Chapter 3

Influence of proinflammatory cytokines on the adhesion of human colon carcinoma cells to lung microvascular endothelium

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ABSTRACT

In this experimental study the influence of surgery-induced pro-inflammatory cytokines on tumour recurrence in the lung was investigated.

A reproducible human in vitro assay was developed to study the adhesion of HT29 colon carcinoma cells to monolayers of microvascular endothelial cells of the lung (HMVEC-L) or human umbilical venous endothelial cells (HUVEC).

Pre-incubation of HMVEC-L with maximally active concentrations of IL-1β and TNF-α, but not with IL-6, resulted in at least 250% adhesion compared to control adhesion ($p \leq 0.01$). The effect of IL-1β and TNF-α was concentration and time dependent. Comparable results were found for HUVEC. Tumour cell adhesion was not increased after pre-incubation of HT29 with TNF-α.

Enzyme immuno assays of cytokine pre-incubated HUVEC and HMVEC-L showed concentration- and time-dependent upregulation of E-Selectin, ICAM-1 and VCAM-1 expression. In addition, LFA-1 and VLA-4 were only expressed on HMVEC-L, creating more binding possibilities for HMVEC-L compared to HUVEC.

Inhibition assays with anti-E-Selectin monoclonal antibody significantly decreased tumour cell adhesion to HUVEC, however, it did not affect tumour cell adhesion to HMVEC-L. Furthermore, anti-ICAM-1 and anti-VCAM-1 antibodies did not affect adhesion.

Our results prove IL-1β and TNF-α promote tumour cell adhesion to HMVEC-L *in vitro* and may therefore account for enhanced tumour recurrence in the lung seen after major surgical trauma. Contrary to adhesion to HUVEC is the adhesion to HMVEC-L E-Selectin independent. Probably, not one but a complex of adhesion molecules is responsible for enhanced adhesion to HMVEC-L.

INTRODUCTION

Locoregional recurrence as well as recurrence to distant sites following intentionally curative surgery is a major problem in colorectal cancer. Recurrence rates up to 40 per cent have been reported in colorectal cancer [1]. Among the sites of recurrences, the most frequent are liver, lung and locoregional sites [2-5].

Resection handling of the tumour can provoke detachment of tumour cells. Circulating tumour cells are often found in patients with gastro-intestinal cancer, not only during resection of the primary tumour [6-8]. Although the amount of circulating tumour cells is enhanced during resection of the primary tumour, it will not entirely explain the high recurrence rate found after intentionally curative surgery. Implantation of circulating tumour cells appears to be highly inefficient and most circulating tumour cells are rapidly destroyed [9,10].

It is possible that the surgical trauma itself influences the development of recurrences. Surgical peritoneal trauma provokes an inflammatory reaction, in which leukocytes are activated and cytokines and reactive oxygen species are released. We recently demonstrated that these factors enhance locoregional tumour recurrences [11,12]. However, these factors are released not only locally but also in the circulation. After major abdominal surgery the pro-inflammatory cytokines interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in peripheral blood are elevated [13-17]. We hypothesize that these factors are not only involved in locoregional tumour recurrence after peritoneal trauma, but also in recurrences at distant sites.

The lung is a frequent site for tumour recurrence in many gastro-intestinal tumours. The process of haematogenous metastasis formation to the lung is a multistep event in which tumour cells first have to detach from the primary tumour, invade the bloodstream, subsequently adhere to the endothelium within the lung and finally invade and multiply to form lung metastases [18,19].

An important step in this process is the adhesion of tumour cells to the endothelium. It is known that the cytokines IL-1β and TNF-α enhance tumour cell adhesion to human umbilical venous endothelial cells (HUVEC) *in vitro* by upregulation of adhesion molecules on HUVEC [20-25]. HUVEC are embryonic macrovascular cells used in many models studying the pathophysiology of the endothelium. However, the adhesion of tumour cells to endothelium does not occur in the macrovascular circulation, but in the microvascular circulation [26]. Microvascular endothelial cells react differently to stimuli compared to macrovascular endothelial cells [27-33]. Furthermore, it is believed that endothelial cells from different organs have their own pattern of adhesion molecules [34-37]. Because of these differences, general extrapolation from data obtained from the HUVEC model to the microcirculation is most certainly invalid.

We developed a reproducible human *in vitro* model to quantify colon carcinoma cell adhesion to human microvascular endothelial cells of the lung (HMVEC-L). This model was used to study the effect of cytokines on the adhesion of colon carcinoma cells to investigate the mechanism of enhanced tumour recurrence in the lung after surgical trauma. Expression of cell adhesion molecules on both endothelial cells and tumour cells was assessed and tumour cell adhesion assays with blocking antibodies to cell adhesion molecules were performed to study the interactions between tumour cells and endothelial cells responsible for enhanced tumour cell adhesion. A comparison was made between tumour cell adhesion to HMVEC-L and HUVEC.

