

## Hemostasis and cancer: tumor cells induce the expression of tissue factor-like procoagulant activity on endothelial cells

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**Background and Objectives.** Clotting activation and thromboembolic manifestations are common features in patients with cancer. The two-way interaction between tumor cells and host cells is of crucial importance in this context. In the present study we investigated the effect of tumor cell-endothelial cell co-culture on the expression of procoagulant activity in the mixed cell populations.

**Design and Methods.** Human tumor cell lines (HL60 promyelocytic leukemia and HeLa uterine cervical cancer) and human umbilical vein endothelial cells (HUVEC) were cultured *in vitro* according to standard procedures. Procoagulant activity was studied in a coagulometer and was found to be tissue factor-like. A calibration curve was obtained with decreasing concentrations of rabbit brain thromboplastin (RBT) and the procoagulant activity of both tumor cells and HUVEC was expressed as RBT U/10<sup>5</sup> cells.

**Results.** Before incubation procoagulant activity (mean±SE) was found to be 0.18±0.04 U in HUVEC, 9.8±1.9 U in HL60 cells, 11.9±2.2 U in HeLa cells, 7.2±1.4 U in a mixed HL60 cell-HUVEC population (ratio 2:1) and 8.5±2.0 in a mixed HeLa cell-HUVEC population (ratio 2:1). Incubation at 37°C for up to 4 hours of tumor cells or HUVEC alone did not produce any change in procoagulant activity. In contrast, co-incubation of tumor cells with HUVEC for 4 hours was followed by a significant increase in procoagulant activity of the mixed cell populations. Addition of supernatants from tumor cells, HUVEC or tumor cell-HUVEC co-cultures to HUVEC or tumor cells showed that the tissue factor-like procoagulant activity generated during coincubation was localized on HUVEC.

**Interpretation and Conclusions.** Our results show that the close interaction of tumor cells with endothelial cells may induce surface expression of tissue factor in the latter. This effect could represent an additional mechanism of clotting activation in patients with cancer.

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Key words: tissue factor, tumor cells, endothelial cells.

## Hemostasis

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Thromboembolic disorders are a common complication in cancer patients and may even be the first sign of malignancy.<sup>1-4</sup> It has been estimated that 50% of all cancer patients, and 95% of those with metastasis, exhibit abnormalities in blood clotting.<sup>5</sup> Tumor-associated activation of blood coagulation may occur by either direct (tumor cell-mediated) or indirect (host cell-mediated) mechanisms. The direct activation of blood coagulation is related to several tumor-associated procoagulant agents, including tissue factor,<sup>6-9</sup> a membrane glycoprotein which binds coagulation factor VII and cancer procoagulant, a cysteine proteinase which directly activates factor X.<sup>4,6,10</sup> On the other hand, the interaction of tumor cells with host cells may induce the expression of procoagulant activity in the latter, thus enhancing clotting activation in cancer patients. This effect has been clearly documented in tumor-associated macrophages as well as in circulating monocytes.<sup>11-12</sup>

Experimental and clinical evidence also suggests that the functions of host endothelial cells may be altered by the presence of a growing tumor. In fact, resting and activated endothelial cells were found to be increased in peripheral blood of cancer patients,<sup>13</sup> several tumor-derived factors may induce tissue factor activity on endothelial cells *in vitro*<sup>14,15</sup> and expression of tissue factor in endothelial cells within the tumor vasculature was found to be correlated with the malignant phenotype in human breast disease.<sup>16</sup> However, the mechanisms of such activation of endothelial cells by tumor cells have not yet been clearly identified.

The purpose of the present study was to elucidate whether the interaction of tumor cells and endothelial cells could induce the expression of procoagulant activity in the mixed cell populations and whether this effect is related to soluble factors released by cells in culture or requires the close interaction between the two cell types.

## Design and Methods

### Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord by digestion with 0.1% collagenase (Sigma, St. Louis, MO, USA) as previously described.<sup>15</sup> Human tumor cell lines (HL60 promyelocytic leukemia and HeLa uterine cervical cancer) were purchased from the American Type Culture Collection (Rockville, MD, USA).

All cell lines were grown in 95% air and 5% CO<sub>2</sub> in tissue culture flasks (Costar, Cambridge, MA, USA) in RPMI-1640 medium (KC Biological, Lenexa, KA, USA), supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1% streptomycin-penicillin. HL60 cells grew in suspension. Confluent monolayers of HUVEC or HeLa cells were washed three times with Ca<sup>2+</sup> - Mg<sup>2+</sup> -free Hanks' balanced salt solution (HBSS) and cells were harvested in the absence of proteolytic enzymes by exposing the monolayers to 5 mM EGTA in HBSS for 1 hour at 37°C. The cells were then washed three times in HBSS and counted in a hemocytometer. Cell viability, as determined by trypan blue exclusion, ranged between 90 and 95% for all cell lines.

