Original article

Red blood cells inhibit tumour cell adhesion to the peritoneum

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Background: **Perioperative blood transfusion has been associated with increased tumour recurrence and poor prognosis in colorectal cancer. Blood loss in the peritoneal cavity might be a tumour-promoting factor for local recurrence. The aim of this study was to investigate whether blood in the peritoneal cavity affects local tumour recurrence.**

Methods: **In an established *in vivo* rat model the effect of 1·5 ml syngeneic whole blood on tumour cell adhesion and tumour growth was investigated. In the same model the effect of 1·5 ml pure red blood cell (RBC) concentrate and 1·5 ml RBC-derived substances on tumour cell adhesion was studied. In an established *in vitro* model the effect of increasing numbers of RBCs (0–250 × 10^6) on tumour cell adhesion and tumour growth was assessed.**

Results: **Both the presence of blood and RBC concentrate in the peritoneal cavity prevented tumour cell adhesion *in vivo* (overall $P \leq 0{\cdot}001$ and $P \leq 0{\cdot}05$ respectively), rather than promoting adherence. RBC concentrate and RBC-derived substances had a comparable inhibitory effect on tumour cell adhesion. In *in vitro* studies RBCs inhibited tumour cell adhesion but not tumour growth.**

Conclusion: **RBC-derived factors prevent tumour cell adhesion to the peritoneum, and consequently tumour recurrence.**

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Introduction

Peroperative blood transfusion, irrespective of whether it is allogeneic or autologous, is associated with enhanced tumour recurrence and poor prognosis in patients with colorectal cancer[1–3]. In the study by Busch *et al.*[2], the association between recurrent disease and blood transfusion appeared to exist only for the incidence of local recurrence and not for the incidence of distant metastases. Furthermore, it was concluded that the circumstances necessitating transfusion were the real determinants of prognosis[2]. Conditions leading to blood transfusion are the location and extent of the primary tumour, skill of the surgeon and length of operation. Blood loss in the peroperative period, however, is the most common reason for transfusion.

The effect of blood loss on tumour recurrence has been studied extensively in experimental animal models. The results of these studies are controversial and report both stimulatory and inhibitory effects on tumour growth, irrespective of whether blood transfusion is given[4–6].

Based on the clinical observation that conditions leading to transfusion are the real determinants of prognosis, as well as the experimental results indicating that blood loss itself is an important factor influencing tumour growth, it was hypothesized that local blood loss, i.e. in the peritoneal cavity, might be a factor influencing local tumour recurrence. The present paper reports an *in vitro* and *in vivo* study investigating the effect of blood and blood products on the establishment of intraperitoneal tumour recurrence.

Materials and methods

Animals

Inbred rats of the WAG strain were obtained from Harlan-CPB (Zeist, The Netherlands) and kept under standard conditions. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee for Animal Research of the Erasmus University Rotterdam.

Tumour cell line

The syngeneic CC531 colon carcinoma was induced in WAG/Rij rats by 1,2-dimethylhydrazine. A cell line was established from this carcinoma and maintained by serial passage after trypsinization in culture medium[7,8]. CC531 cells were cultured in RPMI 1640 medium supplemented with 5 per cent fetal calf serum, L-glutamine 200 mmol/l, penicillin 5000 units/ml and streptomycin 5000 units/ml. All supplements were obtained from Gibco, Paisley, UK. Before use, cells were trypsinized (10 min at 37 °C), centrifuged (5 min at 700*g*), resuspended in RPMI 1640 and counted. Viability was measured by trypan blue exclusion and always exceeded 90 per cent.

Blood products

WAG rats were exsanguinated by cardiac puncture. Red blood cell (RBC) concentrate was obtained by centrifugation of blood and resuspending the pelleted RBCs in RPMI medium to the original volume. Using this suspension, RBC content was collected by lysing RBCs in liquid nitrogen. The resulting homogenate was divided into 1·5-ml aliquots and centrifuged at 10 000 r.p.m. for 15 min to pellet cell fractions.

In vivo experiments

Operative procedure and tumour scoring

Under ether anaesthesia a laparotomy was performed followed by peritoneal inoculation of 10^6 CC531 tumour cells. The abdomen was closed in one layer with silk 2/0 sutures (Braun, Uden, The Netherlands). Three weeks after operation the rats were killed and tumour establishment was scored at multiple intraperitoneal sites. The scoring ranged from 0 to 5 per site, according to the peritoneal cancer index described by Steller[9].

Experimental design

In the first experiment, the effect of 1·5 ml syngeneic whole blood on intraperitoneal tumour recurrence was determined. The control group received 1·5 ml phosphate-buffered saline (PBS). Both groups consisted of ten rats.