MATERIALS & METHODS

Cells

HMVEC-L were purchased from Cambrex (Verviers, Belgium) at passage 4 and maintained in EGM-2-MV Bullet kit according to the manufacturer at 37°C, 95% relative humidity and 5 % CO_2. Confluent monolayers were passaged by 0.025% trypsin / 0.01% EDTA and cells were used up to passage 8.
HUVEC (kindly provided by A. Seynhave, Erasmus University Rotterdam, The Netherlands) were maintained and cultured as described for HMVEC-L.
The human colon carcinoma cell line HT29 was grown in RPMI 1640 medium supplemented with 10% foetal calf serum, glutamine (2 mM) and penicillin (10^5 U/L) and maintained by serial passage after trypsinization using 0.05% trypsin / 0.02% EDTA (all, except penicillin, obtained from Gibco, Breda, the Netherlands; penicillin from Yamanouchi, Leiderdorp, The Netherlands).
One week before the adhesion experiments, tumour cells were slowly adapted to endothelial medium (EGM-2-MV Bullet kit). Before the adhesion assay, tumour cells were trypsinized and maintained in suspension culture for 2 hours to regenerate cell-surface proteins.

Adhesion assay

To quantify tumour cell adhesion to HMVEC-L and HUVEC, a standardised cell adhesion assay was developed according to methods from Van Rossen *ET all* [38]. 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. HMVEC-L and HUVEC reached confluence in 3 to 4 days as determined by light microscopy.

To determine the effect of cytokines on tumour cell adhesion, endothelial monolayers were pre-incubated with varying doses, during varying times with recombinant human IL-1β, TNF-α and IL-6 (R&D Systems, Uithoorn, The Netherlands). Non pre-incubated monolayers served as controls. Not only the effect of endothelial pre-incubation was investigated, but the effect of tumour cell pre-incubation as well. Therefore, tumour cells were pre-incubated with 10 ng/ml TNF-α for 12 hours before the adhesion assay.

To demonstrate specificity of the effects of IL-1β and TNF-α, excess of anti-IL-1β or anti-TNF-α respectively (R&D Systems) was added during one hour at 37°C according to instructions of the manufacturer. Subsequently, endothelial monolayers were pre-incubated with the formed cytokine / anti-cytokine complex.

To quantify tumour cell adhesion, trypsinized 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 in a Heraeus centrifuge and incubated at 37°C for 1 hour. After this, wells were washed twice with medium to remove non-adherent tumour cells. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 nm excitation and 530 nm emission filters.

Inhibition assays

To determine whether E-Selectin plays a role in the adhesion of HT29 cells to HMVEC-L or HUVEC, inhibition experiments were carried out. Endothelial monolayers were pre-incubated with TNF-α. One hour before the adhesion assay was performed, 50 µg/ml of function blocking monoclonal mouse antibodies to human E-Selectin (R&D Systems) were added to endothelial monolayers. Same inhibition assays were carried out with monoclonal mouse antibodies to human ICAM-1 (25 µg/ml) and VCAM-1 (60 µg/ml) (R&D Systems).

Proliferation assays

To establish whether pre-incubation of monolayers HMVEC-L and HUVEC with different cytokines was of influence on endothelial cell growth, a DNA measurement was performed. In this assay, the DNA content was measured using the bisbenzimide fluorescent dye (Roche Diagnostics) as previously described by Hofland *et al* [39]. To do this, 2×10^4

endothelial cells / ml were plated in 24 wells plates and after 2, 4 and 6 days wells were washed and plates were stored at -20°C until analysis. In addition, a pellet of plated cells (day 0) was frozen immediately.

Immunocytochemistry of cytokine receptors

The presence of IL-1β and TNF-α receptors on HMVEC-L was assessed by immunocytochemistry. Cytospins of HMVEC-L were incubated overnight at 4°C with goat antibodies to human IL-1 receptor, TNF receptor type I or II (R&D Systems). Negative controls were incubated with 5% BSA in PBS. The cytospins were subsequently incubated with rabbit anti-goat antibodies, rinsed and finally incubated with goat peroxidase anti-peroxidase complex (Dako Cytomation, Eindhoven, The Netherlands). Cytospins were developed in 3.3' diaminobenzidine tetrachloride.

Immunocytochemistry of adhesion molecules

Endothelial cells and tumour cells were prepared for staining by cytospin preparation, fixed in acetone for 10 minutes and stored at -20°C until use.
The cytospins were incubated for 30 minutes at room temperature with the following primary antibodies: mouse anti-human monoclonal antibodies to E-Selectin (R&D Systems), ICAM-1, VCAM-1 (Dako Cytomation), sLe^a, sLe^x (Sanbio, Uden, The Netherlands), LFA-1 (αLβ2) and VLA-4 (α4β1) (Becton Dickinson, Alphen a/d Rijn, The Netherlands). Negative controls were incubated with PBS. As secondary antibodies, biotinylated goat anti-mouse antibodies were used followed by incubation with Streptavidin-biotinylated alkalin-phosphatase complex. Substrate development was done with New Fuchsine 4%. Cytospins were counterstained with Haematoxiline.
The expression of cell adhesion molecules was quantified by 2 separate observers using semi-quantitative scoring system ranging from no expression (-), weakly positive (±) to positive expression (+).

