

In situ detection of tissue factor in vascular endothelial cells: Correlation with the malignant phenotype of human breast disease

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Expression of tissue factor (TF) in the endothelium has been observed only rarely in human disease and has been thought to be elaborated on the surface of vascular endothelial cells (VECs) *in vitro* as an artifact of tissue culture. Using monoclonal antibodies and a novel probe for functional TF, we have localized TF to the VECs (and tumor cells) within the tumors of seven patients with invasive breast cancer but not in the VECs (or tumor cells) of benign tumors from ten patients with fibrocystic disease of the breast. The potent procoagulant TF was shown to be a marker of the initiation of angiogenesis in human breast cancer. Further evidence that the TF was the demonstration of a similar distribution of cross-linked fibrin only in the VECs of the malignant tumors. We interpret these data as further support for the concept that tumor cells can activate nearby VECs and regulate blood vessel growth *in vivo*. Large clinical-pathologic studies will be necessary to determine whether TF is a useful marker for the "switch to the angiogenic phenotype" in human breast disease and/or correlates with the thromboembolic complications of breast cancer.

Substantial evidence supports the existence of a complex network of positive and negative regulatory signals for the control of angiogenesis in human tumors, which can be derived from the tumor cells themselves, from tumor-associated host macrophages and/or from the extracellular matrix¹. Nevertheless, the sequence of events that leads to the "switch to the angiogenic phenotype" in a previously dormant *in situ* tumor remains largely unknown. Furthermore, in spite of the apparent negative prognostic significance of identifying new blood vessels in such human tumors as breast cancer²⁻⁵, prostate cancer⁶ and brain tumors⁷, most clinical studies have been forced to rely on the rather crude technique of counting microvessels for the quantification of angiogenesis¹.

Tissue factor (TF) is a lipid-dependent glycoprotein that serves as the principal cell-surface receptor for blood coagulation factor VII (and its activated enzymatic form factor rVIIA). As such, TF is the primary regulator of the activation of mammalian blood coagulation⁸. Patients with breast cancer have an increased risk for the development of thrombosis, particularly during hormonal manipulation and/or chemotherapy (reviewed in ref. 9). The mechanisms for the production of this "hypercoagulable state" are unknown, although the neovascularity characteristic of this type of cancer has been suggested as a possible target for tumor-associated vascular endothelial cell (VEC) injury and subsequent thrombosis. Previous studies of tumor tissue from patients with

breast cancer have demonstrated that fibrin deposition occurs principally at the newly vascularized leading edge of the tumor in association with areas of developing necrosis¹⁰. It has also been suggested that this fibrin deposition is a result of increased TF expression within the tumor and associated vasculature, although direct evidence for this has been lacking. Characterization of TF in human blood vessels has been accomplished principally in VECs grown *in vitro*¹¹⁻¹⁹. However, it remains highly controversial as to whether the results of these *in vitro* studies of passaged VECs, in which TF procoagulant activity remains cryptic in the membrane until the cells are "activated" (for example, by high concentrations of cytokines such as interleukin-1 β (IL-1 β) or tumor necrosis factor- α (TNF- α), or by Gram-negative bacterial endotoxin or lipopolysaccharide (LPS)), can be extrapolated to *in vivo* events. Several investigators have drawn attention to the lack of expression of TF in the vascular endothelium in normal vessels and atherosclerotic vessels^{20,21} and the relative resistance of the vascular endothelium to induction of TF even during fatal *Escherichia coli* sepsis in an experimental model²². Although TF mRNA and/or antigen have been identified in the adventitial layer and in mesenchymal-appearing cells of the large blood vessels in some of these studies²⁰⁻²² and in occasional VECs of the placenta²³, little evidence has been presented as to the potential for TF expression in small vessel endothelial cells in either normal or pathologic tissue. Indeed, in baboons injected with *E. coli*,