In the second experiment, tumour cells were allowed to adhere before performing a relaparotomy after 24 h and adding 1·5 ml whole blood. The control group received PBS. Both groups consisted of eight rats.

In the third experiment, the effect of 1·5 ml RBC concentrate on tumour establishment was evaluated. The control group received 1·5 ml PBS; both groups consisted of ten rats.

In the last experiment, a distinction was made between RBCs and RBC contents using the supernatants derived from 1·5 ml lysed RBC concentrate. Both groups comprised nine rats.

In vitro experiments

Mesothelial cell culture

Mesothelial cells were isolated from the small bowel mesentery of rats and identified immunohistochemically as described before[10]. Mesothelial monolayers were established in 24-well plates (Costar, Badhoevedorp, The Netherlands), precoated with collagen type I (Boehringer Mannheim, Mannheim, Germany).

Experimental design

RBCs ranging in concentration from 10 to 250×10^6 were added to the wells coated with mesothelium or collagen type I. In the first experiment RBCs and 40 000 CC531 tumour cells were added simultaneously. In the second experiment the effect of RBCs on tumour growth was evaluated by leaving CC531 cells to adhere overnight, before adding RBCs. To determine a potential direct toxic effect, RBCs were added to a mesothelial monolayer. After 48 h, the plates were collected by washing away superfluous non-adherent cells and kept at −20°C for DNA analysis.

DNA analysis

The total DNA content of each well was measured using the bisbenzimide fluorescent dye (Boehring Diagnostics, La Jolla, California, USA) as described previously by Hofland *et al.*[11]. The DNA measured represents the total amount of cells per well.

Statistical analysis

Statistical analysis was performed using the Kruskal–Wallis analysis of variance to determine overall differences followed by the Mann–Whitney *U* test to compare differences between groups.

Results

In vivo experiments

Effect of whole blood on peritoneal tumour take

Significant differences in tumour take at all peritoneal sites scored were found between the control and experimental groups. Adding 1·5 ml blood to the peritoneal cavity simultaneously with tumour cells inhibited the take of tumour cells (*Table 1*).

When blood was injected after the CC531 tumour cells had adhered, however, no significant differences in tumour take could be detected (results not shown). This indicates that the protective effect of blood is based on preventing tumour cell adhesion rather than affecting tumour growth.

Effect of red blood cells on peritoneal tumour take

Adding 1·5 ml pure RBC concentrate together with tumour cells produced a similar effect to that obtained when whole blood was used. At all scored peritoneal sites there was significantly less tumour take when RBC concentrate was added simultaneously with 10^6 CC531 tumour cells (*Table 2*).

When comparing the effect of RBCs with RBC contents, no differences in tumour take could be scored (results not shown). These results indicate that inhibition of tumour cell adhesion to the peritoneum can be attributed to the content of RBCs rather than the RBCs as a whole.

In vitro experiments

RBCs in large amounts did not have a direct toxic effect on the mesothelial monolayer. A significant inhibition of tumour cell adhesion was observed, irrespective of adherent background, after adding 50×10^6 RBCs or more ($P < 0{\cdot}05$). No further inhibitory effect was seen after adding more than 100×10^6 RBCs (*Fig. 1*). When CC531 cells were left to adhere before adding the RBCs no difference in adherence could be measured (data not shown). These *in vitro* results indicate, as the *in vivo* results, that the observed inhibition of tumour establishment by RBCs is based on impeding tumour cell adhesion rather than affecting tumour growth.

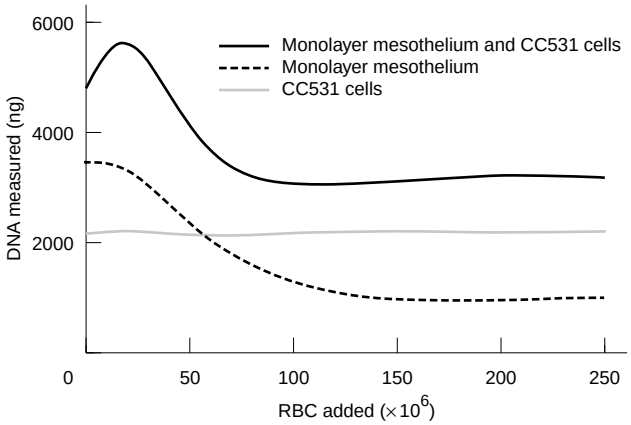

Fig. 1 Tumour cell adhesion represented by total DNA measured. Values are mean(s.d.) ($n = 4$)

