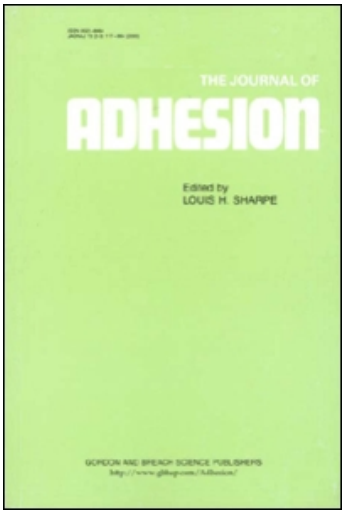

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Influence of Per-Operative Serum on Tumour Cell Adhesion *in Vitro*

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In case of massive tissue damage, the ensuing inflammatory reaction produces a potentially lethal cocktail of active mediators. Beside the influence of these local factors on local tumour recurrence, we hypothesize that during surgery systemically produced mediators are potential stimulators of tumour cell adhesion as well. The aim of this study was to investigate the influence of serum samples, obtained at eight different time periods before, during, and after surgery on the interaction between human colon carcinoma cells and mesothelial cells.

In the control situation, the average adhesion of the Caco-2 cells to the mesothelial monolayers was 29%. No enhancement in adhesion was observed after incubation of the mesothelial cells with the pre- or postoperative serum of the patients.

This study suggests that systemic factors produced directly after surgery are not influencing tumour adhesion to mesothelial monolayers. Therefore, local tumour recurrence is probably only influenced by locally produced factors.

Keywords: Adhesion; Colon cancer; Mesothelial cells; Serum; Surgery

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INTRODUCTION

Local tumour recurrence after curative surgery of colorectal tumours is a persistent problem. The local recurrence rate is almost 50% after curative resection. The most common sites of local recurrence are the site of the primary tumour and the peritoneal surface [1]. There are several theories on the mechanism of local recurrence of spilled tumour cells. According to the theory of metastatic efficiency, implantation of tumour cells onto raw tissue surfaces is an efficient process compared with inefficient implantation [1]. The fibrin entrapment hypothesis proposes that peroperatively spilled tumour cells are trapped in fibrin at the resection site and on damaged peritoneal surfaces [2]. Another hypothesis, on which our study is based, suggests that after surgical trauma the acute inflammatory response will produce a great amount of growth factors and cytokines, which are very beneficial for the healing process, but also for adhesion and growth of tumour cells [3–7]. Surgical handling of the peritoneum is ambiguous regarding cancer. In case of massive tissue damage, the ensuing inflammatory reaction produces a potentially lethal cocktail of active mediators. These mediators are known to influence tumour recurrence to the peritoneum, probably caused by an upregulation of adhesion molecules, such as ICAM-1 and VCAM-1. Besides these locally produced cytokines and adhesion molecules, systemically produced mediators might be potential stimulators of local tumour cell adhesion as well. Recently, a soluble form of ICAM-1 has been described, and elevated levels have been associated with advance disease in gastric, colon, gallbladder, pancreatic, and renal carcinomas [8]. Preoperative serum levels of ICAM-1, E-selectin, and VCAM-1 are also correlated in gastric and colorectal cancer with invasion, lymph node involvement, and distant mestastasis [9,10]. Serum levels of these adhesion molecules, measured at day 7, decreased significantly after radical resection of the tumour compared with preoperative levels. The serum levels of these adhesion molecules remain unchanged in patients with unresectable tumours and in patients who underwent surgery for benign gastric disease [10].

We hypothesized that during surgery, systemically produced mediators might be potential stimulators of local tumour recurrence. Therefore, in the present study, we investigated the influence of serum, obtained at eight different time periods before, during, and after surgery from patients who underwent elective abdominal surgery for colorectal carcinoma, on the interaction between colon carcinoma cells and mesothelial cells. We developed an *in vitro* model, in which we studied the adhesion of colon carcinoma cells on mesothelial cells preincubated with serum from the same patient.

MATERIALS AND METHODS

Serum Samples of Patients, Culture of Omental Mesothelial Cells, and the Caco-2 Cell Line

In total, we studied nine patients undergoing elective abdominal surgery who had given informed consent to donate eight samples of blood at eight different time points and to donate a piece of omental tissue during surgery. The experimental protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam, The Netherlands. Blood was drawn one day before surgery, time point −24, at the beginning of the operation, time point 0, and 2, 4, 8, 12, 24, and 48 h after surgery. One sample of blood was taken at every time point. From each sample we obtained approximately 4 ml of serum.

Mesothelial cells (MC) were obtained from the omental tissue of the same patients. The MC were isolated according to techniques modified from Nicholson *et al.* [11] and Wu *et al.* [12] and cultured in RPMI 1640 medium supplemented with 10% human pooled normal serum, L-glutamin (200 mmol/L), and penicillin (5000 U/ml).