Enzyme Immuno Assay (EIA)

Endothelial and HT29 cells were grown to confluence as described for the adhesion assays in 96-well flat-bottomed multititer plates (Becton&Dickinson). Cells were pre-incubated with either cell culture media alone or combined with IL-1β or TNF-α. Cells were washed with phosphate buffered saline (room temperature, pH 7.4) and fixed in ethanol / methanol for 45

minutes and washed again. Subsequently, wells were incubated for 10 minutes with 1% goat serum to inactivate unspecific binding sites. Mouse monoclonal antibody to E-Selectin, ICAM-1 or VCAM-1 (R&D) in a dilution of 1:500 was added for 1 hour, followed by the addition of a second antibody, biotinylated goat anti-mouse antibody (Sigma) in a dilution of 1:250. Increased sensitivity was obtained using the ExtrAvidin-Peroxidase system (Sigma). Adding diammonium salt in citrate-phosphate buffer with urea hydrogen peroxide developed substrate. 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.

Statistical analysis

All data were analysed 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 with comparable results.

RESULTS

Validation of assay

Labelling tumour cells with calcein-AM did not decrease their viability (>95% using trypan blue). To determine the stability of calcein labelling, fluorescence of the labelled cells and of the supernatant of labelled cells was measured. The fluorescence of the labelled cells stayed constant for at least 60 minutes indicating retention of the dye within the cells (data not shown). This result was also seen in the adhesion assay, where maximal tumour cell adhesion was reached after one hour incubation followed by a decrease at longer incubation times (Figure 1). Therefore, for all subsequent experiments, incubation time was 1 hour. To obtain comparable conditions for HUVEC, all experiments with HUVEC were carried out with 1-hour incubation time as well.

A dilution series with labelled tumour cells on HMVEC-L monolayers showed a linear correlation between cell number and measured fluorescence that was used as a standard to

calibrate the measured fluorescence. In this way the amount of adhered tumour cells in the experimental wells could be determined.

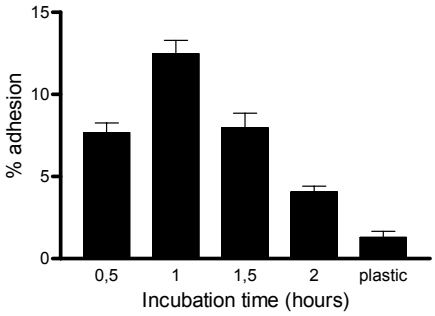

Figure 1. HT29 cells were labelled with calcein-AM and then incubated for 0,5 –2 hours in a well with a monolayer of HMVEC-L or an empty well (plastic) to adhere. Data are expressed as the mean (n=12, % vs total) + SEM.

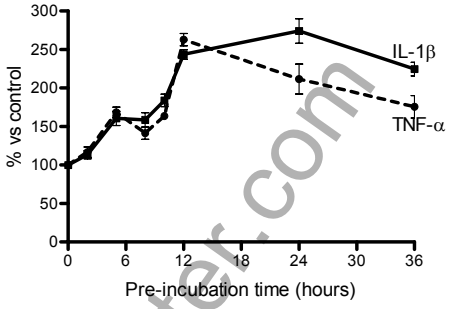

Figure 2. HT29 adhesion to HMVEC-L after pre-incubation of HMVEC-L with 50 ng/ml IL-1b and 1 ng/ml TNFα at varying time intervals. Means (n=6, % vs control) + SEM are shown.

Adhesion to endothelial cells

In all assays, tumour cell adhesion to non pre-incubated HMVEC-L was between 10-20% of the total amount of tumour cells added. Basal adhesion to HUVEC was always significantly lower than to HMVEC-L, between 2 and 10%.

Pre-incubation of HMVEC-L with IL-1β or TNF-α, but not with IL-6 resulted in enhanced tumour cell adhesion. After 2 hours pre-incubation cell adhesion was enhanced reaching a plateau after 12 hours pre-incubation with 1 ng/ml TNF-α (263% vs. control) or 50 ng/ml IL-1β (274% vs. control). After 24 hours, cell adhesion decreased (Figure 2). A small plateau was also seen after 5 hours pre-incubation. This might correlate with E-Selectin dependent adhesion, because E-Selectin expression occurs relatively early compared to ICAM-1 and VCAM-1 expression. For HUVEC, cell adhesion was also higher after pre-incubation with 10 ng/ml IL-1β for 12 hours than for 4 hours, namely 272 and 190 % vs. control respectively. Comparable results were found for pre-incubation with 10 ng/ml TNF-α: 303% after 12 hours and 177% after 4 hours pre-incubation (Figure 3). Therefore, in all subsequent experiments cytokine pre-incubation lasted 12 hours, unless otherwise stated.