Table 1 Effect of whole blood in the peritoneal cavity on local tumour take

Abdominal site	1·5 ml PBS ($n = 10$)	1·5 ml blood ($n = 10$)	P*
Subcutis	3 (2–5)	1 (0–3)	0·001
Mesentery	2 (1–3)	0 (0–1)	< 0·0001
Kidney	3 (1–5)	1 (0–1)	0·0001
Liver	2 (1–3)	0 (0–1)	0·0001
Omentum	3 (2–5)	0 (0–1)	< 0·0001
Retroperitoneum	2 (0–2)	0 (0)	0·0002

Values are median (range) peritoneal cancer index score. PBS, phosphate-buffered saline. *Mann–Whitney *U* test

Table 2 Effect of red blood cells in the peritoneal cavity on local tumour take

Abdominal site	1·5 ml PBS ($n = 10$)	1·5 ml RBCs ($n = 10$)	P*
Subcutis	5 (4–5)	1 (0–3)	< 0·001
Mesentery	2 (1–4)	0 (0–4)	0·002
Kidney	3 (2–5)	1 (0–5)	0·004
Liver	2 (2–4)	0 (0–2)	< 0·001
Omentum	5 (4–5)	0 (0–5)	< 0·001
Retroperitoneum	1 (0–3)	0 (0–4)	0·05

Values are median (range) peritoneal cancer index score. PBS, phosphate-buffered saline; RBC, red blood cell. *Mann–Whitney *U* test

Discussion

Local or regional tumour recurrence, or both, may be a result of residual microscopic disease, direct extension into adjacent organs or intraoperative implantation of exfoliated tumour cells. In the latter process, tumour cells shed into the peritoneal cavity survive and grow into established tumours. Adjuvant measures to kill the exfoliated tumour cells may prevent this mode of recurrence.

The observation that the presence of RBCs in the peritoneal cavity could impede tumour establishment was reason enough to explore this phenomenon. There were primarily three hypotheses proposed for the mechanism of this effect, the first being a growth-limiting effect. The *in vitro* studies demonstrated that there was no decline in total DNA after 48 h caused by RBCs, i.e. no decline in tumour cell number, once the tumour cells had adhered. Furthermore, after adhesion of CC531 cells *in vivo* a growth-inhibiting effect could not be detected. Both experiments prove that RBCs do not inhibit tumour growth, and that the inhibition of tumour take by RBCs takes place at the point of tumour cell adhesion.

The second hypothesis was that tumour cell adhesion could be impeded by mechanical inhibition. Although in the *in vivo* studies this hypothesis was less likely to be relevant, in the *in vitro* studies a significant inhibition of tumour cell adhesion was seen after adding 50×10^6 or more RBCs. This is at least 1250 times more RBCs than tumour cells which may impede tumour cell adhesion through mechanical inhibition.

The third hypothesis was accredited to an unknown function of RBCs, which could be executed by either the RBC membrane or contents. It was demonstrated that the latter produced a similar inhibitory effect on tumour establishment to that of intact RBCs, indicating that mechanical inhibition by an excess of RBCs was not relevant and so the inhibition of tumour cell adhesion to the peritoneum could be attributed to the content of RBCs rather than intact RBCs.

Other than oxygen transporters, RBCs are known to contain several antioxidant protectors. These scavengers catalyse or accelerate the dissociation of oxygen free radicals which play a fundamental role in cellular injury in a wide variety of pathophysiological processes. Following abdominal surgery, the most likely source of these free radicals is a rapid influx of neutrophils and macrophages, recruited to the abdominal cavity as a result of surgical trauma or inflammation[12,13]. The purpose of these reactive cell products is to destroy invading organisms and damaged tissue.

Excessive or inappropriate production of free radicals is associated with morbidity and death after peritoneal trauma and inflammatory disease[14,15]. Diminution of peritoneal trauma by scavengers affecting free radical-mediated injury might contribute to the prevention of implantation of spilled tumour cells.

The results of the present experiments indicate a possible relation between scavengers carried by RBCs and prevention of free radical-mediated injury. In previous *in vivo* studies a significant correlation between the extent of peritoneal trauma and the degree of tumour implantation was shown[16]. The evidence to date supports an effector role of RBCs in actively preventing tumour cell adhesion to the peritoneum, resulting in less tumour take. Additional studies are required to determine which of the three scavengers that are transported in the cytoplasm of RBCs (glutathione peroxidase, catalase and superoxide dismutase) bring about this effect.

The potential for clinical application of scavengers to modify free radical injury, and so inhibit establishment of peroperatively spilled tumour cells, may lead to new therapeutic approaches for the prevention of locoregional recurrence.

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