# Polymorphonuclear Leukocytes Increase the Adhesion of Circulating Tumor Cells to Microvascular Endothelium

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**Abstract.** *Background: Reactive oxygen species (ROS) released from activated polymorphonuclear leukocytes (PMN) during surgery may play a crucial role in the enhanced distant tumor recurrence after surgical trauma. Materials and Methods: The effect of PMN on the adhesion of the human colon carcinoma cells HT29, Caco2 and the pancreatic carcinoma cells PanC1 and BxPC3 to microvascular endothelium (MEC) was studied in a reproducible human in vitro model. Results: Pre-incubation of MEC with tissue plasminogen activator (TPA)-activated PMN resulted in more than 200% increase of tumor cell adhesion to MEC compared to control ($p<0.01$). Exposure of MEC to TPA or non-activated PMN did not significantly affect adhesion. Addition of the antioxidant enzymes superoxide dismutase or catalase significantly decreased tumor cell adhesion to MEC exposed to PMN. Conclusion: These results demonstrate that activated PMN promote tumor cell adhesion to the microvascular wall by production of ROS. This indicates that in tackling the ROS production, preventing tumor recurrence at distant sites, might be feasible.*

Colon cancer recurrence rates after intentional curative surgery have been reported to be up to 40% (1), while pancreatic cancer recurrence rates 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 tumor cells, which can be found in many patients with gastro-intestinal malignancies (3-6).

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Previously conducted *in vivo* studies have shown a relation between surgical trauma and tumor 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, *i.e.* 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 tumor load in a rat model and that the severity of surgical trauma influenced the degree of tumor load. Indeed, laparoscopy, which causes minor trauma, gave significantly less tumor load compared to laparotomy in a rat model (9).

Subsequent *in vivo* experiments have demonstrated that one of the cellular components of blood, *i.e.* the erythrocytes, once introduced in the abdominal cavity after abdominal surgical trauma, 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 have demonstrated that the polymorphonuclear leukocytes (PMN), which are major $O_2^-$ producers, enhanced tumor load. Since a surgical trauma generally elicits an inflammatory exudate consisting of more than 75% PMN lacking erythrocytes, tumor recurrence is promoted. 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 will spread out systemically as well (12-18). Indeed, during and several days after major surgery, the peripheral blood level of elastase, an indicator of PMN activity, is elevated (19, 20). Furthermore, after major abdominal surgery the number of PMN increased not only systemically, but also at distant sites, for example in the lung (21). In haematogenous metastasis, circulating tumor 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); that latter process might be promoted by the occurrence of an acute inflammation dominated by PMN, as previously found for the mesothelium (25-27).

In order to study the underlying mechanism by which PMN induce tumor cell adhesion to microvascular endothelium, a reproducible human *in vitro* model was developed. In this model, two tumor cell types were used, colon and pancreatic, to investigate if PMN affect tumor cell – endothelial cell interactions. The role of PMN-derived ROS was further investigated by the addition of antioxidants in the adhesion assays.

## Materials 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's instructions 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 pancreatic carcinoma cell lines PanC1 and BxPC3 were grown in EGM-2-MV Bullet kit as well in order to create similar conditions, and were maintained by serial passage after trypsinization using 0.05% trypsin / 0.02% EDTA (Invitrogen, Breda, The Netherlands).

Before the adhesion assay, tumor cells were trypsinized and maintained in suspension for 2 h 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 xg for 32 min 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 xg 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 cells 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 ferricytochrome *c* reduction (28). Analogous to our adhesion model, $2x10^5$ PMN with or without 5 μM tissue plasminogen activator (TPA) were added to the experimental wells. After addition of 75 μM cytochrome *c* (Roche Applied Science, Almere, The Netherlands) the change in absorbance at 540 nm (reference) and 550 nm was continuously recorded by a thermostatted Versamax microplate reader (Molecular Devices, Berkshire, United Kingdom) for 125 min at 37˚C. In this model, pH changes, determined with a pH electrode, were not observed.