The human colon adenocarcinoma cell line Caco-2 was a kind gift of W. Dinjens (Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands). The cell line was cultured in RPMI 1640 medium supplemented with 10% human pooled serum, L-glutamin (200 mmol/L), and penicillin (5000 U/ml). All supplements were obtained from Invitrogen (Karlsruhe, Germany). For experiments, the cells were trypsinized (5 min at 37°C), centrifuged (5 min at 450 g), resuspended in RPMI1640, and counted. Viability was measured by trypan blue exclusion and always exceeded 95%.

Adhesion Assay

To quantify tumour cell adhesion to a monolayer mesothelium, a standardised cell adhesion assay was developed according to methods from Catterall *et al.* [13]. Mesothelial monolayers were established in 96 well plates (Canberra Packerd, Groningen, The Netherlands), precoated with collagen type I (15 $\mu g/cm^2$, coating according to instructions of the manufacturer). To do this, confluent cells were washed with phosphate buffered saline (PBS) and harvested with trypsin (0.05%) and EDTA (0.02%), and 2.5×10^4 mesothelial cells were added in 200 µl of medium to each well. The plates were incubated at 37°C, in a humidified atmosphere of 5% CO_2 in air; medium was replaced daily with fresh medium. Monolayers reached confluence in 5 to 7 days as determined by light microscopy. To determine the effect of the serum samples on tumour cell adhesion, mesothelial monolayers were

preincubated overnight with the eight serum samples of the same patient. We used a 10% serum–RPMI medium. The control mesothelial monolayers were incubated overnight in medium with 10% human pooled normal serum.

Tumour cells were labelled with calcein. Before adding to the mesothelial monolayers, the labelled cells were washed twice with RPMI 1640/0.5% BSA to remove free dye. Medium from the experimental wells was removed, and 200 µl RPMI 1640/0.5% BSA containing 30,000 calcein labelled tumour cells was added. Plates were centrifuged for 1 min at 80 g on a Perkin Elmer centrifuge (Perkin Elmer, Edeu Prairie, MN, USA) and incubated at 37°C for 60 min. Thereafter, the medium of each well was removed and washed twice with 200 µl of RPMI 1640/0.5% BSA medium. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 excitation and 530 emission filters. On each plate, a standard was prepared by adding different numbers of labelled tumour cells to the wells. The amount of tumour cells adhered was determined by calibrating the measured fluorescence of the experimental wells on the standard.

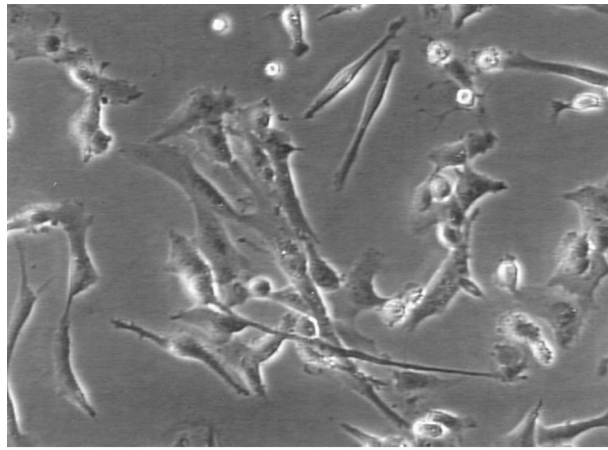
Statistical Analysis

All data were analysed using analysis of variance (ANOVA) to determine overall differences between group means. If the ANOVA was significant on a 5% level, the *post hoc* Newman–Keuls test was carried out to make a comparison between groups. Values represent $\pm$ SEM of $n = 6$ wells per treatment. $P \leq 0.05$ was considered to be statistically significant. Experiments were carried out at least twice with comparable results.

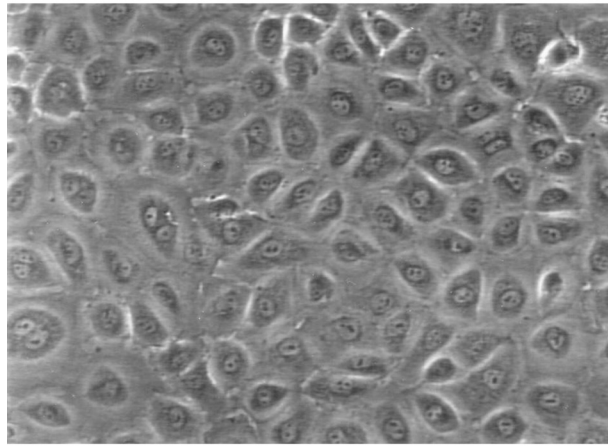
RESULTS

During the first day of culture, the mesothelial cells spread out to make contact to each other to create a monolayer (Figure 1A). After 3–5 days, a monolayer is formed with a typical cobblestone appearance (Figure 1B). The morphology of the monolayers did not change after preincubation with the serum samples.

