4 days as determined by light microscopy.

To determine the effect of ROS on tumour cell adhesion, endothelial monolayers were pre-incubated with varying doses of X and XO, during varying times. Untreated monolayers served as controls. Tumour cells were pre-incubated or not with the X - XO complex for 12 hours before the adhesion assay. Appropriate SOD and/or catalase were added to the model system to assess ROS specificity of the effects.

To quantify tumour cell adhesion, tumour cells (1×10^{6} cells/ml) were labelled with calcein-AM (Molecular Probes, Leiden, The Netherlands) and 3×10^{4} cells per well were added. Plates were centrifuged for 1 minute at $80 \times g$ and incubated at 37°C for 1 hour. After this, the wells were washed twice with medium. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using a wavelength of 485 nm for excitation and 530 nm for emission respectively.

Enzyme immuno assay (EIA)

Endothelial and tumour cells were grown to confluence as described for the adhesion assays in 96-well flat-bottomed microtiter plates (Becton & Dickinson, Erembodegem, Belgium). Cells were pre-incubated with either cell culture medium alone or combined with X and/or XO. Next, the cells were washed with phosphate buffered saline (room temperature, pH 7.4) and fixed in ethanol / methanol for 45 minutes and washed again. Subsequently, non-specific binding sites were blocked by incubating the wells for 10 minutes with 1% goat serum (Sigma-Aldrich, Zwijndrecht, the Netherlands). Mouse monoclonal antibody to E-

Selectin, intracellular adhesion molecule-1 (ICAM-1) or vascular cellular adhesion molecule-1 (VCAM-1) (ITK, Uithoorn, the Netherlands) in a dilution of 1:500 was added for 1 hour, followed by the addition of biotinylated goat anti-mouse antibody (Sigma-Aldrich, Zwijndrecht, the Netherlands) in a dilution of 1:250. Increased sensitivity was obtained using the ExtrAvidin-Peroxidase system (Sigma-Aldrich, Zwijndrecht, the Netherlands). After washing away any free peroxidase, a substrate solution containing 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt in 0.05 M citrate-phosphate buffer with urea hydrogen peroxide buffer with urea hydrogen peroxide was added. Incubation of endothelial cells without the primary antibody served as a negative control. As a positive control, the ExtrAvidin-Peroxidase system was added followed by substrate development without washing away the peroxidase. After 40 minutes the reaction was stopped with sodium fluoride and photometrical evaluation was performed with a computer-controlled ELISA reader at $\lambda = 405$ nm.

Apoptosis

To assess whether superoxide anions caused apoptosis in MEC a cell-death detection ELISA[plus] kit (Roche Applied Science, Almere, the Netherlands) was used for the detection of cytoplasmic histone-associated DNA fragments. In short, endothelial cells were grown to confluence as described for the adhesion assays in 96-well flat-bottomed microtiter plates. The cells were pre-incubated with X and/or XO for 12 hours and then lysated, whereafter 20 μl of the lysate was transferred into Streptavidin-coated microplate wells. Eighty μl of immunoreagent containing biotinylated anti-histone and peroxidase-labeled anti-DNA antibodies was added into the wells followed by incubation on a plate shaker under gently shaking (300 rpm) for 2 h at 15–25°C. Then the wells were washed thoroughly with incubation buffer and 100 μl of 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate was added. Plates were incubated for 15 minutes on a plate shaker at 250 rpm where after photometric analysis at 405 nm was performed.

Proliferation assay

To establish whether pre-incubation of MEC monolayers with superoxide anions was of influence on MEC cell number, the DNA content was determined using the bisbenzimide fluorescent dye (Roche Applied Science, Almere, The Netherlands) as previously described by Hofland *et al* [26]. Therefore, 2×10^4 endothelial cells / ml were plated in 24 wells plates and after 1 day X-XO was added. At day 0, 1, 2 and 3 after the addition of X-XO wells were washed and plates were stored at -20°C until analysis.

Statistical analysis

All data were evaluated using analysis of variance (ANOVA) to determine overall differences between groups. The Dunnett post-test was carried out to compare between groups. $P \leq 0.05$ was considered to be statistically significant. Experiments (n=6) were performed at least twice.

RESULTS

Evaluation of the model

Labelling tumour cells with calcein-AM did not decrease their viability (>95% using trypan blue). Dilution series of labelled tumour cells on endothelial monolayers showed a linear correlation ($r^2 > 0.99$) between cell number and the level of fluorescence (Fig. 1). Thus, by using such standard curves it became possible to estimate the number of adherent tumour cells in the experimental wells from the fluorescence intensity.