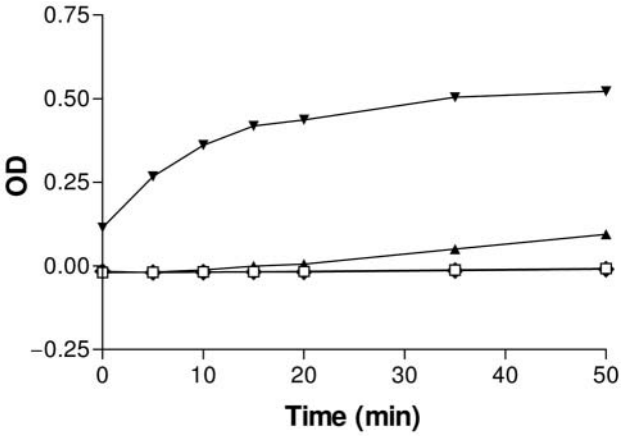


Figure 1. *Production of superoxide anions as measured using the ferricytochrome c reduction assay: control (◆), PMN (▲), TPA (□) or PMN activated with TPA (▼). Data represent mean absorbance values (OD 540-550nm) ± SEM of quadruplicate wells.*

*Adhesion assay.* A standardised cell adhesion assay was developed to quantify tumor cell adhesion to MEC, as described previously (29). Briefly, endothelial monolayers were established in 96-well microtiter plates (Perkin Elmer, Groningen, The Netherlands). To do this, confluent cells were trypsinized and $2x10^4$ 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.

The effect of PMN on tumor cell adhesion was determined using endothelial monolayers pre-incubated with $2x10^5$ PMN per well for 12 h at 37˚C. Before adding the PMN to the wells the PMN were stimulated with 5 μM 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) which converts superoxide anions into molecular oxygen and hydrogen peroxide. Since hydrogen peroxide itself is a strong ROS, catalase (400 U/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands) was also added to decompose the hydrogen peroxide.

In order to quantify tumor cell adhesion, tumor cells ($1x10^6$ cells/ml) were labelled with calcein-AM (Molecular Probes, Leiden, The Netherlands) and $3x10^4$ cells per well were added. Plates were centrifuged for 1 min at 80 xg and incubated at 37˚C for 1 h. After this, wells were washed twice with medium. The remaining fluorescence per well was measured on a Perkin-Elmer plate reader (Wellesley, USA) 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 groups. $P \leq 0.05$ was considered to be statistically significant. Experiments (n=6) were performed at least twice.

