Chapter 5

Polymorphonuclear leukocytes increase the adhesion of circulating tumour cells to microvascular endothelium

M ten Kate, AGJ Aalbers, W Sluiter, LJ Hofland,
P Sonneveld, J Jeekel, CHJ van Eijck

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ABSTRACT

Tumour recurrence after intentionally curative surgery remains a major problem. We hypothesize that reactive oxygen species (ROS) released from activated polymorphonuclear leukocytes (PMN) during surgery play a crucial role in the enhanced tumour recurrence after surgical trauma. Therefore, we studied the effect of activated PMN on the adhesion of tumour cells to microvascular endothelium (MEC) in a reproducible human *in vitro* model. Pre-incubation during 12 hours of MEC with tissue plasminogen activator (TPA) -activated PMN resulted in a significant increase in adhesion to MEC of the human colon carcinoma cells HT29 (341.8% *vs.* control, $p<0.01$), Caco2 (237.5% *vs.* control, $p<0.01$) and of the pancreatic carcinoma cells PanC1 (282.3% *vs.* control, $p<0.01$) and BxPC3 (219.5% *vs.* control, $p<0.01$). Exposure of MEC to TPA or non-activated PMN did not affect tumour cell – endothelial cell interactions.

The addition of the antioxidant enzymes superoxide dismutase (SOD) or catalase gave a significant decrease in tumour cell adhesion to MEC exposed to PMN, although basal levels were not reached, even not with both enzymes. The decline in enhanced adhesion by addition of both antioxidant enzymes amounted to 58.2% of enhanced adhesion after pre-incubation of MEC with activated PMN for BxPC3, 52% for HT29, 41.6% for Caco2 and 38.5% for PanC1 (all $p<0.01$)

In conclusion, the results of the present study show that activated PMN promote the adherence of tumour cells to the microvascular wall by production of ROS. This indicates that by tackling the ROS production preventing tumour recurrence not only locally, but also at distant sites might be feasible.

INTRODUCTION

For colon cancer recurrence rates up to 40% have been reported after intentionally curative surgery [1], for pancreatic cancer reported recurrence rates are even up to 80% [2].
Although surgery remains the only curative option for many gastro-intestinal malignancies, operative trauma in itself may favour the successful implantation of circulating tumour cells that can be found in many patients with gastro-intestinal malignancies[3-6].
Previous *in vivo* studies showed a relation between surgical trauma and tumour recurrence. First, Busch *et al.* found that blood transfusions decrease survival after surgery for colorectal cancer [7]. However, not the blood transfusions themselves but rather the circumstances necessitating them, namely the degree of surgical trauma, turned out to be the real predictors of prognosis. Indeed, in our laboratory van den Tol *et al.* [8] demonstrated that surgical trauma enhanced tumour load in a rat model and that the severity of surgical trauma influenced the degree of tumour load. Indeed, laparoscopy, which causes minor trauma, gave significantly less tumour load compared to laparotomy in a rat model [9].
Subsequent *in vivo* experiments demonstrated that one of the cellular components of blood, i.e. the erythrocytes, once introduced in the abdominal cavity after abdominal surgical trauma, in fact effectively inhibited loco-regional recurrences [10]. The responsible beneficial components of the red blood cells turned out to be the antioxidant enzymes catalase and superoxide dismutase (SOD) [11]. These enzymes neutralize the reactive oxygen species (ROS) O_2^- and H_2O_2. On the other hand, passive transfer experiments demonstrated that the polymorphonuclear leukocytes (PMN) which are major O_2^- producers, enhanced tumour load. Since a surgical trauma generally elicits an inflammatory exsudate consisting of more than 75% PMN lacking erythrocytes, a condition is created that promotes tumour recurrence. Moreover, such an inflammatory reaction caused by abdominal surgical trauma will not be limited to a local reaction in which the mesothelium in the abdomen is involved, but spreads out systemically as well [12-18]. Indeed, during and several days after major surgery, the peripheral blood level of elastase, which is an indicator of PMN activity, is elevated [19,20]. Furthermore, as shown after major abdominal surgery the number of PMN not only increased systemically, but also at distant sites, for example in the lung [21]. In haematogenous metastasis, circulating tumour cells have to overcome many defence mechanisms. If they survive, a fundamental step in the formation of a metastasis is their adherence to the microvascular endothelium of a distant organ [22-24], and that latter process might be promoted by the occurrence of an acute inflammation dominated by PMN likewise as found for the mesothelium [25-27].
In order to study the underlying mechanism by which PMN induce tumour cell adhesion to microvascular endothelium, we developed a reproducible human *in vitro* model. In this model, two tumour cell types were used, namely colon and pancreatic carcinoma cells to

investigate if PMN affect tumour cell – endothelial cell interactions. The role of PMN-derived ROS was further investigated by the addition of antioxidants in the adhesion assays.