In our model, ferricytochrome *c* in the wells with X-XO was reduced at a rate of 0.32 nmol/ml/min as can be calculated from the results presented in figure 2 using a molecular extinction coefficient of ferricytochrome *c* of 13.125 M^{-1} for a light path of 0.625 cm in the microtitre plate. The addition of SOD prevented the reduction of ferricytochrome *c* completely, indicating that the X-XO system indeed mainly generated superoxide and that 400 U/ml SOD is sufficient in this model to dismutate the formed superoxide anions. Interestingly, in the absence of xanthine xanthine oxidase still generated superoxide, but at a lower rate (Fig.2). In the assay with xanthine oxidase only in the absence of fetal calf serum, we found no superoxide production (data not shown), while in the presence of fetal calf serum, but without the addition of xanthine oxidase and extra xanthine, MEC also were found to produce some superoxide (Fig.2). This made it likely that the fetal calf serum of the medium contained the necessary substrate xanthine, and that MEC contain some endogenous xanthine oxidase (Fig.2).

Adhesion to microvascular endothelial cells

Basal adhesion, i.e. adhesion to non-pre-incubated MEC, was between 20 and 30% of added cells for HT29 and Caco2. For PanC1, basal adhesion was between 10 and 20%.

Pre-incubation of MEC with the X-XO complex enhanced tumour cell adhesion (Fig.3). For PanC1, this enhancement occurred after 12 hours pre-incubation and was increasing with longer pre-incubation times reaching a maximum of 180% vs. control (untreated MEC) after 24 hours of pre-incubation (p<0.01). Comparable results were found for Caco2 after X-XO pre-incubation of MEC with a tumour cell adhesion of 164% compared to basal adhesion (p<0.01). Maximal adhesion for HT29 occurred already after 12 hours pre-incubation of MEC with X-XO and was 167% *vs.* control (p<0.01).
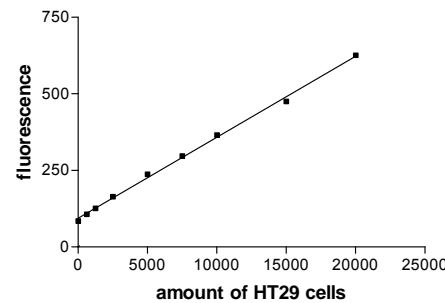

Figure 1. Calibration curves showing the fluorescence measurements (OD 485-530 nm) of calcein-AM labelled tumour cells in order to quantify the amount of tumour cells. Data represent mean ± SEM of duplicate wells.
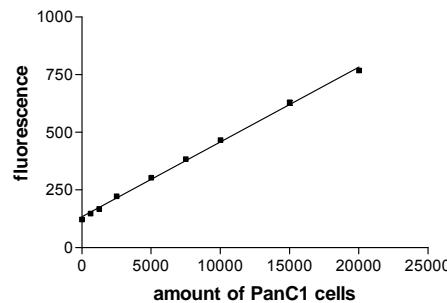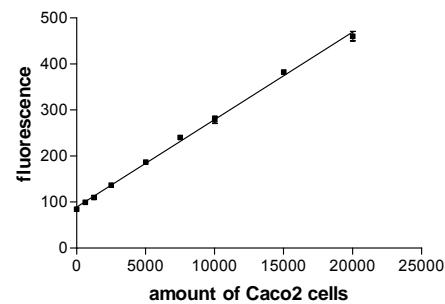
Pre-incubation with X alone did not influence tumour cell adhesion for all three cell lines (Fig.4). However, pre-incubation with XO alone did enhance the adhesion of HT29 to 172%, of Caco2 to 170% and of PanC1 to 128% *vs.* control (all p<0.01) (Fig.4). This was not surprising, since XO in medium alone did produce superoxide anions (Fig.2), probably because fetal calf serum in the medium contains X, acting as a substrate for XO.

Pre-incubation of HT29, Caco2 and PanC1 with the X-XO complex for 12 hours did not enhance their adhesion to untreated or pre-treated MEC statistically significantly (Fig.5; only data for HT29 are shown).

To verify if superoxide is the relevant ROS causing the enhanced adhesion we evaluated the effects of SOD in this model (Fig.4). Addition of SOD did decrease the enhanced adhesion of Caco2 to X-XO – treated MEC to nearly basal levels, from 158% to 116% (p<0.01). Comparable results were observed for HT29. SOD also decreased adhesion of PanC1 to X-XO – treated MEC, from 299 to 213% (p<0.05). Since superoxide anions spontaneously dismutate into the stronger ROS hydrogen peroxide that may affect MEC on its turn, we studied the effect of catalase next. The results showed that catalase inhibited the enhanced tumour cell adhesion after X-XO – pre-incubation effectively as well, i.e. for HT29 adhesion decreased from 167 to 141% (p<0.05), for Caco2 from 158 to 113% (p<0.01) and for PanC1 from 299 to 163% (p<0.01). In combination both antioxidant enzymes did not gave an additional effect. The addition of SOD or catalase to untreated MEC did not decrease basal adhesion alone (Fig.4), indicating that the low level of superoxide production of MEC in culture medium was insufficient to act as an autocrine stimulus (Fig.2).