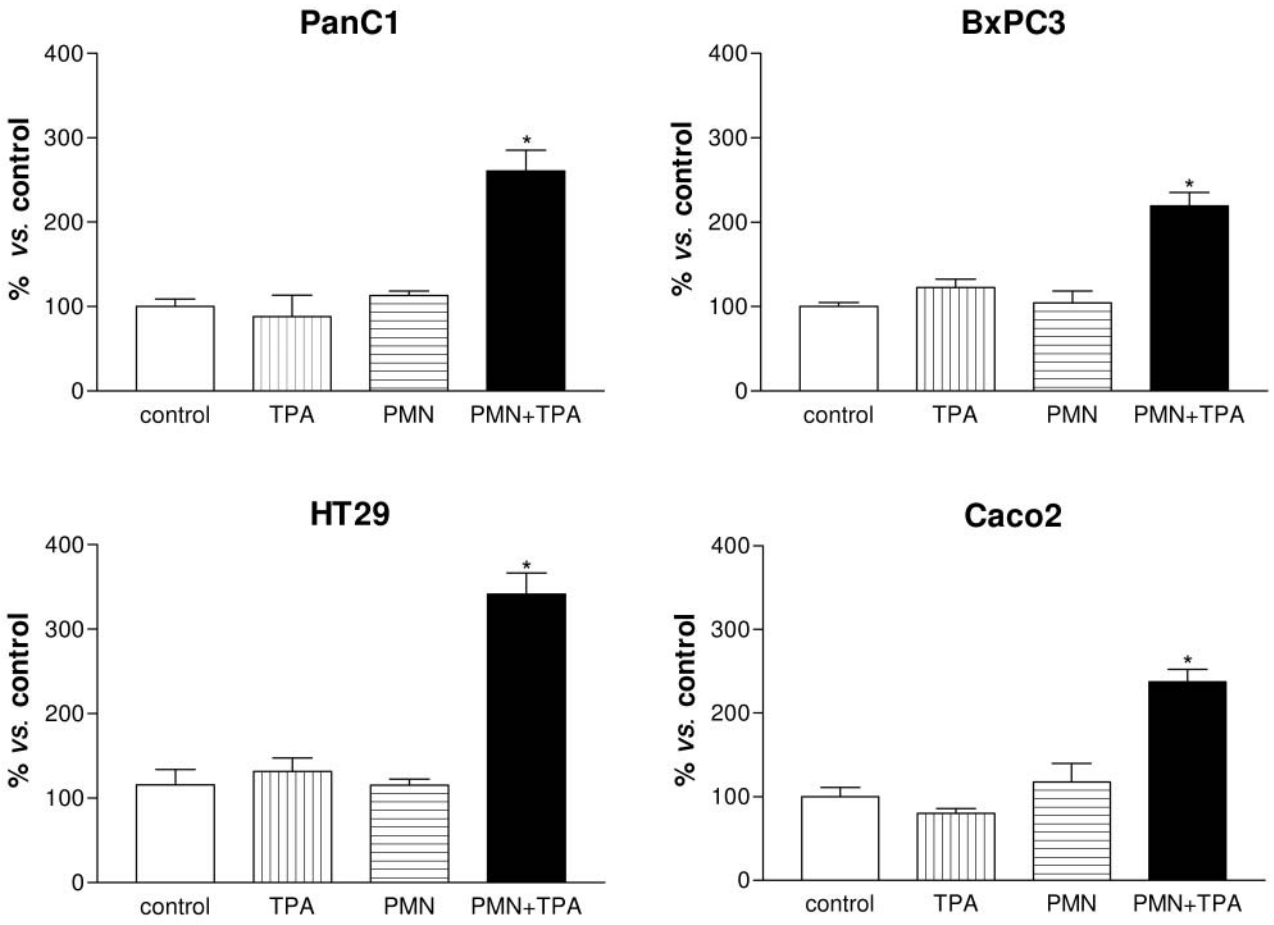


Figure 2. *Cell adhesion of pancreatic (PanC1, BxPC3) and colon (HT29, Caco2) carcinoma cell lines to MEC after 12 h pre-incubation of MEC with TPA, PMN or PMN activated with TPA (PMN+TPA). Means (n=6; % vs. control) ± SEM are shown. *p<0.01 vs. control.*

## Results

*Evaluation of the model and superoxide anion production.* Labelling tumor cells with calcein-AM did not decrease their viability (>95% using trypan blue). A dilution series of labelled tumor 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 tumor 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 (Figure 1). TPA-stimulated PMN produced superoxide at a rate of 0.22 nmol/ml/min, as 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 any of the tumor cell lines (Figure 2). If the endothelial monolayer was pre-incubated with TPA-stimulated PMN, a considerable increase in tumor cell adhesion was found (Figure 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 enhancement found was 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