### Tumor cell-endothelial cell co-culture

Cells were resuspended in pre-warmed (37°C), serum-free RPMI-1640 medium at the final concentration of 10<sup>6</sup> cells/mL and incubated for up to 4 hours at 37°C in plastic tubes. Experiments were performed by incubating each cell line alone and also by studying, in parallel, mixed cell populations, obtained by adding HL60 cells to HUVEC (ratio 2:1) and HeLa cells to HUVEC (ratio 2:1). Aliquots of the different cell suspensions were removed after appropriate incubation times (0 min, 120 min and 240 min) for the assays of procoagulant activity (see below). At the end of incubation cells were counted again; no significant change was ever observed compared to pre-incubation values, indicating that in these experimental conditions no significant adhesion of cells to the test tube occurred. Furthermore, in crossover experiments HUVEC, tumor cells or tumor cell-HUVEC co-cultures (ratio 2:1) were resuspended in serum-free RPMI-1640 medium at the final concentration of 10<sup>6</sup> cells/mL and incubated for 4 hours at 37°C. The cells were then sedimented by centrifugation at 900 × g for 10 min at room temperature. Supernatants were collected and used to resuspend HUVEC or tumor cells cultured in parallel and pelleted in a similar way. Incubation was then resumed and continued for 4 additional hours at 37°C. Procoagulant activity of HUVEC and tumor cells was determined at the end of the second incubation.

### Assay of procoagulant activity

Procoagulant activity of HUVEC, tumor cells or of mixed cell populations was studied as previously described.<sup>6</sup> Briefly, 100 μL of serum-free RPMI-1640 medium or of cell suspension (10<sup>6</sup> cells/mL in the same culture medium) were incubated with 100 μL of human normal plasma for 2 min at 37°C in an Elvi 820 coagulometer (Logos, Milan, Italy). The reaction was started by addition of 100 μL of 0.025 M CaCl<sub>2</sub> and the time required for clotting was recorded. The dependence on factor VII or factor X was assayed by using human factor VII-deficient plasma or factor X-deficient plasma (Sigma).

The procoagulant activity of all cell lines was found to be tissue factor-like and was expressed as specific activity in arbitrary units as already described.<sup>6</sup> Briefly, a calibration curve was obtained with decreasing concentrations (from 10<sup>-1</sup> to 10<sup>-6</sup>) of rabbit brain thromboplastin (RBT, Sigma) and the procoagulant activity of HUVEC, tumor cells or of mixed cell populations was expressed as RBT U/10<sup>5</sup> cells. One unit corresponds to the procoagulant activity of the 10<sup>-3</sup> dilution of RBT.

### Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) for repeated measures. A probability value of less than 0.05 was considered statistically significant.

## Results

### Procoagulant activity of tumor cells and of HUVEC

In basal conditions HL60 cells and HeLa cells were able to shorten significantly the recalcification time of human normal plasma, while HUVEC showed a much less evident effect (Table 1). Mixed tumor cell/HUVEC populations (ratio 2:1) possessed a procoagulant activity which was slightly lower than that of tumor cells alone. No activity was ever present in factor VII-deficient plasma or in factor X-deficient plasma, suggesting that the procoagulant activity of all cell lines was attributable to membrane-bound tissue factor. The activity was then expressed as RBT U/10<sup>5</sup> cells as described in the Design and Methods.

### Effect of tumor cell-HUVEC co-incubation on tissue factor expression

Incubation at 37°C for up to 4 hours of tumor cells or HUVEC alone did not produce any change in procoagulant activity (Table 2). In contrast, when tumor cells were co-incubated with HUVEC, tissue factor expression in the mixed cell populations was substantially unchanged after 120 min, but after a 4

**Table 1.** Effect of tumor cells, HUVEC and of mixed cell populations ( $10^6$  cells/mL) on the recalcification time of different human plasma substrates.

	Recalcification time (sec.)*		
	Normal plasma	Factor VII deficient plasma	Factor X deficient plasma
HL60	48-56	>300	>300
HeLa	45-55	>300	>300
HUVEC	114-155	>300	>300
HL60/HUVEC (ratio 2:1)	54-63	>300	>300
HeLa/HUVEC (ratio 2:1)	52-61	>300	>300
RPMI-1640 medium	>300	>300	>300

\*Range of values obtained with duplicate measurements in 3 independent experiments.

**Table 2.** Effect of incubation of tumor cells, HUVEC or of mixed cell populations on cell-associated procoagulant activity (RBT U/ $10^5$  cells) (means  $\pm$  S.E. of data from 5 independent experiments).