Loading of the tumour cells with calcein did not affect their viability (>95% using trypan blue). To determine the stability of calcein uptake, the release of fluorescence in the supernatant of labelled cells after an incubation time of up to 120 min was measured. The fluorescence of the washed cells remained constant for at least 90 min, indicating retention of the dye in the cells (data not shown). A dilution



(a)



(b)

FIGURE 1 Growth features of mesothelial cells. During the first days of culture, the mesothelial cells spread out to make contact with each other to form a monolayer (A). After 3–5 days there is a monolayer: notice the typical cobblestone appearance (B).

series was made using labelled Caco-2 cells on a mesothelial monolayer. There was a direct relationship between the cell number added and measured fluorescence, resulting in a linear correlation, which was used as a standard to calibrate the measured fluorescence. In this way the number of adhered tumour cells in the experimental wells could be determined.

Tumour cell adhesion to a mesothelial monolayer in medium with 10% human pooled serum was relatively slow and temperature dependent. Maximum adhesion was seen after 60 min. At this time point a steady-state situation was achieved, and cell adhesion did not increase thereafter. The tumour cells remained rounded up during these adhesion assays. After more than 60 min, the first signs of spreading out could be detected under the light microscope. Therefore, for all subsequent experiments, 60 min was taken as a cutoff point.

The bar graphs in Figure 2 show the results of adhesion after preincubation of the mesothelial cells with the different serum samples

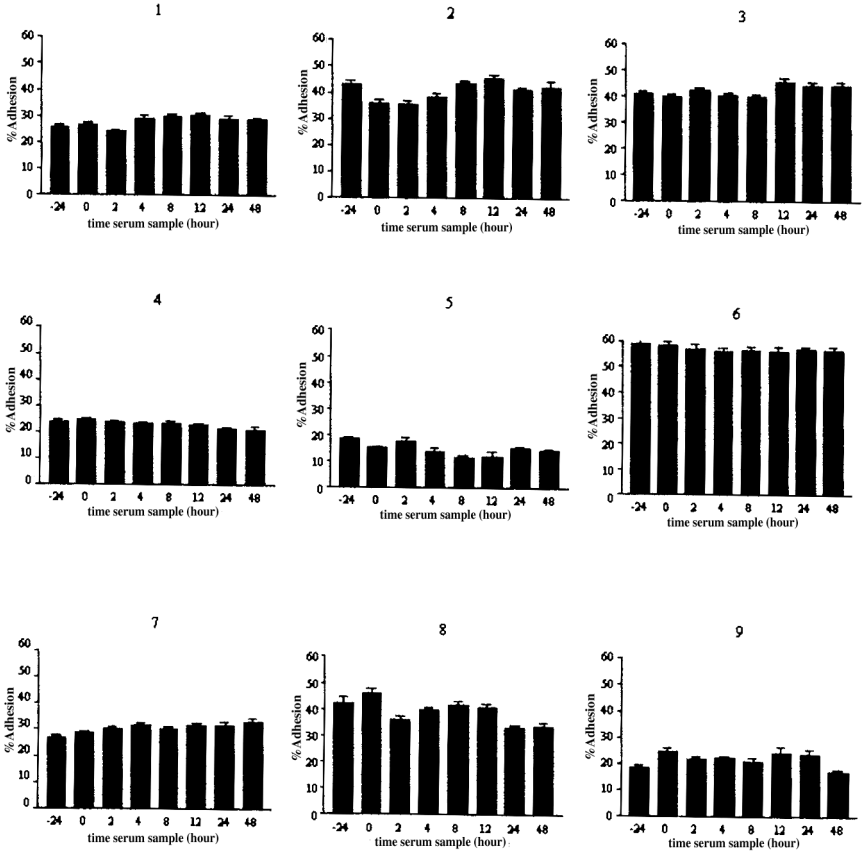


FIGURE 2 Bargraphs showing the adhesion of Caco-2 cells to monolayers mesothelium of nine different patients preincubated with serum samples of the same patient at eight different periods. Data are expressed as the mean ($n = 8$) +SEM.

