Expression of tissue factor pathway inhibitor (TFPI) in human breast and colon cancer tissue

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Summary

Activation of blood coagulation, a phenomenon frequently observed in breast and colon cancer patients, contributes to tumour progression. The principal initiator of blood coagulation activation in cancer patients is tissue factor (TF), while tissue factor pathway inhibitor (TFPI) is the main inhibitor of the TF-dependent pathway of blood coagulation. Previous immunohistochemical studies revealed no expression of TFPI in human cancer cells. The aim of the study was to evaluate the expression of TFPI protein and mRNA in breast and colon cancer tissues. A total of 108 cancer tissues (from primary tumours and metastatic lymph nodes) were obtained from 87 patients during surgical treatment. Immunohistochemical studies using a polyclonal anti-TFPI antibody were performed including a semiquantitative analysis. The in situ hybridisation method employed single-stranded DNA oligonucleotide (probe sequence: 5’Biotin-CCACCATACTTGAAACGTTCACACT-Biotin3’) directed against TFPI mRNA. Strong or medium expression of TFPI protein was observed in cancer cell bodies in all breast cancers and in most (39/66 cases) colon cancers examined. Weaker expression of TFPI was detected in cancer cells localised in lymph node metastatic foci of breast cancer. Endothelial cells were also TFPI-positive. TFPI mRNA was demonstrated in all cases of breast and in approximately 80% cases of colon cancer cells. TFPI mRNA and protein are present in association with colon and breast cancer cells, suggesting that the protein may play a role in cancer biology. The presence of TFPI in association with breast cancer cells localised in regional lymph nodes may indicate its role in lymphatic spread.

Keywords

Breast cancer, coagulation inhibitor, colon cancer, TFPI, tissue factor pathway inhibitor

Introduction

Abnormalities in the haemostatic system laboratory tests are frequent findings in patients suffering from malignancy (1, 2). Activation of blood coagulation derived from cancer-dependent stimuli leads to increased incidence of thromboembolic complications in cancer patients (1–7). Colon and breast cancers belong to the most common malignancies in humans. Hypercoagulability in cancer patients may be, at least in part, a result of inadequate activity of coagulation inhibitors.

One of the most important inhibitors of blood coagulation is tissue factor pathway inhibitor (TFPI), a member of Kunitz-type serine protease inhibitor superfamily (8). TFPI is synthesised by endothelial cells (ECs) of small blood vessels (4, 8–11). However, TFPI mRNA was also demonstrated in placenta, lung, liver, heart, kidney, skeletal muscles, pancreas and brain (4, 12). Small amounts of TFPI are synthesised by activated macrophages, monocytes and fibroblasts (10). In humans, the major portion of TFPI is associated with the EC membrane through a glycosyl phosphatidylinositol (GPI)-anchored protein in a manner not dependent on glycoaminoglycans (GAGs) (13, 14). In a smaller proportion it binds to plasma lipoproteins. It is also stored in blood platelets, and circulates in plasma in a free form (12, 14). TFPI inhibits the activity of both coagulation factor Xa and tissue factor/factor VIIa (TF/VIIa) complex (15). Patients with various neoplasms, especially at an advanced stage of the disease, exhibited increased plasma concentrations of TFPI in comparison with healthy subjects (16–21). Interestingly, heparin administration leads to higher release of TFPI in cancer patients than in subjects who do not suffer from malignancy (18). Moreover, an increased content of factor Xa/TFPI complex was demonstrated in the plasma of patients with solid tumours in comparison to healthy individuals (20). High plasma TFPI concentration may indicate a host compensatory mechanism resulting from a hypercoagulable state in cancer patients. Anticancer treatment (surgery, radiotherapy or chemotherapy) commonly leads to the normalisation of TFPI plasma levels (21, 22).
**Objectives**

The role of TFPI in the pathophysiology of cancer is not completely defined yet. Previous immunohistochemical (IHC) studies performed by other investigators revealed no expression of TFPI in different human cancer cells, whereas cancer cells in experimental models were characterised by TFPI expression (11, 23). The present study was conducted to analyse the presence of TFPI mRNA and protein in situ in cancer tissue fragments obtained from two of the most commonly diagnosed malignant tumours in humans – colon and breast cancer. In the case of breast cancer, TFPI expression was investigated in both primary tumours and regional lymph node metastases.