Superoxide anion production
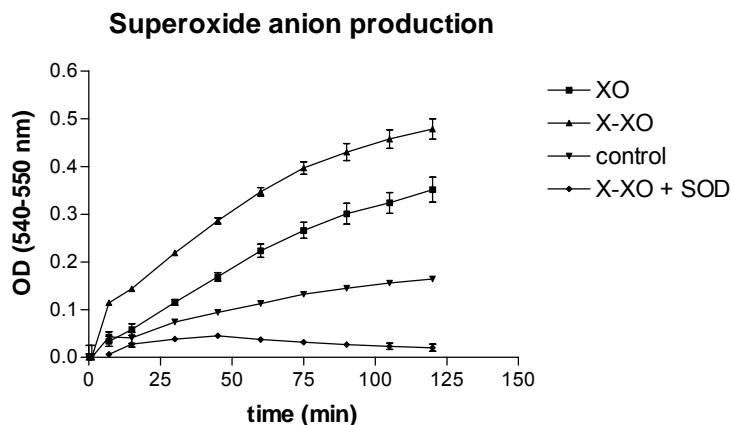
Figure 2. Validation of superoxide anion production by the ferricytochrome *c* reduction assay. X-XO was added to the wells with or without 400U/ml SOD immediately followed by the addition of 75 μM cytochrome *c*. Continuous measurements at 550-540 nm were carried out for 125 minutes. Data represent mean absorbance values (OD 540-550nm) ± SEM of quadriplate wells.
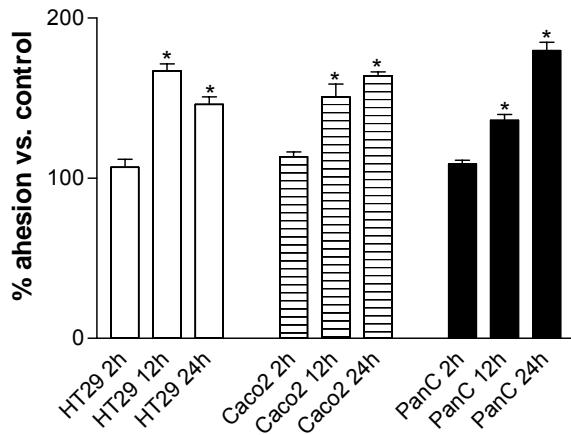
Figure 3. Tumour cell adhesion to MEC after pre-incubation of MEC with X-XO at varying time intervals. Means (n=6; % *vs.* control) ± SEM are shown.
* p<0.01 *vs.* control.
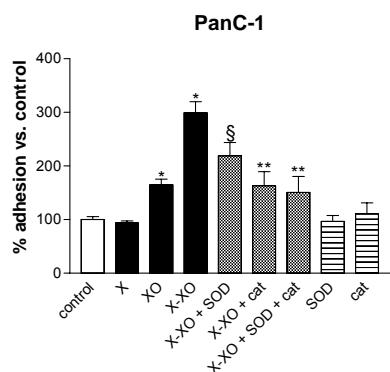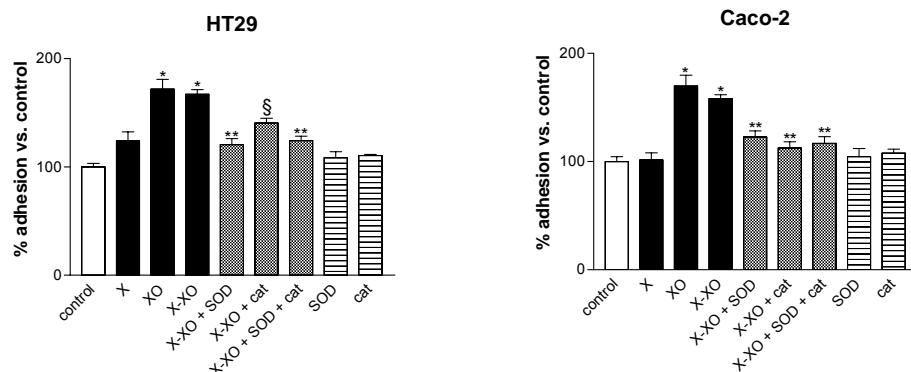
Figure 4. Adhesion of HT29 (a), Caco2 (b) and PanC1 (c) after 12 hours pre-incubation of MEC with X, XO and X-XO. The antioxidant enzymes SOD, catalase (cat) and the combination of both were added during the pre-incubation. Measurement of adhesion to an empty well (plastic) was determined as a negative control. Means (n=6; % vs. control) ± SEM are shown. * p<0.01 *vs.* control;
** p<0.01 *vs.* X-XO; § p<0.05 *vs.* X-XO.
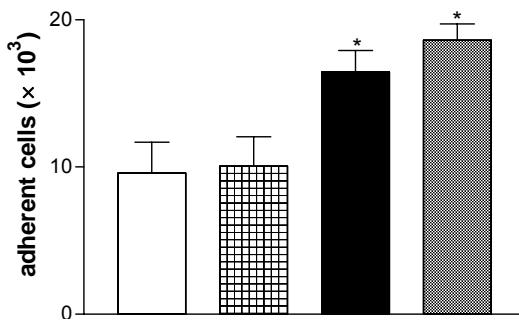
Figure 5. Adhesion of untreated HT29 versus HT29 pre-incubated with X-XO for 12 hours (HT29+X-XO) to untreated endothelium (control) and endothelium pre-incubated with X-XO (X-XO). Means (n=6; % vs. control) ± SEM are shown. * p<0.01 *vs.* control.