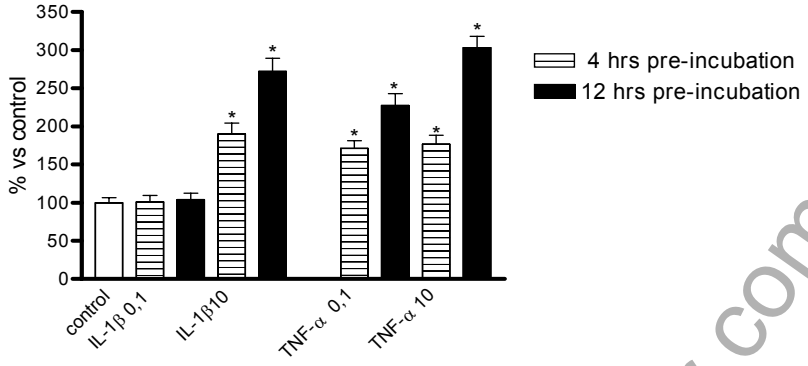

Figure 3. HT29 adhesion to HUVEC after 4 and 12 hours pre-incubation of HUVEC with 0,1 or 10 ng/ml IL-1β or TNF-α. Means (n=6, % vs control) + SEM are shown. * = p<0,01.

The enhanced cell adhesion was dose dependent (Figure 4). Maximal HT29 cell adhesion was achieved after pre-incubation with 50 ng/ml IL-1β or with 1 ng/ml TNF-α. Pre-incubation with the combination of IL-1β and TNF-α did not enhance the adhesion any further (data not shown). The enhanced adhesion after cytokine pre-incubation could be inhibited with a thousand fold excess of anti-IL-1β or anti-TNF-α, confirming that indeed the enhanced adhesion is caused by the concerning cytokine (data not shown).

Adhesion of HT29 to HUVEC pre-incubated with IL-1β, TNF-α or IL-6 resulted in comparable results as seen with HMVEC-L. However, cytokine pre-incubation increases adhesion percentages more for HUVEC than for HMVEC-L, probably because the control adhesion, i.e. the basal adhesion, to HUVEC is significantly lower compared to HMVEC-L (data not shown).

Since not only the endothelium is exposed to factors released during surgery, but also the circulating tumour cells, we also investigated the influence of exposing HT29 to TNF-α on tumour cell adhesion. We could not detect a significant difference in basal adhesion between normal and TNF-α stimulated tumour cells (Figure 5). Furthermore, there was no significant difference in adhesion to stimulated HMVEC-L. Therefore, increased adhesion under influence of TNF-α seems to be mainly dependent on endothelial changes, not of changes in tumour cells.

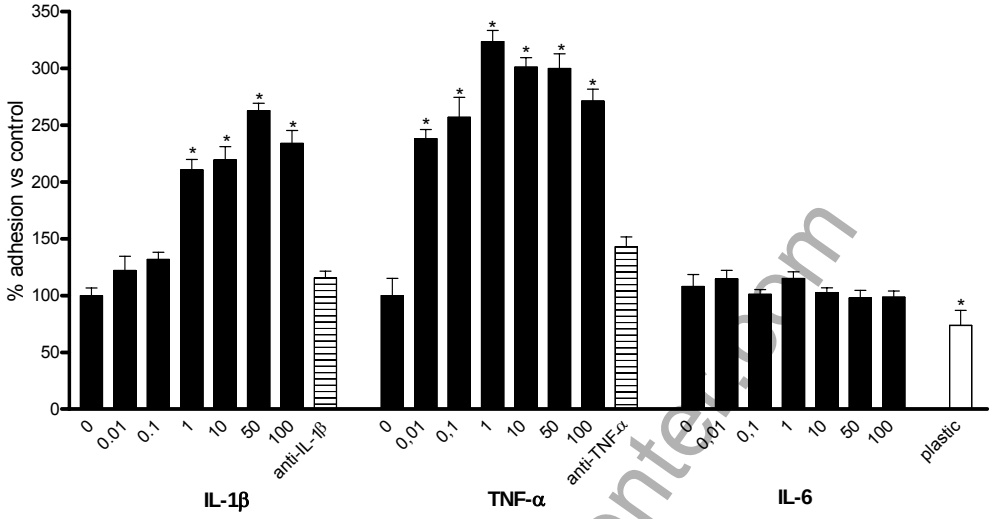

Figure 4. HT29 adhesion to HMVEC-L after preincubation with IL-1β, TNF-α and IL-6 at different concentrations (ng/ml). A thousand fold excess of anti-IL-1β and anti-TNF-α antibodies were added. Means (n=6, % vs control) + SEM are shown. * = p<0,01.