**Materials and methods**

A total of 108 tissue fragments were obtained at surgical treatment of 87 previously untreated patients with breast or colon cancer, fixed in a buffered 4% formalin solution and embedded in paraffin.

Studies were performed on:

- 21 fragments of primary breast cancer and 21 fragments of regional axillary node metastatic breast cancer tissue obtained from the same patients (invasive ductal carcinoma: G1 histopathologic grade of malignancy – 2, G2 – 9 and G3 – 24 cases, respectively)
- primary colon cancer – 66 cases (adenocarcinoma: G2 histopathologic grade of malignancy – 47 cases and G3 – 19 cases, respectively).

Control fragments of respective normal tissues derived from the tumour-free surgical margins. Microwave oven (600W) pretreatment in citrate buffer (pH=6) for 3 minutes for antigen retrieval was performed. Polyclonal antibody against homogeneous recombinant human TFPI (24) was produced in rabbits, and anti-TFPI IgG was purified from immune sera by protein A-Sepharose chromatography. By immunoblotting, the anti-TFPI IgG failed to recognise human recombinant TFPI-2.

Of note, the anti-TFPI IgG failed to recognise human recombinant TFPI-2.

The staining procedures and controls for the avidin–biotin complex technique (ABC) using reagents (Vectastain Kits, Vector Laboratories, Burlingame, CA, USA) were reported previously (26). Antibodies were tested on control and tumour tissues at concentrations that provided maximum staining intensity with minimal background staining. Controls consisted of omission of the primary antibody from the procedure. The results of staining of the above neoplastic tissues were compared with respective normal tissues processed simultaneously. Antigen staining was detected by the dark brown reaction product that appeared with antibody labelling in the ABC immunostaining procedure. Immunohistochemically TFPI-positive specimens (12 cases from each localisation, totalling 36 cases) were further evaluated for the presence of TFPI mRNA by the in situ hybridisation (ISH) method. Single-stranded DNA oligonucleotide (probe sequence: 5’Biotin-CCACCATCTTGAAC-GTTCACACT- Biotin3’) directed against locus NM 006287 of TFPI mRNA was employed. The probe was synthesised by Sigma-Aldrich (Poznan, Poland). The ISH protocol of R&D Systems (R&D Systems, Minneapolis, MN, USA) was utilised. Hybrids were detected with a rabbit anti-biotin monoclonal antibody according to the ABC technique as in the IHC studies mentioned above (26) associated with ImmunoMax amplification technique (27, 28). Negative controls included hybridisation without addition of the molecular probe and incubation of slides in RNase A solution (R&D Systems) before hybridisation. Reaction results were visualised as a dark brown reaction product.

By itself, IHC does not allow a quantitative survey. A semiquantitative analysis of TFPI IHC expression was performed according to the Remmele and Stegner scale (29) with our own modification in order to verify IHC findings (►Table 1). Numerical values were assigned to both percentage of cancer cells with positive staining (A) and the intensity of staining (B). The immunoreactive score (IRS) was a product of the multiplication of both values (IRS = A x B). The results ranged from 0 to 12 (Table 1). IRS was assessed exclusively for cancer cells. IRS value ranges between 1–4, 5–8, and 9–12 were interpreted as weak, medium and strong expression of TFPI, respectively. Visual assessment of the protein expression was performed in 10 random high-power fields. The specimens were assessed by two independent blinded observers. The study protocol was approved by local Ethics Committee at Medical University, Bialystok, Poland. The informed content was obtained from the patients.