MATERIAL AND METHODS

Cell lines

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 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.
The human colon carcinoma cell lines HT29 and Caco2 and the human pancreas carcinoma cell lines PanC1 and BxPC3 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 (Invitrogen, Breda, the Netherlands).
Before the adhesion assay, tumour cells were trypsinized and maintained in suspension for 2 hours to regenerate cell-surface proteins.

PMN isolation

Isolation of PMN was achieved by the Hypaque-Ficoll separation technique. Human blood was drawn from healthy volunteers by venipuncture. The blood was layered over the PMN isolation medium (Polymorphprep, Axis-shield, Norway) and centrifuged at 460 × *g* for 32 minutes at 21°C to allow for density separation of cell populations. Thereafter, the PMN layer was carefully aspirated and washed with 0.45% NaCl to restore osmolality. The PMN were centrifuged at 400 × *g* for 10 min at 21°C followed by the addition of red cell lysis buffer (30 min, 4°C). Finally cells were washed and resuspended in culture medium (EGM-2-MV Bullet kit). Purity of the final PMN suspension was evaluated by examination of Giemsa stained smears, which revealed to be more than 97% PMN. By trypan blue exclusion viability of more than 97% was observed.

Ferricytochrome c reduction assay

Production of superoxide anions generated by the PMN in this model was assessed using the ferricytochrome *c* reduction assay [28]. Analogous to our adhesion model, 2×10^5 PMN with or without 5 *µ*M tissue plasminogen activator (TPA) were added to the experimental wells. After addition of 75 *µ*M cytochrome *c* (Roche Applied Science, Almere, The Netherlands) the change in absorbance at 540 nm (reference) and 550 nm was continuously recorded by the thermostatted Versamax microplate reader (Molecular Devices) for 125 minutes at 37°C. In this model pH changes, determined with a pH electrode, were not observed.

Adhesion assay

A standardised cell adhesion assay was developed to quantify tumour cell adhesion to MEC as described elsewhere [29]. 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 MEC 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 confluency in 3 to 4 days as determined by light microscopy.
To determine the effect of PMN on tumour cell adhesion, endothelial monolayers were pre-incubated with 2×10^5 PMN per well during 12 hours at 37°C. Before adding the PMN to the wells they were stimulated with 5 *µ*M TPA. Untreated monolayers served as controls.
Appropriate antioxidant enzymes were added to the model system to assess ROS specificity of the effects. As superoxide anion scavenger we used superoxide dismutase (400 U/ml) (Roche Applied Science, Almere, The Netherlands) that converts superoxide anions into molecular oxygen and hydrogen peroxide. Since hydrogen peroxide itself is a strong ROS, we added catalase (400 U/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands) as well to decompose the hydrogen peroxide.
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 × *g* and incubated at 37°C for 1 hour. After this, wells were washed twice with medium. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 nm excitation and 530 nm emission filters.

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.

RESULTS

Evaluation of the model and superoxide anion production

Labelling tumour cells with calcein-AM did not decrease their viability (>95% using trypan blue). Dilution series of labelled tumour cells on MEC monolayers showed a linear correlation ($r^2 > 0.99$) between cell number and the level of fluorescence. 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, both MEC (control) and MEC with TPA did not produce superoxide anions as measured with the ferricytochrome *c* reduction assay (Fig. 1). TPA-stimulated PMN produced superoxide at a rate of 0.22 nmol/ml/min as can be calculated from the results presented in figure 1 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. Untreated PMN produced only a minor fraction of the amount produced by TPA-stimulated PMN.