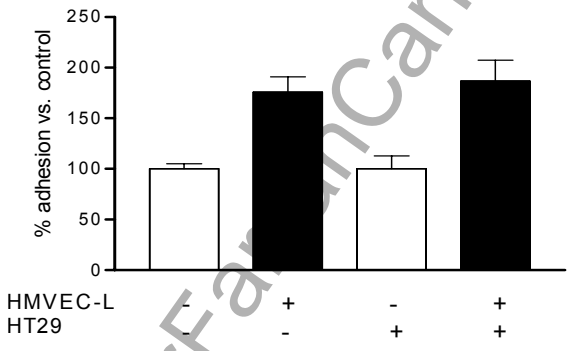

Figure 5. HT29 adhesion to HMVEC-L. The effect of pre-incubation (+) with 1 ng/ml TNF-α of HMVEC-L and / or HT29 on tumour cell adhesion. Means (n=6, % vs. control) + SEM are shown. * = p<0,01.

Expression of cell adhesion molecules

Table 1 shows the expression of cell adhesion molecules on HMVEC-L, HUVEC and HT29. According to these results it is possible that HT29 tumour cells can bind to E-Selectin and ICAM-1 on both endothelial cells via sLe a and LFA-1 respectively. Furthermore, there are binding possibilities between HMVEC-L and HT29 via LFA-1 on HMVEC-L and ICAM-1 on HT29, and via VLA-4 on HMVEC-L and VCAM-1 on HT29. Thus, these results show more binding possibilities for HMVEC-L than for HUVEC.

We looked further at the E-Selectin expression on both endothelial cell lines by EIA, a semi-quantitave assay. The staining intensity (in optical density units measured at 405 nm) of E-Selectin expression on unstimulated HMVEC-L was 0,2. The negative control gave an intensity of 0.108, so there is a slight basal expression on HMVEC-L contrary to the immunocytochemistry results that showed no basal E-Selectin expression. Pre-incubation with IL-1β for 12 hours gave an increased expression from 1 ng/ml with maximal expression at 100 ng/ml (294% vs. control, $p<0.01$) (Figure 6). For TNF-α, increased intensity occurred already at 0.01 ng/ml (148% vs. control, $P<0.01$). Again, maximal intensity was reached at the highest concentration, 100 ng/ml (316% vs. control, $p<0.01$). Pre-incubation with IL-6 did not influence optical density. For HUVEC, basal intensity for E-Selectin was 0.142 whereas the negative control was 0.085, indicating that HUVEC has a low basal E-Selectin expression. Pre-incubation for 12 hours with TNF-α 1ng/ml gave an increase of 324% vs. control ($p<0.01$). Comparable results were found with IL-1β (data not shown).

Kinetics of the cytokine-induced cell surface expression of E-Selectin on HMVEC-L showed enhancement already after 4 hours pre-incubation with IL-1β or TNF-α (Figure 7). Maximal expression was seen after 8 hours pre-incubation followed by a slight decline with still a significant enhanced expression after 24 hours pre-incubation. If the enhanced tumour cell adhesion to HMVEC-L is E-Selectin dependent, the EIA results are conflicting with the adhesion assays, as maximal adhesion was not reached until after 12 hours pre-incubation, while maximal E-Selectin expression was seen already after 8 hours pre-incubation.

Therefore, we looked further at the expression of other adhesion molecules on HMVEC-L. ICAM-1 is upregulated by both TNF-α and IL-1β, which is concentration and time dependent. Unlike E-Selectin, maximal ICAM-1 expression occurs not until after 12 hours pre-incubation (Figure 7). Cytokine pre-incubation only causes a slight increase in VCAM-1 expression on HMVEC-L (Figure 7).

We also investigated the influence of cytokines on adhesion molecule expression on HT29 cells (Figure 8). None of the examined adhesion molecules, except ICAM-1, was upregulated by 10 ng/ml TNF-α. ICAM-1 expression shows a slight increase under influence of TNF-α, the expression amount to 137% compared to basal expression.

