INTRODUCTION

Fibrin deposits in tumor beds are intriguing phenomena. It has been suggested that fibrin plays a role as a provisional matrix in which the tumor grows and induces development of a vascular network. On the other hand, fibrin possibly protects the tumor nodule from host defense mechanisms. We therefore investigate whether tumor cells can induce a procoagulant response in endothelial cells leading to fibrin formation. For our studies we employed a modification of the matrix model of Montesano in which sprouting of endothelial cell aggregates can be followed. This system allows us to study in vitro the involvement of coagulation in tumor growth and angiogenesis.

MATERIALS AND METHODS

Endothelial cells

Bovine Capillary Endothelial cells (BCE), kindly provided by Dr M. Furie, University of New York, Stony Brook, USA were routinely cultured on gelatin-coated culture flasks (25 cm²) in Mini-
nal Essential Medium α-modification (Gibco) with 15% Donor Calf Serum (DCS) (Flow Laboratories). No other supplements were added.

Tumor cells
Colorectal tumor cell line HT29 (Fogh et al., 1977) cells were cultured in Dulbecco's Modified Essential Medium (Flow Laboratories) supplemented with 10% bovine calf serum (BCS) (Hyclone, Greiner).

In vitro coculture of endothelial and tumor cells in a collagen matrix
This system is a modification of the model of Montesano (1992).

Endothelial cells (BCE) were cultured until confluency (1.8×10⁶ cells), trypsinized and transferred to a 35 mm dish coated with 0.5% agarose to prevent adherence and promote the formation of endothelial aggregates. After 24 hours the aggregates were separated from single cells by gravitational force, followed by embedding in a collagen type I gel (Vitrogen, Collagen Corp, Palo Alto) in a 96-well plate (11 ± 5 aggregates per well) in the presence or absence of HT29 cells (300 cells per well). The tumor cells were embedded in vitrogen as single cells and during the incubation formed very young spheroids. The cultures were incubated for 48 hours in a humidified chamber at 37°C and 5% CO₂. Control cultures were, respectively, BCE aggregates or HT29 tumor spheroids alone embedded in vitrogen and incubated for 48 hours.

Morphological studies
The endothelial cell aggregate cultures with or without the tumor cells were examined under a phase contrast microscope (×250) and photographs were taken. Then the cultures were fixed in 2.5% glutaraldehyde in α-MEM buffer and processed according to standard procedures for transmission electron microscopy. Briefly, the cultures were postfixed in 1% osmium tetroxide (OsO₄) and embedded in Epon 812. Ultrathin sections were cut, counterstained with uranyl acetate and lead citrate, and evaluated with a Philips CM10 transmission electron microscope (80 keV). Micrographs were taken.

Chromogenic determination of factor X/Xa
Factor X
Per well 25 µl RVV (Russell's Viper Venom; 1 vial containing 1 mg factor X activating protein in 0.1 mmol/l NaCl, dissolved in 15 ml distilled water), 25 µl Tris buffer (0.05 mol/l, 20 mg/l polybrene, pH 7.8) and 25 µl CaCl₂ (0.01 mol/l) are added and the mixture is incubated for 15 minutes at 37°C. Then 50 µl of the chromogenic substrate S2337 (1 vial containing 30 µmol/l dissolved in 20 ml distilled water) is added to the well. After 60 minutes incubation at 37°C, 25 µl citric acid GR (4%) is added and the extinction measured at 405 nm.

Inhibition of factor X/Xa by an anti-X antibody
To identify factor X, an antibody against factor X (Anti-Human Coagulation Factor X of the Central Laboratory for Bloodtransfusion, Amsterdam, the Netherlands) was used. The endothelial cells were incubated with HgCl₂ or Iodoacetamide for 30 minutes prior to the amidolytic assay with S2337, and absorption measured at 405 nm.

Chromogenic determination of Factor VII
To determine whether tissue factor is responsible for factor X/Xa expression, we used a previously described assay (Van Dam-Mieras et al., 1992). This assay was based on the dependency of factor VII activity on tissue factor. The assay, using chromogenic substrate S2765 was carried out as described by the manufacturer (COA-Set FVII, Kabivitrum, Amsterdam, the Netherlands), with adapted incubation times for the collagen gels (incubation times were doubled).

In vitro cocultures of endothelial and tumor cells in separate compartments
In order to verify that the procoagulant vesicles were indeed originating from the endothelial cells, the following assay was performed. Endothelial cells were cultured in 6-well culture plates until confluency. In each well an insert (0.45 µm, Falcon, Becton and Dickinson BV, Etten-Leur, the Netherlands) was placed in which HT29 tumor cells were grown. Tumor cells were not able to pass through the pores within 24 hours. At different time intervals the cocultures were evaluated for the formation of vesicles.

Similarly, endothelial cell monolayers were incubated with conditioned medium from the tumor cells and evaluated for the occurrence of vesicles.