Adhesion to microvascular endothelial cells

Basal adhesion, i.e. adhesion to non-pre-incubated MEC, was between 10 and 15% of added cells for HT29 and Caco2. For PanC1, basal adhesion was between 5 and 10% and for BxPC3 this was between 20 and 30% (data not shown).
Exposure of MEC for 12 hours to resting PMN did not significantly increase adhesion for all 4 tumour cell lines (Fig.2). If the endothelial monolayer was pre-incubated with PMN that had been stimulated by TPA, a considerable increase in tumour cell adhesion was found (Fig.2). The highest increase was found for HT29, namely 341.8% *vs.* control ($p<0.01$). For PanC1 the enhancement was 282.3% *vs.* control ($p<0.01$) and for Caco2 237.5% *vs.* control ($p<0.01$). BxPC3 showed the smallest increase, namely 219.5% *vs.* control ($p<0.01$). To

exclude the possibility that the found enhancement is caused by the activation of MEC by TPA, we pre-incubated MEC with TPA alone as well. As shown in figure 2 that was apparently not the case, since none of the cell lines demonstrated a significant difference in adhesion to MEC pre-incubated with TPA compared to their basal adhesion (p>0.05).

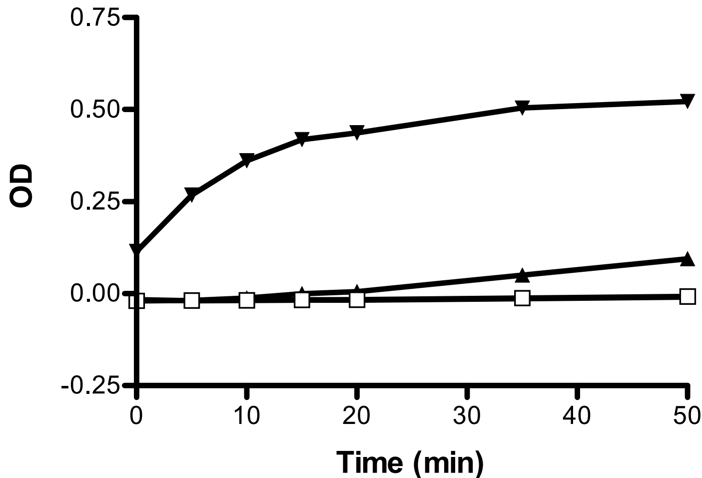

Figure 1. Production of superoxide anions measured by the ferricytochrome *c* reduction assay: control (♦), PMN (▲), TPA (□) or PMN activated with TPA (▼). Data represent mean absorbance values (OD 540-550nm) ± SEM of quadruplicate wells.

Antioxidants and tumour cell adhesion

We evaluated the effects of the antioxidant enzymes SOD and catalase in this model to study if PMN caused the enhanced adhesion of the various tumour cells to MEC by their production of ROS (Fig.3). The results showed that addition of SOD did decrease the enhanced adhesion of HT29 to MEC (p<0.01), of (p<0.01), of Caco2 (p<0.05) and of BxPC3 (p<0.01), which makes it likely that superoxide contributed to the increased adherence of the tumour cells but was not the only actor because adhesion did not return to basal levels.
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 decreased the enhanced tumour cell adhesion after pre-incubation with

activated PMN effectively as well, i.e. significantly for HT29 ($p<0.01$), for PanC1 ($p<0.01$), for Caco2 ($p<0.01$) and for BxPC3 ($p<0.05$) *vs.* control (fig.3).

In combination both antioxidant enzymes decreased the enhanced tumour cell adhesion even further (all *vs*. control, $p<0.01$). BxPC3 displayed the largest reduction of 151.1%. This reduction resulted in a tumour cell adhesion of 68% *vs.* control and therefore 32% below the basal adhesion ($p<0.01$). Apparently, the low level of ROS produced by the MEC themselves as shown earlier is contributing to the basal adhesion of BxPC3 as well.