**Results**

**IHC studies**

**Colon cancer**

TFPI expression was detected in association with cancer cells in most cases of colon cancer (approx. 70%) (►Fig. 1A). However, the intensity of TFPI staining was heterogeneous (►Table 2). The vast majority of TFPI-positive colon cancer cells (approximately

<table>
<thead>
<tr>
<th>A value</th>
<th>Percentage of cancer cells with positive staining</th>
<th>B value</th>
<th>Intensity of cancer cells’ staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no staining</td>
<td>0</td>
<td>no staining</td>
</tr>
<tr>
<td>1</td>
<td>up to 10%</td>
<td>1</td>
<td>weak</td>
</tr>
<tr>
<td>2</td>
<td>11% – 50%</td>
<td>1.5</td>
<td>weak and medium</td>
</tr>
<tr>
<td>3</td>
<td>51% – 80%</td>
<td>2</td>
<td>medium</td>
</tr>
<tr>
<td>4</td>
<td>more than 80%</td>
<td>2.5</td>
<td>medium and strong</td>
</tr>
<tr>
<td></td>
<td>(12 cases from each localisation, totalling 36</td>
<td>3</td>
<td>strong</td>
</tr>
<tr>
<td></td>
<td>cases)</td>
<td></td>
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90%) were characterised by medium or strong IRS value. More intense staining was visualised in less malignant neoplasms. However, in contrast to the above, in one case of G3 colon cancer, stronger expression was detected as well. Larger clusters of cancer cells were TFPI-negative. Expression of TFPI was also demonstrated in ECs of small blood vessels. There was either no or merely subtle expression of TFPI in normal epithelium of the colon (Fig. 1D).

Breast cancer
Cancer cells of all primary breast cancer tissues examined were characterised by strong (IRS 9–12, more than 60% of cases) or medium (IRS=5–8, approx. 40% of cases) expression of TFPI (Table 3, Fig. 1B). The expression of TFPI was particularly pronounced in small foci of cancer cells and at the host-tumour interface. Of note, breast cancer cells in regional lymph nodes exhibited positive staining for TFPI than cancer cells from the primary tumour, although with weaker intensity (Table 3, Fig. 1C). Namely, weak (IRS=1–4, approx. 30% of cases) and medium (IRS=5–8, approx. 70% of cases) TFPI expression was observed in association with cancer cells, whereas no strong expression of TFPI was revealed at all (Table 3). Furthermore, strong expression of TFPI was demonstrated in ECs lining small blood vessels that supplied tumour tissue. TFPI antigen was also detected in tumour infiltrating macrophages (TAMs). TFPI was present in normal breast tissue as well; however, its expression was much less intense (Fig. 1E). No expression of TFPI was detected in normal lymphatic tissue (Fig. 1F).

Figure 1: Specific staining by the ABC peroxidase technique using polyclonal antibody against TFPI. Solid line arrows show staining (brown reaction product) for TFPI of tumour cell bodies in G2 colon cancer primary tumour (A), G2 breast cancer primary tumour (B), G2 breast cancer metastases in axillary lymph node (C). No staining for TFPI was observed in normal colon tissue (D) and normal lymph node tissue (F), whereas normal breast tissue (E) revealed slight staining for TFPI (light brown). Dotted arrows indicate staining for TFPI in association with endothelial cells in colon (A) and breast (B) tumour tissue. Hematoxylin counterstain; original magnification x 400 (A, B), x 200 (C, D, F) or x 100 (E).
ISH studies

TFPI mRNA was detected in cancer cell bodies in all primary tumour breast cancer cases and tumour cells of metastatic lymph nodes examined (Fig. 2A). Most of the colon cancer specimens examined (10/12, approx. 80%) were found to express mRNA for TFPI, implying induced synthesis of the protein by cancer cells (Fig. 2A). Respective normal tissues were TFPI mRNA-negative (Fig. 2D-F).