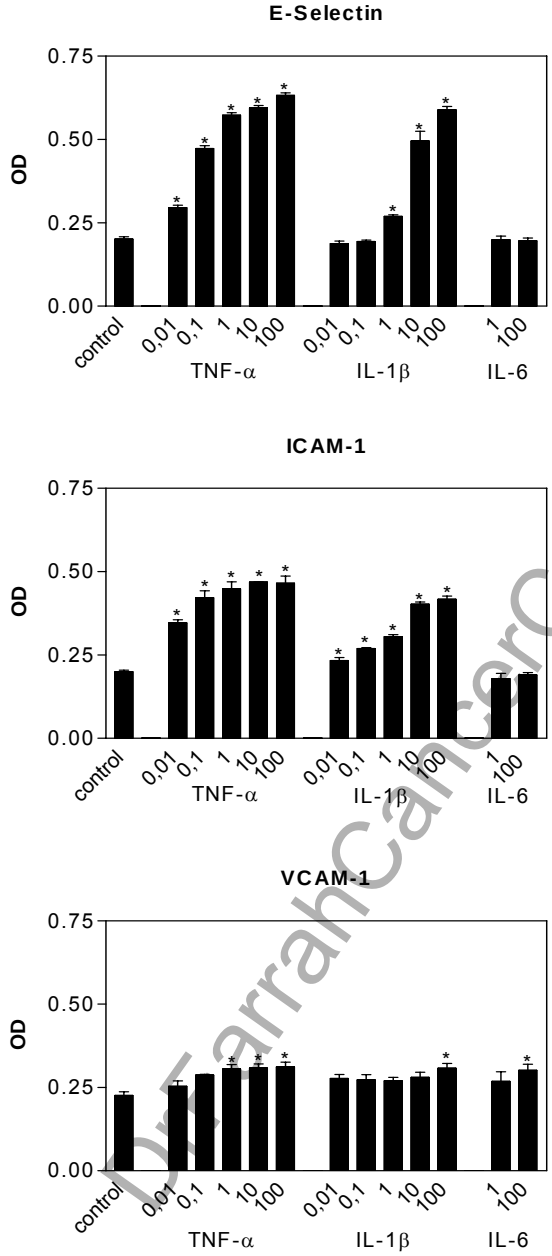

Figure 6. Dose response of adhesion molecule expression on HMVEC-L pre-incubated for 12 hours with TNF-α, IL-1β or IL-6. Dose response of adhesion molecule expression on HMVEC-L. After 12 hours pre-incubation with the cytokines TNF-α, IL-1β or IL-6 at concentrations ranging from 0,01 till 100 ng/ml, EIA was performed. Bars represent the mean absorbance values (OD 405 nm) ± SD of quadriplate wells. * = p<0,01.

Tabel 1. Cell adhesion molecules expressed by HMVEC-L, HMVEC-L preincubated with 10 ng/ml IL-1β, HUVEC and HT29. Comparable results were found with TNF-α (data not shown). Known ligands for E-Selectin are sLea and sLex, for ICAM-1 LFA-1 (= αLβ2 complex) and for VCAM-1 VLA-4(=α4β1 complex)

Cell adhesion molecule	HMVEC-L	HMVEC-L + IL-1β	HUVEC	HT29
E-Selectin	-	+	+	-
ICAM-1	+	+	+	+
VCAM-1	-	+	-	+
sLe^a	-	-	-	+
sLe^x	-	-	-	+/-
LFA-1	+		-	+
VLA-4	+		-	-

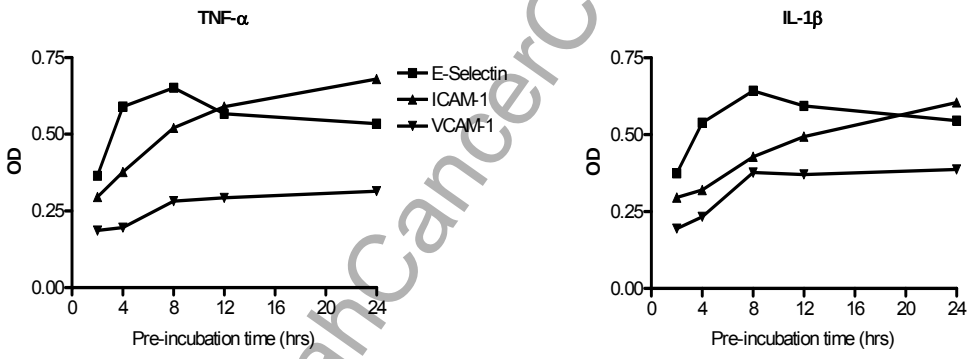

Figure 7. Kinetics of adhesion molecule expression on HMVEC-L assessed by EIA. HMVEC-L monolayers were pre-incubated with TNF-α (1ng/ml) or IL-1β (10ng/ml) for varying times. Data represent mean absorbance values (OD 405nm) ± SD of quadriplate wells.

Effect of E-Selectin, ICAM-1 or VCAM-1 blockade

Addition of E-Selectin antibody to HMVEC-L monolayers after 4 hours pre-incubation with TNF-α did not either decrease basal adhesion nor stimulated adhesion of HT29 cells (Figure 9). Similar results were seen after 12 hours pre-incubation with TNF-α. However, addition of this antibody to TNF-α stimulated HUVEC monolayers decreased tumour cell adhesion with 63,8% ($p<0,01$) (According to the manufacturer maximal inhibition of ca. 60% can be achieved using this antibody). This antibody did not affect basal adhesion to HUVEC.

ICAM-1 and VCAM-1 antibodies did not influence tumour cell adhesion to both unstimulated as TNF-α stimulated HMVEC-L or HUVEC (data not shown).