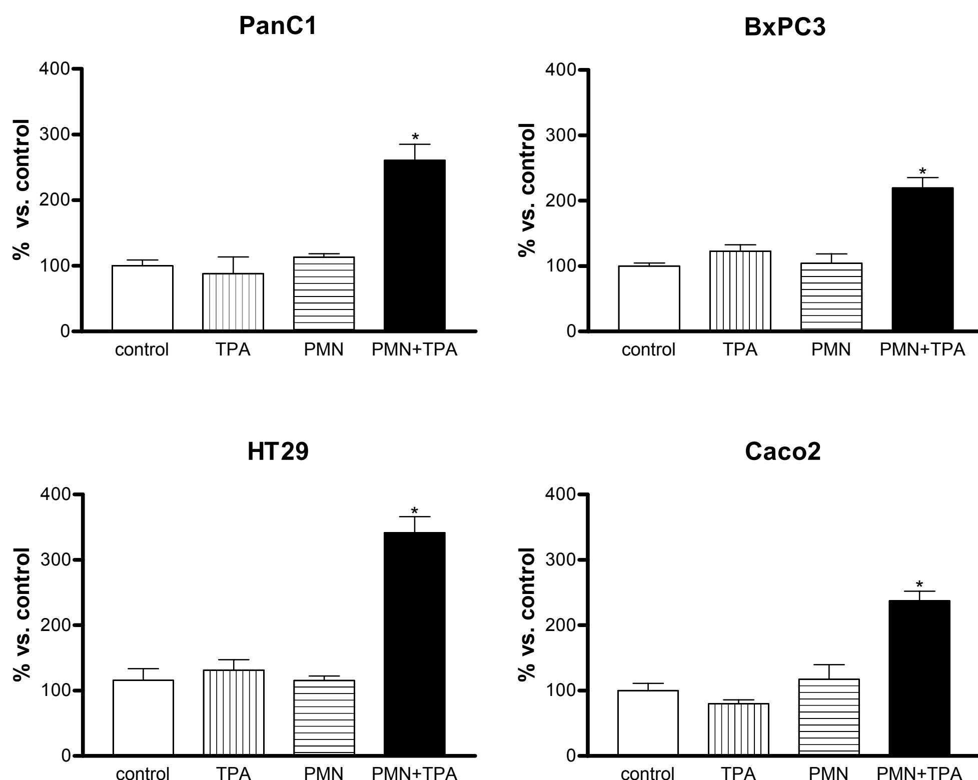

Figure 2. Cell adhesion of the pancreatic (PanC1, BxpC3) and colon (HT29, Caco2) carcinoma cell lines to MEC after 12 hours pre-incubation of MEC with TPA, PMN or PMN activated with TPA (PMN+TPA). Means (n=6; % *vs.* control) ± SEM are shown. * $p<0.01$ *vs.* control.