	Time of incubation at 37°C		
	0'	120'	240'
HL60	9.8 $\pm$ 1.9	10.7 $\pm$ 1.6	10.3 $\pm$ 1.9
HeLa	11.9 $\pm$ 2.2	10.8 $\pm$ 1.8	12.3 $\pm$ 2.6
HUVEC	0.18 $\pm$ 0.04	0.21 $\pm$ 0.06	0.20 $\pm$ 0.06
HL60/HUVEC (ratio 2:1)	7.2 $\pm$ 1.4	8.2 $\pm$ 1.7	13.8 $\pm$ 3.4*
HeLa/HUVEC (ratio 2:1)	8.5 $\pm$ 2.0	10.2 $\pm$ 2.5	16.8 $\pm$ 4.1**

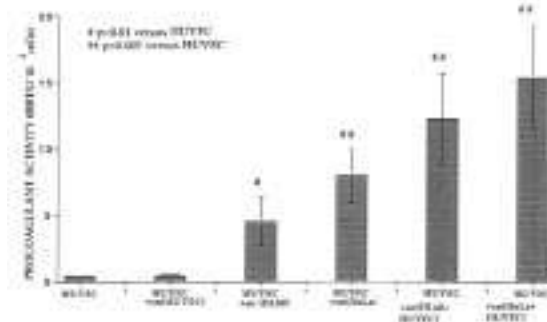
\* $p < 0.05$  versus 0' values; \*\* $p < 0.02$  versus 0' values.

hour incubation at 37°C it was significantly increased. These results demonstrate that co-incubation of tumor cells with HUVEC for an appropriate time may produce an increase in the clot promoting activity of the mixed cell populations.

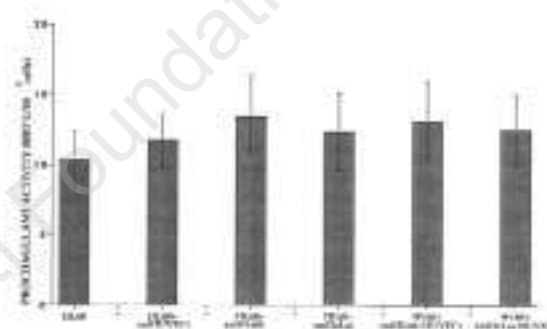
#### Localization of tissue factor generated during tumor cell-HUVEC co-incubation

As described in the Design and Methods, supernatants of tumor cells, HUVEC or tumor cell/HUVEC co-cultures were used to resuspend HUVEC or tumor cells cultured in parallel and the procoagulant activity was determined at the end of a second 4-hour incubation at 37°C.

The effects of the different supernatants on the procoagulant activity of HUVEC are reported in Figure 1. This activity was significantly increased after addition of supernatants from tumor cells incubated alone, but the increase was even greater



**Figure 1.** Effect of supernatants from HUVEC, tumor cells or tumor cell-HUVEC co-cultures on the procoagulant activity of HUVEC (means  $\pm$  S.E. of data from 5 independent experiments).



**Figure 2.** Effect of supernatants from HUVEC, tumor cells or tumor cell-HUVEC co-cultures on the procoagulant activity of HL60 cells (means  $\pm$  S.E. of data from 5 independent experiments).

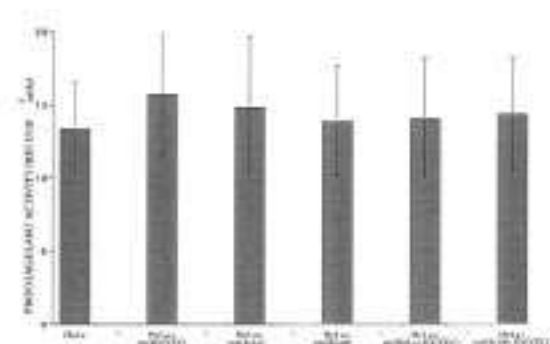
when supernatants from tumor cell-HUVEC co-cultures were used.

In contrast, supernatants from HUVEC, tumor cells or tumor cell-HUVEC co-cultures had no appreciable effect on procoagulant activity of HL60 cells (Figure 2) or of HeLa cells (Figure 3).

These data suggest that tissue factor generated during tumor cell-HUVEC co-incubation is localized on endothelial cells.

#### Discussion

Our study has shown that the procoagulant activity of the cell lines investigated was exclusively related to membrane-bound tissue factor and that in basal conditions HUVEC showed a very low procoagulant capacity with the same characteristics. Tissue factor expression on both tumor cells and HUVEC was unchanged when the cells



**Figure 3.** Effect of supernatants from HUVEC, tumor cells or tumor cell-HUVEC co-cultures on the procoagulant activity of HeLa cells (means $\pm$ S.E. of data from 5 independent experiments).

were incubated alone for up to 4 hours, indicating that in these experimental conditions no spontaneous activation of cells occurred. As far as endothelial cells are concerned, our data confirm the results of a previous study,<sup>17</sup> in which cell associated procoagulant activity was found to be low and stable when these cells were incubated alone.