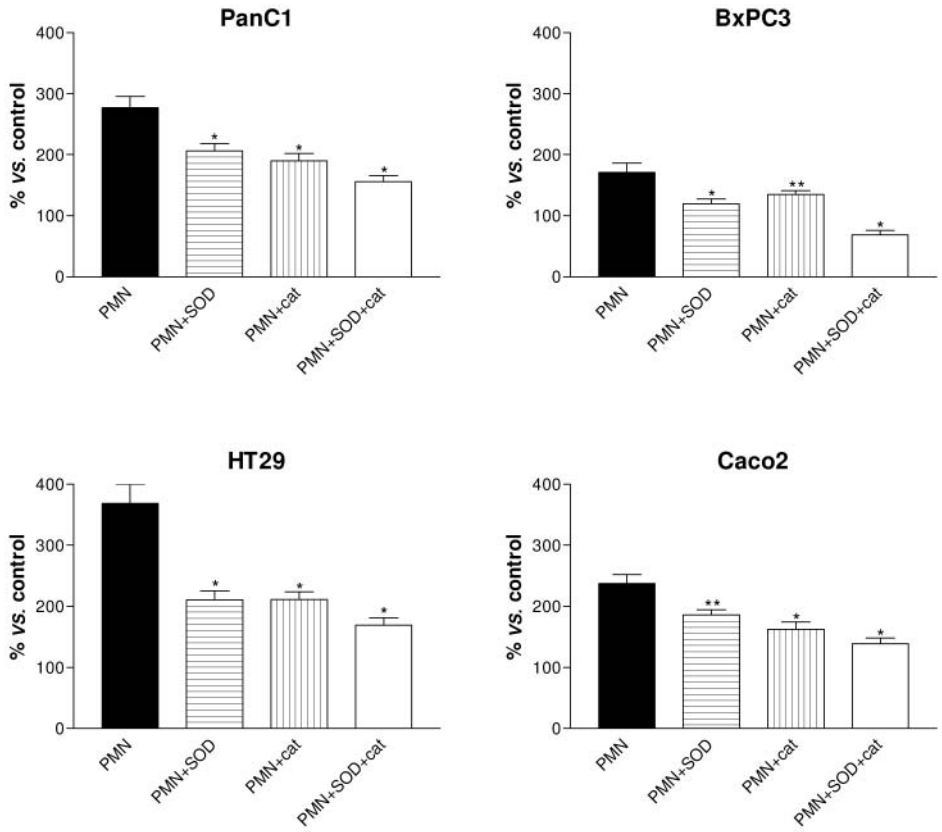


Figure 3. *Cell adhesion of pancreatic (PanC1, BxPC3) and colon (HT29, Caco2) carcinoma cell lines to MEC after pre-incubation of MEC with PMN activated with TPA (PMN). Superoxide dismutase (SOD), catalase (cat) and a combination of both were added during the pre-incubation. Means (n=6; % vs. control) ± SEM are shown. *p<0.01 vs. PMN; **p<0.05 vs. PMN.*

difference in adhesion to MEC pre-incubated with TPA compared to their basal adhesion ($p>0.05$).

*Antioxidants and tumor cell adhesion.* The effects of the antioxidant enzymes SOD and catalase in this model were evaluated to study if PMN caused the enhanced adhesion of the various tumor cells to MEC by their production ROS (Figure 3). The results showed that addition of SOD did decrease the enhanced adhesion of HT29 ($p<0.01$), PanC1 ($p<0.01$), Caco2 ($p<0.05$) and BxPC3 ($p<0.01$) to MEC, which makes it likely that superoxide contributed to the increased adherence of the tumor cells but was not the only factor because adhesion did not return to basal levels.

Since superoxide anions spontaneously dismutate into the stronger ROS hydrogen peroxide, which may in turn affect MEC, the effect of catalase was also studied. The results showed that catalase also decreased the enhanced tumor cell adhesion after pre-incubation with activated PMN effectively, *i.e.* significantly for HT29 adhesion ($p<0.01$), for PanC1 ($p<0.01$), for Caco2 ($p<0.01$) and for BxPC3 ($p<0.05$) *vs.* control (Figure 3).

In combination both antioxidant enzymes decreased the enhanced tumor cell adhesion even further (all *vs.* control, $p<0.01$). BxPC3 displayed the largest reduction of 151.1%. This reduction resulted in a tumor 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, contributed to the basal adhesion of BxPC3 as well.

## Discussion

Previous experimental and clinical studies have suggested that surgical trauma promotes tumor recurrence (7-8, 30-31). 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, the local and systemic inflammatory reaction activates polymorphonuclear leukocytes with the release of ROS (18, 34). It was hypothesized that circulating tumor cells, which are found

in the majority of cancer patients (3-4, 6), are more successful in forming metastases under the influence of ROS produced by these PMN. Since tumor cell adhesion to the microvascular endothelium forms a crucial step in tumor 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 demonstrate that exposure of MEC to TPA-activated PMN significantly enhance the adhesion of both colon and both pancreatic carcinoma cell lines under study. Exposure of MEC to TPA or unstimulated PMN alone did not influence tumor cell – endothelial cell interactions. One should expect that exposure of MEC to TPA, which has wide ranging and generally non-specific effects as a cellular activator, would influence tumor cell adhesion. However, in this experimental study no enhancement in tumor cell adhesion after exposure of MEC to TPA alone was detected.

At least two questions remain: (i) which is the relevant ROS, and (ii) whether knocking down the source of ROS or inactivating the ROS themselves prevent tumor recurrence.

From the experiments with SOD and catalase it became clear that each antioxidant enzyme decreased the enhanced tumor cell adhesion significantly. This means that not only superoxide anions, but also hydrogen peroxide are equally involved in this phenomenon. In all cell lines addition of both antioxidant enzymes simultaneously decreased the adhesion even more than that of each enzyme alone. This indicates that perhaps a third kind of ROS, *i.e.* 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, 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 tumor cell lines under investigation did not return to basal levels with the addition of both antioxidant enzymes. Therefore, it is likely that activated PMN produced other factors like cytokines and proteases, besides ROS, that can interfere with tumor cell – endothelial cell interactions. Indeed, activated PMN produce several pro-inflammatory cytokines like IL-1β and TNF-α (35) which are known to increase tumor cell adhesion (36).

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.* (37) have previously shown 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 does not affect tumor cell adhesion to MEC. Obviously, further studies to dissect the mechanisms by which the PMN modulate tumor cell – endothelial cell interactions are necessary.

## Conclusion

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

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