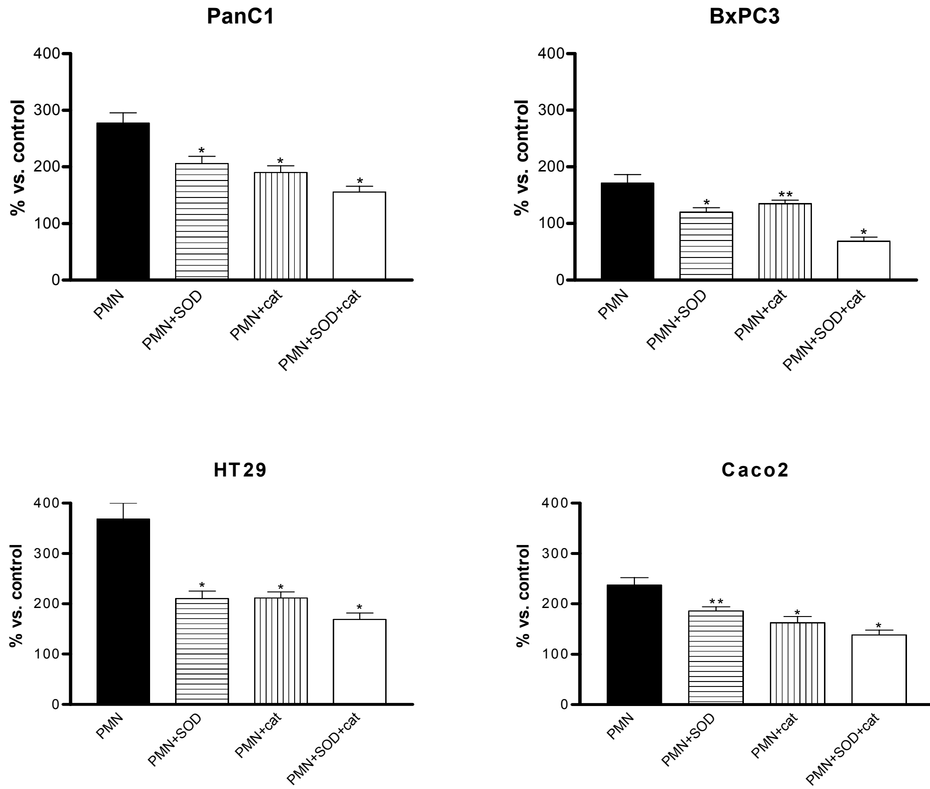

Figure 3. Cell adhesion of the pancreatic (PanC1, BxpC3) and colon (HT29, Caco2) carcinoma cell lines to MEC after pre-incubation of MEC with PMN activated with TPA (PMN). The antioxidant enzymes SOD, catalase (cat) and the combination of both were added during the pre-incubation. Means (n=6; % vs. control) ± SEM are shown.

DISCUSSION

Previous experimental and clinical studies have suggested that surgical trauma promotes tumour recurrence [8,30-32]. The exact mechanism by which surgical trauma promotes recurrence is not completely understood, but a number of *in vivo* studies suggest that the inflammatory sequelae produced by the surgical trauma play an important role. Among other mechanisms, this local and systemic inflammatory reaction activates polymorphonuclear leucocytes with the release of ROS [33-36]. We hypothesized that circulating tumour cells, which are found in the majority of cancer patients [4,37,38], are more successful in forming metastases under influence of ROS produced by these PMN. Since tumour cell adhesion to the microvascular endothelium forms a crucial step in tumour recurrences at distant sites, we studied if the release of ROS by PMN is indeed of essential importance in these interactions. The results of the present study demonstrated that exposure of MEC to TPA-activated PMN enhanced the adhesion of both colon and both pancreatic carcinoma cell lines under study significantly. Exposure of MEC to TPA or unstimulated PMN alone did not influence tumour cell – endothelial cell interactions.

At least 2 questions remain: (i) which is the relevant ROS and (ii) can knocking down the source of ROS or inactivating the ROS themselves prevent tumour recurrence?

From the experiments with SOD and catalase it became clear that each antioxidant enzyme decreased the enhanced tumour cell adhesion significantly. This means that not only superoxide anions, but also hydrogen peroxide are equally involved in this phenomenon. Addition of both antioxidant enzymes simultaneously decreased the adhesion even more than by one of each enzyme alone. This indicates that perhaps a third kind of ROS, namely a highly reactive hydroxyl radical, is of relevance as well. 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 with the exception of BxPC3, in the presence of activated PMN the adherence to MEC of the tumour cell lines under investigation did not return to basal levels by the addition of both antioxidant enzymes. Therefore, it is likely that activated PMN produce other factors like cytokines and proteases besides ROS that can interfere with tumour cell – endothelial cell interactions. Indeed, activated PMN produce several pro-inflammatory cytokines like IL-1β and TNF-α [39] of which we know that they are able to increase tumour cell adhesion [40].

Besides the activation of MEC by factors released by TPA-activated PMN, binding of the activated PMN to MEC may initiate a plethora of processes in the MEC through signalling of their cellular adhesion molecules. These processes may create a paracrine loop with a further increase in inflammation. Indeed, Boehme *et al* [41] have shown previously that PMN need to be activated to induce shedding of adhesion molecules in the medium by endothelial

cells, which is in accordance with our present finding that untreated PMN did not affect tumour cell adhesion to MEC. Obviously, further studies to dissect the mechanisms by which the PMN modulate tumour cell – endothelial cell interactions are necessary.

In conclusion, the results of the present study suggest that PMN activated by surgical trauma may promote tumour recurrence at distant sites by the production of ROS in concert with other unrevealed factors. We found here that at least three types of ROS play a role therein, i.e. superoxide,anions hydrogen peroxide, and the highly reactive hydroxyl radical. Therefore, inactivating those ROS by increasing the antioxidant status of the patient seems a feasible way of at least reducing tumour recurrence at distant sites.

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