Statistics
Statistical analyses were performed using the Wilcoxon Mann Whitney test.

RESULTS
Endothelial cell aggregates (BCE cells) showed sprout formation when cultured in collagen type I gel in the presence of Donor Calf Serum, as has been described by Montesano (Fig. 1a). It has been described that the model developed by Montesano (1992) is a valid in vitro model for studying angiogenesis. When endothelial cells and tumor cells were co-cultured in the collagen gel, extracellular vesicle-like structures were found on the endothelial sprouts (Fig. 1b,c,d). No vesicles could be observed on the control sprouts, which is in agreement with the results of Montesano. These vesicles were fairly large (0.5-2 µm) and showed a remarkable resemblance to the procoagulant vesicles formed upon stimulation of erythrocytes and thrombocytes with, for instance, a calcium ionophore (Schroit and Zwaal,
Fig. 1. (A) Sprouting endothelial (BCE) aggregates in collagen type I matrix and (B) sprouting endothelial aggregates cocultured with HT29 tumor spheroids (arrows). ×200. (C) Ultrastructural micrographs show numerous microvesicles (arrows) on endothelial sprouts cocultured with tumor spheroids, with loss of the endothelial cell integrity. ×8,050. (D) Vesicles are shed from the endothelial cell membrane (arrow) ×18,550.
The latter are known to display a procoagulant character, most probably due to perturbations in the asymmetric phospholipid distribution in the membrane. We therefore investigated whether the vesicles, induced on endothelial sprouts upon coculturing with tumor cells, exhibited procoagulant characteristics. We argued that, as serum (which is a source for coagulation factors like factor X), is present in culture media, the formation of a procoagulant surface would most probably lead to the binding of coagulation factors to that cell surface and therefore we decided to search for a procoagulant effect using S2337, a chromogenic factor X substrate. The results of the factor X and factor Xa assays are summarized in Fig. 2. Upon measurement of both X and Xa, a significant \( P<0.001 \) increase of factor X/Xa expression is found for endothelial aggregates co-cultured with tumor cells. The amidolytic activity, displayed after coculture of endothelial and tumor cells, is more than the sum of the individual values from the endothelial cell aggregate cultures and the tumor cell cultures (Fig. 2). Thus, apparently, co-culturing of endothelial cell aggregates with tumor cells results in apotential increase in amidolytic activity, suggestive of factor X/Xa.

To investigate whether the vesicles indeed were originating from the endothelial cells we also studied the effect of cocultivation, using separate compartments, for endothelial and tumor cells. Also in this separate compartment system vesicle formation occurred on the endothelial cells after exposure to HT29 tumor cells. Tumor-conditioned medium alone did not induce vesicle formation.

Addition of anti-factor X antibody, prior to testing for amidolytic activity, completely blocked the factor X/Xa-like activity (Table 1). In a control experiment, we have shown that the antibodies used here inhibited human as well as bovine factor X and factor Xa. These results confirmed that indeed factor X activity is being measured in the amidolytic assay.

We then investigated whether tissue factor exposure can explain the procoagulant activity on endothelial sprouts. Tissue factor activity is assayed by a chromogenic factor VII assay. The assay is based on the dependency of factor VII activity on tissue factor. Complex formation between tissue factor and factor VII results in factor VII activation and subsequently this complex activates factor X either directly or indirectly via factor IX activation.

**Table 1. Identification of factor X with an anti-X antibody**

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<tr>
<th></th>
<th>Factor X expression</th>
<th>With anti-X</th>
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<tr>
<td></td>
<td>(i.u./ml)</td>
<td>(i.u./ml)</td>
</tr>
<tr>
<td>BCE sprouts</td>
<td>45±11.8</td>
<td>2±1.9*</td>
</tr>
<tr>
<td>BCE sprouts with HT29</td>
<td>113±18.2</td>
<td>5±3.2*</td>
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<tr>
<td>tumor cells</td>
<td></td>
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</tbody>
</table>

To identify factor X, antibody against factor X is added to the aggregates 60 minutes prior to the amidolytic assay. Absorptions are measured at 405 nm as described in Materials and Methods. Arbitrarily 100 i.u./ml is defined as the amount of factor X corresponding to an absorption value of 1.000 \( P<0.05 \).
Table 2. Chromogenic activity recorded in the factor VII assay

<table>
<thead>
<tr>
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<th>With thromboplastin addition (i.u./ml)</th>
<th>Without thromboplastin addition (i.u./ml)</th>
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<tr>
<td>Collagen gel</td>
<td>10±2.8</td>
<td>1±1.8</td>
</tr>
<tr>
<td>BCE sprouts without HT29 tumour cells</td>
<td>13.5±4.1</td>
<td>1±1.9</td>
</tr>
<tr>
<td>HT29 tumour cells in collagen gel</td>
<td>8±3.2</td>
<td>0.8±1.8</td>
</tr>
<tr>
<td>BCE sprouts with HT29 tumour cells</td>
<td>14.6±4.9</td>
<td>0.9±2.1</td>
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To determine the presence of tissue factor in the cultures, the two-step VII/VIIa amidolytic assay, as outlined in Materials and Methods, is performed. Absorptions are measured at 405 nm and arbitrarily 100 i.u./ml is defined as the amount of factor VIIa corresponding the absorption value of 1.000 (*P<0.05).