Discussion

Activation of blood coagulation in cancer patients proceeds not only intravascularly, but also extravascularly at the tumour site (2). The presence of components of the TF-dependent pathway of blood coagulation (e.g. TF, factor VII, factor X) was observed in colon and breast cancer tissues (30–32). The activity of the aforementioned factors is believed to contribute to regulation of the processes of apoptosis, cell migration, angiogenesis and metastasis formation (3, 33–37). Furthermore, activation of blood coagulation leads to thrombin generation and fibrin formation, both of which facilitate tumour growth through several interrelated mechanisms (2, 30, 38).

Data concerning TFPI expression in loco in tumour tissue are contradictory. Namely, Werling et al. (11) demonstrated no TFPI presence in cancer cells of colon, breast and renal cancer, non-small cell lung cancer and lymphoma tissue. However, the authors did not report a number of cases on which the study was performed (11). In contrast, TFPI mRNA and protein was observed in colon, breast and pancreatic cancer cell lines (23). Similar to the latter in vitro experiments, the present study revealed the presence of TFPI antigen in cancer cells in most cases (45/66) of colon cancer and in all cases of breast cancer. Methodological discrepancies among IHC studies may be responsible for the different results concerning the presence of TFPI in cancer tissue. In the present study, in contrast to the earlier study mentioned above (11), an antigen retrieval method was included, which allowed unrestricted antigen access for the antibody. Furthermore, a large number of the examined tissues (in total 108 fragments) provides more reliable results. TFPI associated with cancer cells derives from different sources. Namely, it may be of blood-borne origin or be synthesised by cancer cells themselves. ISH studies were conducted to attempt to resolve this issue. Interestingly, TFPI mRNA was demonstrated in cancer cells of breast cancer and colon cancer, which may suggest a role for TFPI in the biology of the neoplasms. Of interest, experimental in vitro studies revealed that colon cancer cell lines exhibiting a high potential of metastatic dissemination to the liver were characterised by low TFPI content, along with high TF mRNA, protein expression and activity (39). However, elevated expression of TF, together with normal expression of TFPI in a metastatic colon cancer cell line, was also documented (39). It is conceivable that TFPI activity may, at least in part, counterbalance TF and Xa biological activities since its presence was documented in 70% of colon cancer cases and in all cases of breast cancer. Despite the fact that TFPI has no effect on cancer cell proliferation (40), it is possible that the inhibitor may stimulate local cancer progression. Of particular interest are results of a study demonstrating ECM-bound TFPI via an interaction with TF/VIIa complex localised on cancer cells that facilitated tumour cell adhesion and migration (41). In a highly aggressive melanoma, it was demonstrated that the procoagulant function of TF is regulated by TFPI and this activity is essential for perfusion of vasculogenic mimicry channels formed by TF-expressing melanoma cells, which play an important role in supplying blood for the growing tumour (42). The presence of TFPI was also demonstrated in ECs of small blood vessels supplying neoplasms and in TAMs. Such localisation of the protein was reported previously (11) and may reflect a counterbalance mechanism, secondary to activation of coagulation observed in neoplastic tissue (2, 30). Furthermore, the presence of TFPI in association with ECs is not surprising since ECs of small blood vessels are the main source of TFPI synthesis (9, 10). It is noteworthy that thriving angiogenesis is a prerequisite for successful tumour growth, and newly formed blood vessels consist only of EC linings (3). Thus, TFPI may inhibit angiogenesis indirectly through the inhibition of TF proangiogenic activity, or directly, since TFPI was demonstrated to inhibit ECs motility and formation of capillary-like structures by the ECs (43). Activated macrophages were also documented to synthesise TFPI (9, 11). Keeping in mind that TAMs provide their components to form a complete coagulation pathway, the presence of TFPI may reflect a response to activated blood coagulation in the close vicinity of TAMs.

Table 2: Semiquantitative analysis of immunohistochemical expression of TFPI in colon cancer cells.