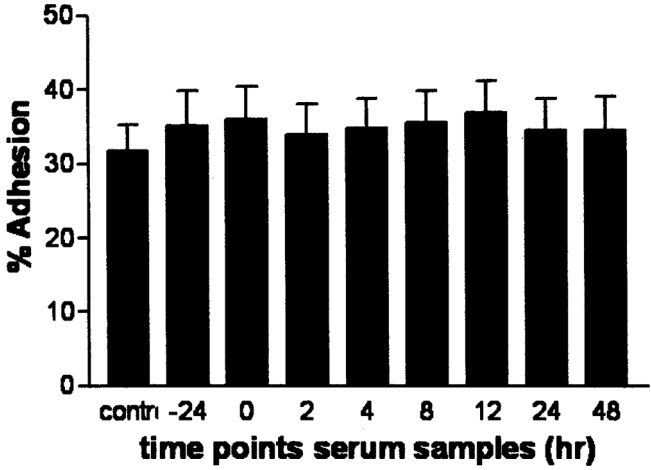


FIGURE 3 Mean adhesion of Caco-2 cells to monolayer mesothelium preincubated with serum samples of the nine patient at eight different periods. Data are expressed as the mean ($n = 8$) +SEM. The first bar shows the control adhesion of Caco-2 cells on mesothelial cells incubated in human-pooled serum overnight.

of the nine individual patients. Compared with the percentage adhesion observed with serum obtained 1 day prior to surgery (−24 h), no statistically significant changes in the percentage adhesion were observed after incubation with serum at the start of the surgical procedure (0 h) and 2, 4, 8, 12, 24, and 48 h during and after surgery. As shown in Figure 3, the adhesion of Caco-2 cells to the mesothelial cells from the different patients varies between 20 and 50%. The variability of the adhesion of Caco-2 cells to the different donors was also observed within incubation with pooled human serum, suggesting intrinsic differences between the mesothelial cells obtained from different patients in their capacity to cause Caco-2 adhesion. Figure 3 show the mean adhesion of the nine patients, including the control with human pooled serum. No significant difference in adhesion was observed between the control group and the serum samples taken from the patients.

DISCUSSION

Tumour metastasis following curative surgery has been a relentless hurdle for patients with colorectal cancer. Tumour recurrence after surgery has been studied in various animal models. These studies

concluded that tumour recurrence is enhanced by surgical trauma and that the expanse of tumour growth is dependent on the severity of surgical trauma [14–19]. Despite all these animal models, the mechanism of tumour recurrence is still unclear. Which factors are involved and whether it is a systemic or local stimulated process are still open to question.

Local tumour recurrence is a multistep process in which several factors are involved. Serum is the total complex of cytokines, soluble adhesion molecules, and growth factors. In this model we have chosen to use whole serum, because it is not known if tumour recurrence is influenced by systemic factors. It is important to investigate first if the serum is influencing tumour adhesion before you extract different factors from the serum and study them individually.

In this study we have evaluated whether systemic influences on local tumour recurrence can be involved. In our cell adhesion model, postoperative serum did not enhance adhesion of Caco-2 cells to incubated mesothelium. This suggests that systemic factors are not a main cause influencing the adhesion of spilled tumour cells.

However, several other explanations for the absence of an influence of postsurgical serum on tumour cell adhesion need to be discussed as well.

1) The concentration of factors that could potentially influence tumour cell adhesion may be too low. In previous studies we showed that 1 ng/ml IL-1β or TNF-α can enhance tumour cell adhesion to the mesothelium [20]. The concentration of IL-6 in serum in normal healthy patients is $<$100 pg/ml, and levels of IL-1β and TNF-α are not detectable in serum [21].
2) The absence of enhancement of tumour adhesion may be explained by blockade of ICAM-1 and VCAM-1 receptors by soluble ICAM-1 and VCAM-1. *In vivo*, the function of soluble ICAM-1 and VCAM-1 is that they shed the tumour cells to block the counterligands on immunocompetent recognition lymphocytes and in that way promote metastasis development [8,9].

In addition, another interesting observation is that inflammatory cytokines such as IL-1β or TNF-α are detectable in higher concentrations ($>$1000 pg/ml) in the drainage fluid of the thorax and abdominal cavity [21]. Taking this into consideration, local tumour recurrence may be more potently influenced by locally produced factors.

The basal adhesion of colon carcinoma cells to the mesothelium, after incubation with serum a day before surgery and control human

pooled serum, varies between 20 and 60%. Variations in basal adhesion are caused by interindividual differences of mesothelial cells, which is the only variable in this model, but of the utmost importance *in vivo*. These differences of the mesothelium may lead to the interindividual variations in tumour recurrence after curative colon surgery.

In conclusion, tumour recurrence might be influenced by substances of the cocktail of intraabdominal-produced mediators, which have conflicting demeanor regarding wound healing and local tumour recurrence. In addition to the influence of these locally produced factors, this study shows that systemic factors produced directly after surgery are not a main factor influencing tumour cell adhesion. More research is required to investigate if systemic factors can stimulate distant metastasis and in which manner local tumour recurrence is influenced. Such knowledge may lead to novel therapeutic options using compounds interfering with this process.

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