Mechanism of adhesion

To study if pre-incubation with X-XO influences the number of MEC we determined the course in the amount of DNA during an observation period of 3 days. The results showed a significant decline in the amount of DNA, not until 2 days of incubation with X-XO (Fig.6a). During the first 24 hours of culture in presence or absence of X-XO however, the number of MEC as reflected by the amount of DNA did not change significantly. Photographs of the endothelial monolayers with or without pre-incubation with X-XO for 12 hours also show that the number of endothelial cells is comparable (Fig.6b). On the other hand, pre-incubation with X-XO did lead to an increased apoptosis rate of 7.9 times the normal apoptosis rate (p<0.01), while XO only stimulated the apoptosis rate 3.7 fold (p<0.01) (Fig.7).

It was previously shown [27-29] [30] that the adhesion molecules E-Selectin, ICAM-1 and VCAM-1 on MEC and the ligands lymphocyte function-associated antigen-1 (LFA-1), very late activation antigen (VLA-4) and CD44 on tumour cells play an important role in tumour cell adhesion to MEC and that the expression of these molecules can be induced by apoptosis [31,32].

In this model we found that non-stimulated MEC and tumour cells did express E-Selectin, ICAM-1 and VCAM-1 (Fig.8 and 9). After 8 hours of pre-incubation with X-XO, enhanced E-Selectin expression on MEC was observed with a peak expression after 12 hours of 1.66 times the expression on non-stimulated MEC (p<0.01). Increased ICAM-1 and VCAM-1 expression on MEC was observed later, namely after 12 hours pre-incubation. While ICAM-1 expression then increased still further to a maximum of 170% *vs.* control (p<0.01), VCAM-1 expression declined after its peak expression of 149% *vs.* control at 12 hours of pre-

incubation (Fig.6). None of the adhesion molecules under study showed enhanced expression on HT29 (Fig.9), Caco2 and PanC1 by X-XO pre-incubation (data not shown).
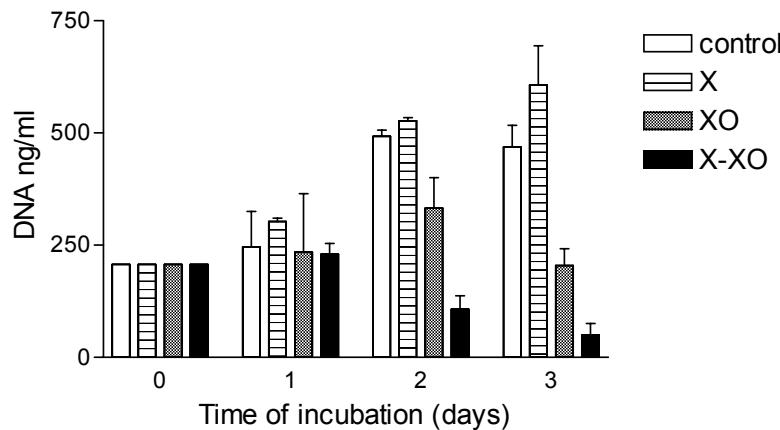