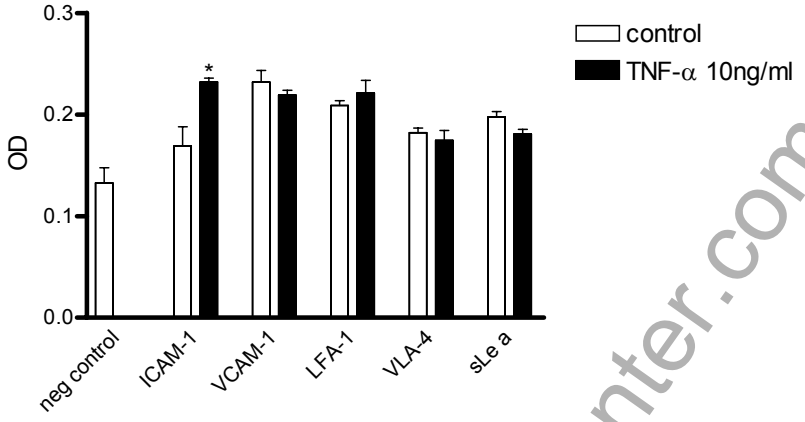

Figure 8. Adhesion molecule expression on HT29 colon carcinoma cells. After 12 hours pre-incubation with 10 ng/ml TNF-α, EIA was performed. Bars represent the mean absorbance values (OD 405 nm) ± SD of quadriplate wells. * = p<0,01 TNF-α vs. control.

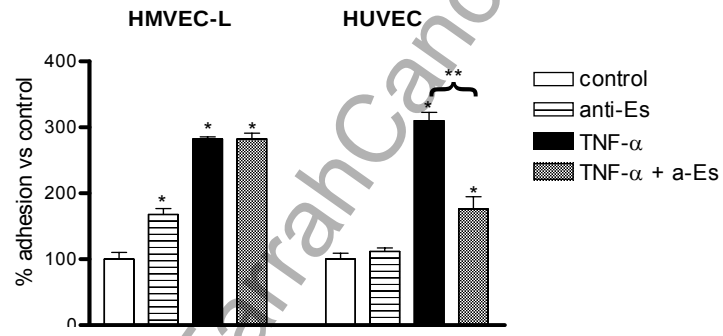

Figure 9. HT29 tumour cell adhesion to HMVEC-L or HUVEC. Prior to tumour cell adhesion, endothelial cells were pre-incubated for 12 hours with TNF-a 1 ng/ml and/or for 1 hour with anti-Eselectin (anti-Es) 60mg/ml (according to instructions of the manufacturer). Data are expressed as the mean (n=6) +SEM. * = p<0,01 vs control ; ** = p<0,01 vs TNF-a.

Cell proliferation

Pre-incubation with IL-6 did not modify total HMVEC-L DNA content and therefore did not affect cell growth. Pre-incubation with IL-1β for more than 4 days enhances proliferation of HMVEC-L, whereas pre-incubation for more than 4 days with TNF-α decreases cell growth.

These effects are not seen for shorter pre-incubation times. In HUVEC, pre-incubation with IL-1β seems to increase cell growth, though not statistical significant. Pre-incubation with TNF-α shows decreased cell growth after 3 days (Figure 10).

Because cytokine pre-incubation in the adhesion assays never lasted more than 12 hours, we conclude that the enhancement of tumour cell adhesion to HMVEC-L or HUVEC was not influenced by changes in HMVEC-L growth.

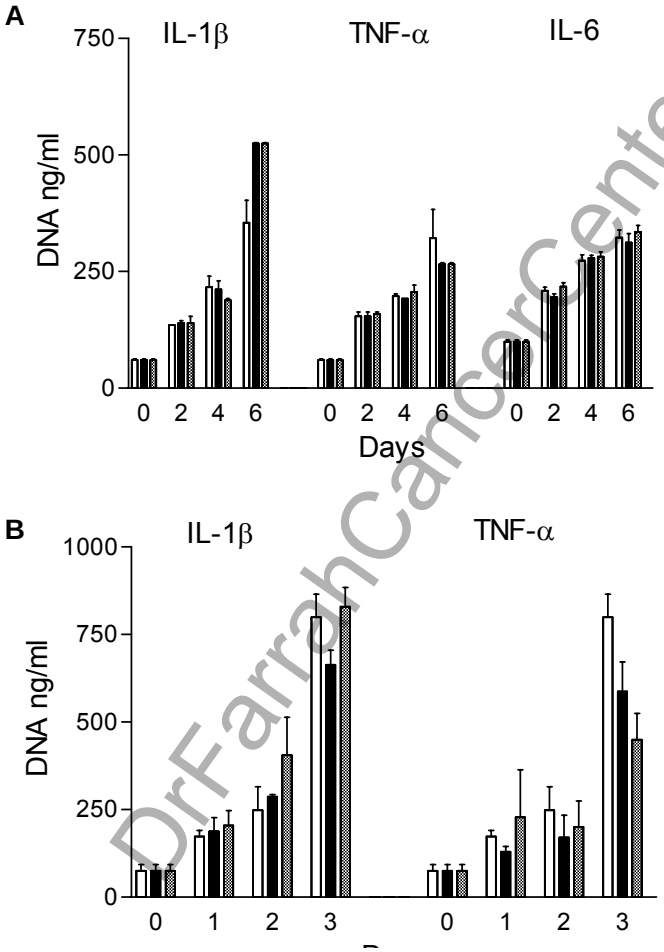

Figure 10. A) HMVEC-L and b) HUVEC proliferation assays. Open bars represent proliferation whithout cytokine incubation, closed bars represent proliferation with 1ng/ml cytokine incubation for HMVEC-L and 0,1 ng/ml for HUVEC and checked bars with 50 ng/ml cytokine incubation for HMVEC-L and 10ng/ml for HUVEC.