Our study has also shown that co-incubation of tumor cells with HUVEC for 4 hours is followed by a significant increase in procoagulant activity of the mixed cell populations. This activity was identical to that observed in basal conditions, i.e. it was totally dependent on factor VII (*data not shown*). Therefore, although tissue factor mRNA levels were not measured, this procoagulant activity may be attributed to membrane-bound tissue factor.<sup>6</sup> Different hypotheses may be put forward to explain this effect. Tumor cells are known to produce and release various cytokines, including interleukin (IL)-1 and tumor necrosis factor (TNF), which can induce procoagulant activity on endothelial cells.<sup>11</sup> Furthermore, several other tumor cell-derived factors have been identified which demonstrate pleiotropic activities on endothelial cells and may induce upregulation of tissue factor expression and potentiation of TNF activity; these include vascular endothelial growth factor,<sup>18</sup> endothelial-monocyte activating peptides I and II,<sup>14</sup> and other less well characterized agents.<sup>15</sup> On the other hand, endothelial cells activated by IL-1 or TNF are themselves the source of different cytokines, including various chemokines, colony-stimulating factors, IL-6 and IL-1 itself;<sup>19</sup> this can lead to amplification of cytokine release in tumor cell-HUVEC co-cultures with further activation of endothelial cells and pos-

sibly also of tumor cells themselves, as it is known that expression of tumor cell procoagulant activity may be increased by cytokines such as TNF, interferon (IFN)- $\alpha$  or IFN- $\gamma$ .<sup>20</sup>

The great complexity of the two-way interaction between tumor cells and endothelial cells and the consequent possible modulation of procoagulant activity of both cell types may well explain why in previous studies the mechanisms responsible for the increase in procoagulant activity of the mixed cell populations remained largely obscure.

The crossover experiments presented in this paper may help to clarify some of these issues. Supernatants of tumor cells cultured alone produced a significant increase in procoagulant activity of HUVEC, but the increase was even greater when supernatants from tumor cell-HUVEC co-cultures were used. In contrast, tumor cell procoagulant activity was not significantly modified by addition of supernatants from HUVEC, tumor cells or tumor cell-HUVEC co-cultures. These data demonstrate that upregulation of tissue factor expression on endothelial cells is responsible for the increase in procoagulant activity generated during tumor cell-HUVEC co-incubation. Evaluation of tissue factor pathway inhibitor on endothelial cells before and after their interaction with tumor cells could be of importance to elucidate the mechanisms responsible for modulation of tissue factor activity and may be the subject of future investigations. In any case, we have clearly shown that products released by tumor cells in culture are partly responsible for the increase in procoagulant activity, but that close interaction between the two cell types is essential for a complete activation of endothelial cells to occur, which requires cell-cell contact and cross-talk. Although the nature of cytokines or other factors involved remains to be determined, our observation is of interest because it could offer an explanation for the enhanced expression of tissue factor activity found on the endothelium within tumor vasculature,<sup>9</sup> where a close interaction exists between tumor cells and endothelial cells.

One can also speculate that overexpression of tissue factor inside tumor microvessels could even affect tumor growth, as it is known that tissue factor may play an important role in angiogenesis and in cancer progression.<sup>21-23</sup>

In conclusion, co-incubation of tumor cells with endothelial cells may induce surface expression of tissue factor in the latter. Products released by tumor cells in culture are partly responsible for this effect, but the close interaction between the two cell types leads to further upregulation of tissue

factor expression on endothelial cells. This effect could represent an additional mechanism of clotting activation in patients with cancer and could even affect tumor progression and angiogenesis.

#### Contributions and Acknowledgments

AM was responsible for the conception and design of the investigation. AT performed the laboratory experiments. AM and AT wrote the manuscript. GG revised it critically for important intellectual content and was responsible for final approval of the version.

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#### Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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#### PEER REVIEW OUTCOMES

##### Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Vicente Vicente, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Prof. Vicente and the Editors. Manuscript received January 10, 2002; accepted April 11, 2002.

##### What is already known on this topic

There is much evidence indicating that tumor cells and host cells interact to induce clotting activation in patients with cancer.

##### What this study adds

This study investigates the effect of tumor cell-endothelial cell coculture on the expression of procoagulant activity (tissue factor) on the endothelial cells.

##### Potential implications for clinical practice

These *in vitro* data suggest that the close interaction of tumor cells with endothelial cells could represent a mechanism of clotting activation in patients with cancer.

Vicente Vicente, Deputy Editor