Figure 6a. MEC proliferation assay. DNA in ng/ml of MEC after 0, 1, 2 and 3 day(s) incubation with X, XO and X-XO. Bars represent means (n=4).
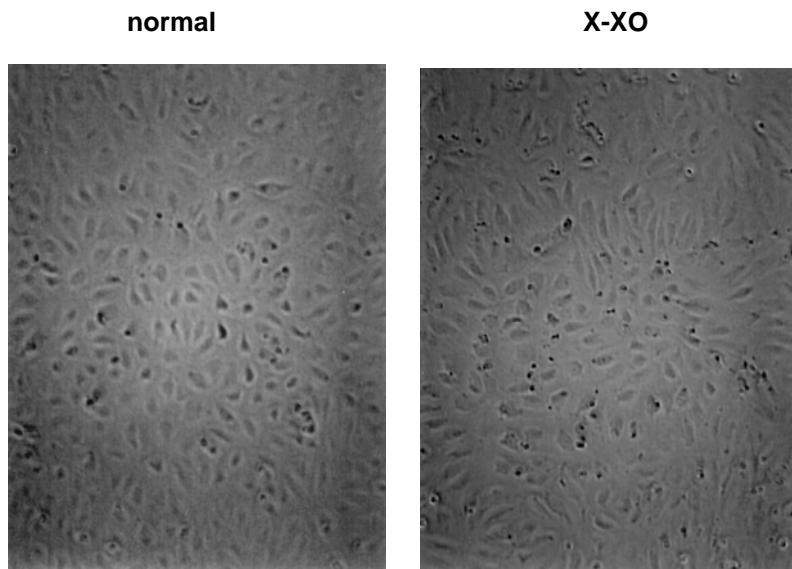

Figure 6b. Photographs of MEC monolayers: in medium (normal) and in medium pre-incubated with X-XO for 12 hours. Comparable number of cells are seen.
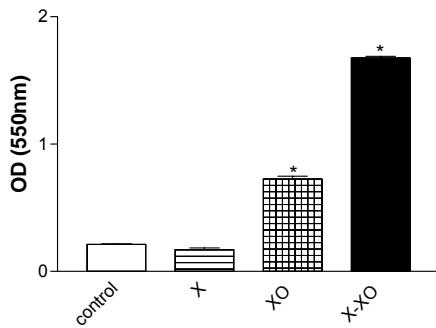
Figure 7. Measurement of apoptosis by ELISA in MEC after pre-incubation with X, XO and X-XO for 12 hours. Data represent mean absorbance values (OD 550nm) ± SD of triplate wells. * $p < 0.01$ *vs.* control.
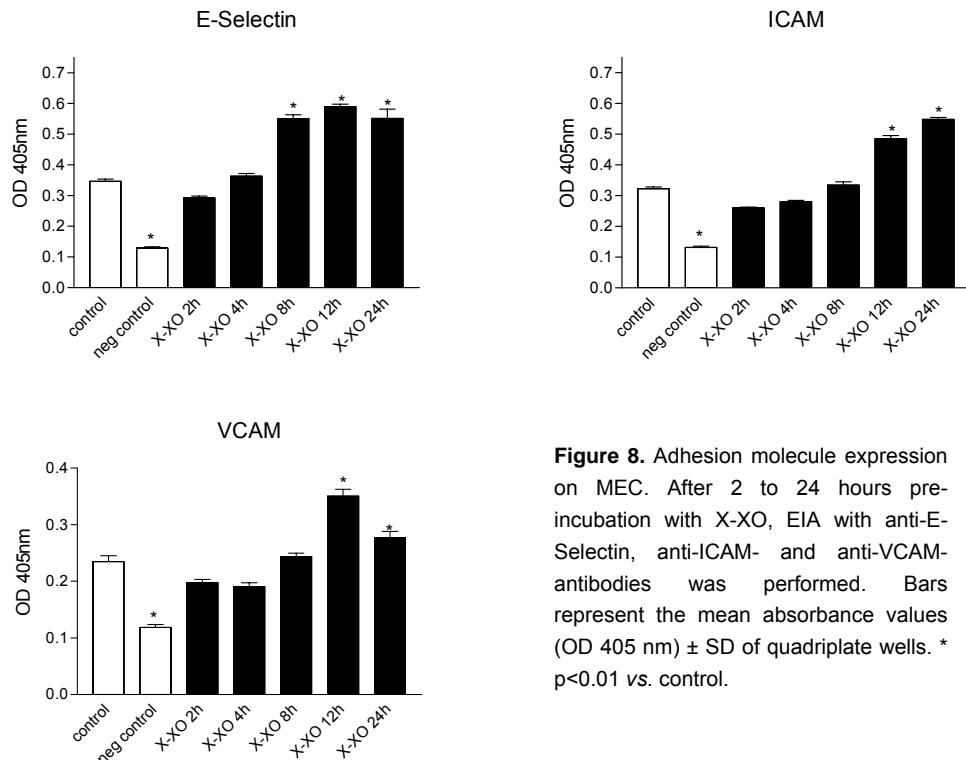