Table 2 demonstrates that, when exogenous tissue factor was added to the system, almost no factor VII activity was found, either on the collagen gels or on the different cell types. Upon addition of tissue factor a similar factor VII activity was found in all experiments. These results show that the increase in procoagulant effect found on endothelial cell sprouts after cocultivation with tumor cells could not be explained by a tissue factor exposure.

As a factor X-activating cancer procoagulant (a cysteine protease) has been described by Gordon (Gordon, 1975; Gordon and Cross, 1981; Gordon, 1987), we subsequently studied the effect of incubating endothelial aggregates with HgCl₂ and Iodoacetamide prior to the assay of factor X activity. Both the factor X and Xa expression were significantly inhibited by these compounds (Fig. 3), which indicated that the binding to the surface seemed to be dependent on the availability of -SH groups. The results do not point in the direction of a cancer procoagulant activity.

**DISCUSSION**

Upon stimulation with HT29 tumor cells, the endothelial cell aggregates formed sprouts on which vesicle-like structures were seen. In order to verify whether these vesicles were indeed originating from the endothelial cells, we also studied the effect of cocultivation, using separate compartments for endothelial and tumor cells. The results confirmed that the vesicles were originating from the endothelial cells. However, an additional effect was seen. In order to obtain vesicle formation, it was necessary that tumor cells were present in the culture system, although direct contact with the endothelial cells was not required. This suggests two possibilities. Either a highly unstable factor is continuously generated by the tumor cells or a component, that needs the presence of the tumor cell in order to exert its activity, is synthesized by these tumor cells. Further experiments will be needed to elucidate this.

The formation of these extracellular microvesicles on endothelial cells and cell sprouts induced by tumor cells in vitro, has not been described before, although vesicles shed by tumor cells themselves have been described. However, these vesicles are much smaller than the vesicles we observed (Dvorak et al., 1983; Falanga and Gordon, 1985; Gordon et al., 1975; Gordon and Cross, 1981; Gordon, 1987; Gordon and Mourad, 1991). The endothelial derived vesicles resembled the vesicles that can be formed upon activation by erythrocytes and thrombocytes. The formation of these vesicles has been associated with stimulatory events that induce a change in the asymmetric distribution of phospholipids over the inner and outer leaflet of the plasma membrane. Because of these membrane perturbations these vesicles are procoagulant in nature (Schroit and Zwaal, 1991).

Our experiments using the factor Xa-like activity assay would lend support for the procoagulant nature of the vesicles on endothelial sprouts and it is tempting to speculate that here also membrane perturbations are involved. The observation that anti-factor X antibodies completely blocked factor X activity showed that our assay specifically measured factor Xa.

We first investigated if tissue factor exposure was involved, by using the two-stage assay for factor VIIa. The results showed that the observed effect could not be explained by tissue factor exposure. We then investigated if our results could be explained by a cancer procoagulant, described as present in several tumor cell types, including HT29 (Gordon, 1987). This cancer procoagulant is a cysteine protease, having SH-groups in the catalytic site. It directly activates factor X without the involvement of the factor VII/Tissue Factor complex (Gordon, 1991). However, the results with the SH-inhibiting reagents HgCl₂ and Iodoacetamide indicated that cancer procoagulant did not provide a straightforward explanation for our results since not only Xa expression was significantly reduced after addition of HgCl₂ or Iodoacetamide but factor X expression as well. This points to an inhibiting effect of SH-modification on the interaction of factor X/Xa with the endothelial cell surface. Further experiments will be needed to elucidate the underlying mechanisms of this phenomenon.

In vivo findings support the hypothesis that procoagulant activity leading to fibrin formation not only supports haemostasis but is a relevant abnormality in malignant growth (Trouseau, 1872; Bick, 1992; Donati et al., 1981; Gordon, 1987; Paku and Paweletz, 1991; Carty et al., 1992). In many malignant situations the intravascular coagulation syndrome is manifested only at local levels and does not lead to clinically manifest symptoms. Both the hidden coagulation abnormalities and the role fibrin seems to play in tumor growth and metastasis, emphasizes the importance of in vitro studies on tumor cell-endothelial cell interactions regarding haemostatic abnormalities. In turn, this could lead to better intervention in the treatment of malignancies.

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**REFERENCES**


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