<table>
<thead>
<tr>
<th>TFPI expression</th>
<th>IRS value</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Weak</td>
<td>1–4</td>
<td>6</td>
</tr>
<tr>
<td>Medium</td>
<td>5–8</td>
<td>29</td>
</tr>
<tr>
<td>Strong</td>
<td>9–12</td>
<td>10</td>
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Table 3: Semiquantitative analysis of immunohistochemical expression of TFPI in primary tumour breast cancer cells and tumour cells of metastatic foci in the axillary lymph nodes.

<table>
<thead>
<tr>
<th>TFPI expression</th>
<th>IRS value</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumour</td>
<td>(n=21)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Weak</td>
<td>1–4</td>
<td>6</td>
</tr>
<tr>
<td>Medium</td>
<td>5–8</td>
<td>15</td>
</tr>
<tr>
<td>Strong</td>
<td>9–12</td>
<td>0</td>
</tr>
<tr>
<td>Axillary metastatic foci (n=21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Weak</td>
<td>1–4</td>
<td>6</td>
</tr>
<tr>
<td>Medium</td>
<td>5–8</td>
<td>15</td>
</tr>
<tr>
<td>Strong</td>
<td>9–12</td>
<td>0</td>
</tr>
</tbody>
</table>
The absence or weak expression of TFPI in some tumour cells of colon cancer may indicate a relative deficiency of TFPI in some areas of nascent tumours, which may facilitate blood coagulation activation. In this regard, of interest is the observation regarding the presence of TFPI in neoplastic cells, particularly in more differentiated colon cancers in contrast to less differentiated ones (exhibiting more aggressive phenotype). It cannot be excluded that the heterogeneous TFPI expression in association with cancer cells may result from the lack of appropriately formed TF/factor VIIa/factor Xa complex, which is a prerequisite for TFPI binding. In fact, non-uniform TF expression in breast and colon cancer cells was reported by others (30–32).

Interestingly, plasma TFPI was demonstrated to exert an anti-metastatic effect in experimental studies (40, 44). The dominant effect of TFPI is exerted at an early phase of metastatic dissemination when cancer cells are circulating in the bloodstream. TFPI may also inhibit TF-driven metastases (45). Tumour cell TF-mediated thrombin generation and tumour cell-induced platelet activation and aggregation (TCIPA) contribute to metastatic spread via various mechanisms (46, 47). One of the mechanisms of TFPI anti-metastatic function results from neutralisation of TF/VIIa or TF/VIIa/Xa complex activity (41).

The dissemination of cancer cells may proceed either through blood-borne metastases or spreading to regional lymph nodes via lymphatic ducts. Therefore, an attempt was made to assess the TFPI expression in the lymph node metastases. Interestingly, the protein was revealed in association with cancer cells in axillary lymph node metastatic foci of breast cancer, which may also sug-
What is known about this topic?
- Many blood coagulation components play a role in cancer progression.
- In colon and breast cancer patients increased plasma concentrations of tissue factor pathway inhibitor (TFPI) were detected in comparison with healthy subjects. It was particularly pronounced in patients presenting at an advanced stage of the disease.
- Data on the presence of TFPI in human cancer tissue, in relation to experimental models, are not consistent. Previous immunohistochemical studies performed by other investigators revealed no expression of TFPI in cancer cells of different malignant tumor types, whereas cancer cells in experimental models were characterised by TFPI expression.

What does this paper add?
- The presence of TFPI mRNA and expression of the protein in association with neoplastic cells of human colon and breast cancers suggests its role in cancer biology.
- The presence of TFPI in association with breast cancer cells localised in regional lymph nodes may indicate its role in lymphatic spread.

Conclusions
The presence of TFPI mRNA and protein in colon and breast cancer tumour cells suggests that the protein may play a role in cancer biology. The presence of TFPI in breast cancer cells localised in regional lymph nodes may point to a role of the protein in lymphatic spread.

References