Figure 8. Adhesion molecule expression on MEC. After 2 to 24 hours pre-incubation with X-XO, EIA with anti-E-Selectin, anti-ICAM- and anti-VCAM-antibodies was performed. Bars represent the mean absorbance values (OD 405 nm) ± SD of quadriplate wells. * $p < 0.01$ *vs.* control.
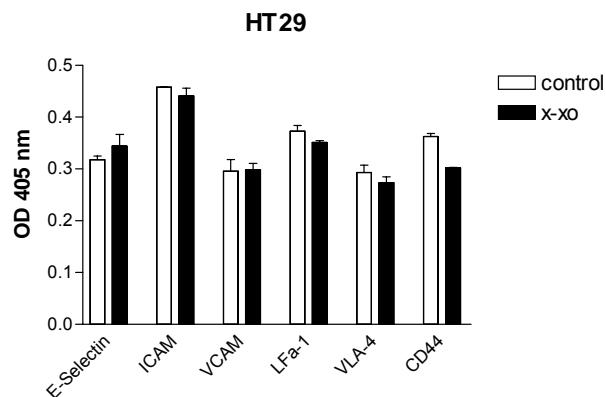
HT29

Figure 9. Adhesion molecule expression on HT29 colon carcinoma cells. After 12 hours pre-incubation with X-XO, EIA was performed. Bars represent the mean absorbance values (OD 405 nm) ± SD of quadriplate wells.

DISCUSSION

ROS are known to play an important role in loco-regional tumour recurrence after surgical trauma [8,9]. In preliminary *in vivo* studies, we were able to detect a significant increase of ROS in peritoneal lavage fluid as well as in plasma after surgery, proving that indeed surgery induces not only a local enhancement of ROS, but also systemically (data not shown). So the inflammatory response after surgical trauma does not confine locally, but spreads out systemically and therefore it is interesting to investigate the role of ROS in the development of distant metastases after surgery.

Therefore, the xanthine – xanthine oxidase complex was used to generate superoxide anions and in this way the influence of superoxide anions on tumour cell – endothelial cell interactions was studied. Exposure of microvascular endothelium to superoxide anions gave a substantial enhancement in tumour cell adhesion to the exposed endothelium comparable to the results found with PMN exposure, while exposure of tumour cells to superoxide anions had no effect on their adhesion to untreated endothelial cells.

We found that exposure of the microvascular endothelial cells to superoxide anions led to an up-regulation of the adhesion molecules E-Selectin, ICAM-1 and VCAM-1 on these cells. Enhancement of adhesion molecules on endothelial cells by exposure to ROS was also found by Bradley [33] and Lo [34]. Both found significant increased ICAM-1-expression after pre-

incubation with the ROS, although a relation with tumour adhesion was not investigated. Terada [35] did not found an up-regulation of ICAM-1 on the endothelium after a short pre-incubation period of 30 minutes with xanthine oxidase. This period of time is too short for completing synthesis of functional adhesion molecules, which is in accordance to our observation that a continuous exposure of the endothelium to ROS lasting minimally 12 hours is necessary before endothelial cells show any increased expression of cellular adhesion molecules.

Exposure of the endothelium to superoxide anions resulted in a major increase of apoptosis. Apoptosis finally will result in cell death leading to loss of binding sites on the endothelium, but exposure of the underlying extracellular matrix as a substrate for binding sites for circulating tumour cells. However, it does not seem very likely that binding sites on the extracellular matrix contributed to the findings of the present study, because the pre-incubation of the endothelial cells lasted only 12 hours during which the number of endothelial cells did not decrease.

The fact that endothelial cells undergoing apoptosis release interleukin -1 β (IL-1β) that via a paracrine loop in turn stimulates the expression of adhesion molecules on the endothelial cells [36,37] suggests that the following sequence of events for the recurrence of tumour cells at distant sites occur. Surgical trauma during the excision of a (primary) tumour leads to the activation of PMN. At distant sites these phagocytes by their massive production of ROS induce apoptosis of microvascular endothelium. Subsequently, by the (local) release of IL-1β the endothelial cells stimulate the expression of at least three major cellular adhesion molecules on their own cell membrane to which circulating tumour cells now easily can adhere and next form a metastasis.

The addition of SOD and/or catalase to ROS-exposed MEC decreased the enhanced tumour cell adhesion significantly. Since both antioxidant enzymes decreased the adhesion to similar levels this means that not only superoxide anions, but also hydrogen peroxide are equally involved in this phenomenon. This indicates that in fact a third kind of ROS, namely the highly reactive hydroxyl radical, is the actual reactant. To generate hydroxyl radicals both superoxide and hydrogen peroxide are needed in the so-called transition metal catalyzed Haber-Weiss reaction, and thus depleting one or the other ROS completely prevents the generation of the hydroxyl radical. Of note is that the addition of either SOD or catalase did not decrease the adhesion to basal levels. Incomplete scavenging of ROS by the antioxidant enzymes cannot account for that, because we showed here that adding SOD to the xanthine-xanthine oxidase complex completely inhibited the generation of superoxide. Presumably the local increase in tension of molecular oxygen as a by-product of the inactivation of superoxide and hydrogen peroxide by SOD and catalase, or xanthine oxidase itself may have contributed to the incomplete reduction in the expression of the adhesion molecules.