DISCUSSION

During and after major abdominal surgery, the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 are released locally and to a lesser degree systemically. In previous *in vitro* studies, cytokines produced after surgical trauma were found to enhance local tumour recurrence. In this *in vitro* study, we demonstrate that pre-incubation of microvascular endothelial cells of the lung with the cytokines IL-1β and TNF-α, but not with IL-6 significantly increase the adhesion of the HT29 human colon carcinoma cells to the microvascular endothelium. Therefore, cytokines released during major abdominal surgery may enhance distant recurrences.

During surgery not only endothelial cells are exposed to cytokines, the disseminated tumour cells are exposed as well. It was not known yet how tumour cells respond to circulating cytokines. Maybe the circulating cytokines also upregulate adhesion molecules on tumour cells by which tumour cell adhesion to endothelial cells under influence of cytokines will be even more efficient.

Although we found a small increase in ICAM-1 expression on HT29 cells after stimulation with TNF-α, we could not detect an increase in adhesion of cytokine - stimulated tumour cells to the endothelium compared to unstimulated tumour cells. Therefore, the observed enhanced tumour cell adhesion after TNF-α pre-treatment seems to be mainly dependent on endothelial changes, not of changes in tumour cells.

The phenomenon of enhanced tumour cell adhesion under influence of surgery was also observed by Higashiyama et al [40]. Surgical stress given to mice before melanoma cell inoculation significantly enhances pulmonary metastasis. However, this enhancement was only seen when surgical stress lasted more than 2 hours. It is known that cytokine production correlates with the duration of surgery i.e. longer operation time gives remarkably more cytokine production and therefore a role of cytokines was suggested. Indeed, administration of an anti-TNF-α monoclonal antibody inhibited the enhanced metastasis.

The increased adhesion we found was time-dependent, suggesting that IL-1β and TNF-α activate protein synthesis leading to adhesion molecule upregulation. In previous studies [20,23,41-43], increased adhesion of HT29 to cytokine stimulated HUVEC was indeed adhesion molecule dependent. The enhanced adhesion was caused by upregulation of the adhesion molecule E-Selectin on HUVEC and could be inhibited by anti-E-Selectin antibody.

Although the adhesion of HT29 cells to HUVEC is E-Selectin dependent, this does not account for the adhesion to HMVEC-L, since addition of an antibody to E-Selectin does not influence tumour cell adhesion to HMVEC-L. Therefore, adhesion can be brought about by either another solitary adhesion molecule complex or a combination of adhesion molecule complexes.

Indeed, the kinetics of E-Selectin expression on HMVEC-L argues against a solitary role of E-Selectin in the adhesion of HT29 to HMVEC-L, because maximal adhesion was not reached until after 12 hours pre-incubation, while maximal E-Selectin expression was seen already after 8 hours pre-incubation.

With regard to the immunocytochemistry results, possible candidates responsible for the enhanced tumour cell adhesion to HMVEC-L could be ICAM-1, VCAM-1, but also their counterparts LFA-1 and VLA-4, which are also expressed by HMVEC-L. Inhibition assays with anti-ICAM-1 and anti-VCAM-1 antibody ruled out a solitary role of one of these adhesion molecules in adhesion. Therefore, the adhesion of tumour cells to the endothelium can be rather intricate, involving several different adhesion molecule complexes.

The difference in basal adhesion and E-Selectin dependent or independent HT29 adhesion we found between HUVEC and HMVEC-L, indicates that these endothelial cell types are definitely not comparable. According to Paget's 'seed and soil' hypothesis, particular tumour cells have their preferential organs to metastasise to, which cannot be explained by simple anatomical or mechanical hypotheses. Each organ microenvironment has different characteristics, like a different pattern of endothelial adhesion molecules, reacting in a particular way on stimuli. The lung microenvironment is unique with many alveolar capillaries consisting of lung microvascular cells with specific adhesion molecules [44]. This can explain why many gastrointestinal carcinomas preferentially metastasise to the lung. Therefore, it is a prerequisite to study endothelial cells derived from the vascular bed of interest rather than extrapolate from results obtained with HUVEC.

In conclusion, our data suggest that disseminated tumour cells may be able to home more efficiently in the lungs by the release of pro-inflammatory cytokines during surgical trauma, which should be validated in animal models. Contrary to the adhesion of colon carcinoma cells to HUVEC that is E-Selectin dependent, it is not known which adhesion molecule or complex of adhesion molecules are responsible for the adhesion of colon carcinoma cells to HMVEC-L.

The apparent relationship between the inflammatory response after surgical trauma and distant tumour recurrence necessitates further unravelling of the mechanisms involved.

This may bring about a treatment modality to reduce distant recurrences and benefit a significant proportion of patients in terms of survival or quality of life.

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