In conclusion, the results of the present study suggest that ROS as a result of surgical trauma influence tumour recurrence at distant sites by increasing binding sites for tumour cells on the endothelium. This indicates that by tackling the production of ROS preventing tumour recurrence not only locally, but also at distant sites might be feasible.

REFERENCE LIST

1. Midgley R, Kerr D. Colorectal cancer. *Lancet* 1999; **353**: 391-9.
2. Link KH, Kornmann M, Staib L, Redenbacher M, Kron M, Beger HG. Increase of survival benefit in advanced resectable colon cancer by extent of adjuvant treatment: results of a randomized trial comparing modulation of 5-FU + levamisole with folinic acid or with interferon-alpha. *Ann Surg* 2005; **242**: 178-87.
3. August DA, Ottow RT, Sugarbaker PH. Clinical perspective of human colorectal cancer metastasis. *Cancer Metastasis Rev* 1984; **3**: 303-24.
4. Wayne JD, Abdalla EK, Wolff RA, Crane CH, Pisters PW, Evans DB. Localized adenocarcinoma of the pancreas: the rationale for preoperative chemoradiation. *Oncologist* 2002; **7**: 34-45.
5. Bouvy ND, Marquet RL, Jeekel J, Bonjer HJ. Laparoscopic surgery is associated with less tumour growth stimulation than conventional surgery: an experimental study. *Br J Surg* 1997; **84**: 358-61.
6. Busch OR, Hop WC, Hoynck van Papendrecht MA, Marquet RL, Jeekel J. Blood transfusions and prognosis in colorectal cancer. *N Engl J Med* 1993; **328**: 1372-6.
7. van den Tol PM, van Rossen EE, van Eijck CH, Bonthuis F, Marquet RL, Jeekel H. Reduction of peritoneal trauma by using nonsurgical gauze leads to less implantation metastasis of spilled tumor cells. *Ann Surg* 1998; **227**: 242-8.
8. van Rossen ME, Stoop MP, Hofland LJ, van Koetsveld PM, Bonthuis F, Jeekel J et al. Red blood cells inhibit tumour cell adhesion to the peritoneum. *Br J Surg* 1999; **86**: 509-13.
9. van Rossen ME, Sluiter W, Bonthuis F, Jeekel H, Marquet RL, van Eijck CH. Scavenging of reactive oxygen species leads to diminished peritoneal tumor recurrence. *Cancer Res* 2000; **60**: 5625-9.
10. Weese JL, Ottery FD, Emoto SE. Do operations facilitate tumor growth? An experimental model in rats. *Surgery* 1986; **100**: 273-7.
11. Shiromizu A, Suematsu T, Yamaguchi K, Shiraishi N, Adachi Y, Kitano S. Effect of laparotomy and laparoscopy on the establishment of lung metastasis in a murine model. *Surgery* 2000; **128**: 799-805.
12. Aosasa S, Ono S, Mochizuki H, Tsujimoto H, Osada S, Takayama E et al. Activation of monocytes and endothelial cells depends on the severity of surgical stress. *World J Surg* 2000; **24**: 10-6.
13. Baigrie RJ, Lamont PM, Kwiatkowski D, Dallman MJ, Morris PJ. Systemic cytokine response after major surgery. *Br J Surg* 1992; **79**: 757-60.
14. Ure BM, Niewold TA, Bax NM, Ham M, Zee DC, Essen GJ. Peritoneal, systemic, and distant organ inflammatory responses are reduced by a laparoscopic approach and carbon dioxide versus air. *Surg Endosc* 2002; **16**: 836-42.
15. Pross M, Mantke R, Kunz D, Reinheckel T, Halangk W, Lippert H et al. Reduced neutrophil sequestration in lung tissue after laparoscopic lavage in a rat peritonitis model. *World J Surg* 2002; **26**: 49-53.
16. Yokota K, Nishihira T, Shineha R, Sayama J, Nitta Y, Kimura M et al. Association between elevated plasma granulocyte colony-stimulating factor and the degree of surgical stress in patients undergoing gastrointestinal surgery. *Surg Today* 1995; **25**: 579-84.
17. Varga G, Gal I, Roth E, Lantos J, Jaberansari MT. Inflammatory mediators and surgical trauma regarding laparoscopic access: neutrophil function. *Acta Chir Hung* 1997; **36**: 368-9.
18. Miyaoka K, Iwase M, Suzuki R, Kondo G, Watanabe H, Ito D et al. Clinical evaluation of circulating interleukin-6 and interleukin-10 levels after surgery-induced inflammation. *J Surg Res* 2005; **125**: 144-50.
19. Hildebrandt U, Kessler K, Plusczyk T, Pistorius G, Vollmar B, Menger MD. Comparison of surgical stress between laparoscopic and open colonic resections. *Surg Endosc* 2003; **17**: 242-6.
20. Noshima S, Morita N, Kobayashi Y, Hayashi D, Okamura K, Takahashi T et al. Surgical stress and the development of complications in relation to polymorphonuclear leukocyte elastase (PMNE) levels. *Surg Today* 1997; **27**: 135-9.

21. Oka Y, Murata A, Nishijima J, Yasuda T, Hiraoka N, Ohmachi Y et al. Enhanced attachment and elastase-releasing capacity of neutrophils after surgery. *Am J Surg* 1994; **167**: 405-11.
22. Fosse E, Opdahl H, Sunde S, Aasen AO, Svennevig JL. Granulocytes in bronchial lavage fluid after major vascular surgery. *Acta Anaesthesiol Scand* 1987; **31**: 33-7.
23. Weiss, L. Principles of Metastasis. 1985. New York, Academic Press Inc.
24. Markert M, Andrews PC, Babior BM. Measurement of O2- production by human neutrophils. The preparation and assay of NADPH oxidase-containing particles from human neutrophils. *Methods Enzymol* 1984; **105**: 358-65.
25. Catterall JB, Gardner MJ, Jones LM, Thompson GA, Turner GA. A precise, rapid and sensitive in vitro assay to measure the adhesion of ovarian tumour cells to peritoneal mesothelial cells. *Cancer Lett* 1994; **87**: 199-203.
26. Hofland LJ, van Koetsveld PM, Lamberts SW. Percoll density gradient centrifugation of rat pituitary tumor cells: a study of functional heterogeneity within and between tumors with respect to growth rates, prolactin production and responsiveness to the somatostatin analog SMS 201-995. *Eur J Cancer* 1990; **26**: 37-44.
27. Chirivi RG, Nicoletti MI, Remuzzi A, Giavazzi R. Cytokines and cell adhesion molecules in tumor-endothelial cell interaction and metastasis. *Cell Adhes Commun* 1994; **2**: 219-24.
28. Giavazzi R. Cytokine-mediated tumor-endothelial cell interaction in metastasis. *Curr Top Microbiol Immunol* 1996; **213 (Pt 2)**: 13-30.
29. Orr FW, Wang HH, Lafrenie RM, Scherbarth S, Nance DM. Interactions between cancer cells and the endothelium in metastasis. *J Pathol* 2000; **190**: 310-29.
30. ten Kate M, Hofland LJ, van Grevenstein WM, van Koetsveld PV, Jeekel J, van Eijck CH. Influence of proinflammatory cytokines on the adhesion of human colon carcinoma cells to lung microvascular endothelium. *Int J Cancer* 2004; **112**: 943-50.
31. Hebert MJ, Gullans SR, Mackenzie HS, Brady HR. Apoptosis of endothelial cells is associated with paracrine induction of adhesion molecules: evidence for an interleukin-1beta-dependent paracrine loop. *Am J Pathol* 1998; **152**: 523-32.
32. Chandra D, Ramana KV, Friedrich B, Srivastava S, Bhatnagar A, Srivastava SK. Role of aldose reductase in TNF-alpha-induced apoptosis of vascular endothelial cells. *Chem Biol Interact* 2003; **143-144**: 605-12.
33. Bradley JR, Johnson DR, Pober JS. Endothelial activation by hydrogen peroxide. Selective increases of intercellular adhesion molecule-1 and major histocompatibility complex class I. *Am J Pathol* 1993; **142**: 1598-609.
34. Lo SK, Janakidevi K, Lai L, Malik AB. Hydrogen peroxide-induced increase in endothelial adhesiveness is dependent on ICAM-1 activation. *Am J Physiol* 1993; **264**: L406-L412.
35. Terada LS, Hybertson BM, Connelly KG, Weill D, Piermattei D, Repine JE. XO increases neutrophil adherence to endothelial cells by a dual ICAM-1 and P-selectin-mediated mechanism. *J Appl Physiol* 1997; **82**: 866-73.
36. Chandra D, Ramana KV, Friedrich B, Srivastava S, Bhatnagar A, Srivastava SK. Role of aldose reductase in TNF-alpha-induced apoptosis of vascular endothelial cells. *Chem Biol Interact* 2003; **143-144**: 605-12.
37. Hebert MJ, Gullans SR, Mackenzie HS, Brady HR. Apoptosis of endothelial cells is associated with paracrine induction of adhesion molecules: evidence for an interleukin-1beta-dependent paracrine loop. *Am J Pathol* 1998; **152**: 523-32.