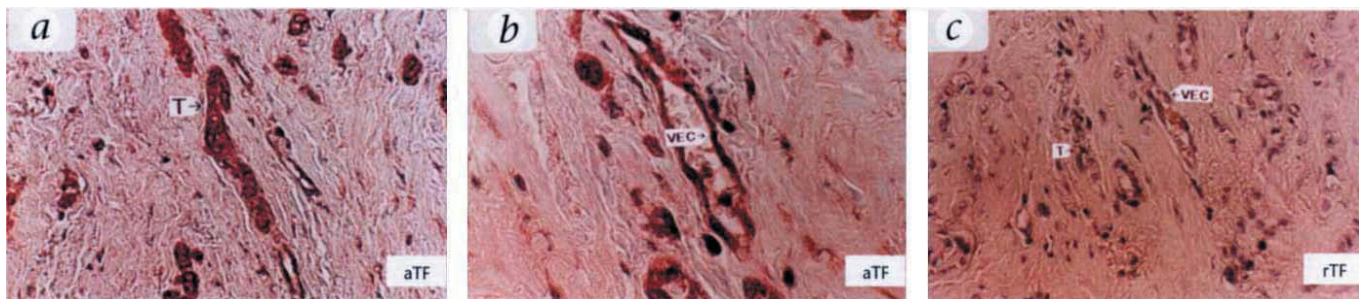


Fig. 1 *In situ* detection of tissue factor antigen in invasive breast cancer. Tissue sections, processed as described in Methods, reacted with a mAb to human TF (aTF), demonstrating in *a*, TF in the tumor cells (T), $\times 40$ and *b*, TF in the VECs, $\times 40$. *c*, specificity control, antihuman TF mAb preabsorbed with $10\times$ molar excess of rTF, $\times 40$.

only splenic VECs were observed to express TF antigen²², supporting the theory that VECs *in vivo* do not contain TF except under unusual conditions.

Very recently, Zhang *et al.* demonstrated that transfecting an antisense DNA sequence for the TF gene into Meth-A sarcoma cells resulted in tumor cells incapable of developing TF procoagulant activity, a marked reduction in their ability to secrete vascular endothelial growth factor (VEGF) *in vitro* or *in vivo* and in tumors with a markedly reduced angiogenic response when implanted in immunodeficient mice²⁴. Their intriguing data suggest the possibility that TF expression in tumors may have a direct and/or indirect effect on neovascularization. Earlier studies from our laboratory of clotting protein deposition *in situ* in patients with lung cancer demonstrated that both tumor cells and tumor-associated macrophages (TAMs) expressed TF, whereas cross-linked fibrin (XLF) was associated predominantly

with the TAMs (ref. 25). In some specimens, however, TF appeared to be expressed in the intima of tumor-associated blood vessels, raising the question as to whether VEC tissue factor generation might also be a marker for the "switch to the angiogenic phenotype." To test this hypothesis, we examined both antigenic and functional TF in human breast tissue obtained from patients with malignant or benign breast disease. Small vessel VECs were identified by their staining for von Willebrand factor (vWF). We demonstrated the presence of TF in human VECs (in association with vWF) in tissue from patients with invasive breast cancer but not in benign fibrocystic disease. In addition, tumor cell expression of TF also appeared to have specificity for the malignant phenotype, because we were unable to observe significant antigenic or functional TF in benign breast tumors. The distribution of TF paralleled that of XLF in the malignant tumors and was not observed in the benign tumors. These results suggest the possibility that VEC tissue factor, in addition to its role in the pathophysiology of the clotting abnormalities in breast cancer, may be useful as a diagnostic and prognostic marker in human breast tumors.

Von Willebrand factor antigen expression in breast disease

We began our studies by localizing small-vessel VECs in both infiltrating breast cancer and fibrocystic disease of the breast. A highly specific marker for VECs, von Willebrand factor (vWF) antigen, was demonstrated readily in the cells lining the small vessels in sections of both infiltrating breast cancer and fibrocystic disease of the breast (data not shown). Nonimmune rabbit serum, which served as the negative control for these experiments, produced no staining of the VECs in the tissue. It should be noted that in all tumors examined (three out of seven), vWF antigen was demonstrated consistently and only in association with the VECs.

Tissue factor antigen expression in breast disease

Tissue factor antigen was detected consistently in both the tumor cells (Fig. 1*a*) and in the endothelial cells lining tumor-associated vessels (Fig. 1*b*) by immunohistochemical staining of formalin-fixed sections of infiltrating breast cancer. All seven tumors stained for TF antigen with a similar distribution. Preabsorption of the TF monoclonal antibody with $10\times$ molar excess of recombinant TF (rTF; as a specificity control) eliminated the staining of TF in the tumor cells and in the VECs (Fig. 1*c*). No TF antigen was detected in tissue from ten patients with benign, fibrocystic disease of the breast. A representative section is illustrated in Fig. 2. Neither the hyperplastic ductal epithelium (Figs. 2, *a* and *b*) nor the VECs lining the vessels associated with this benign breast tumor (Fig. 2*c*) contained demonstrable TF antigen.

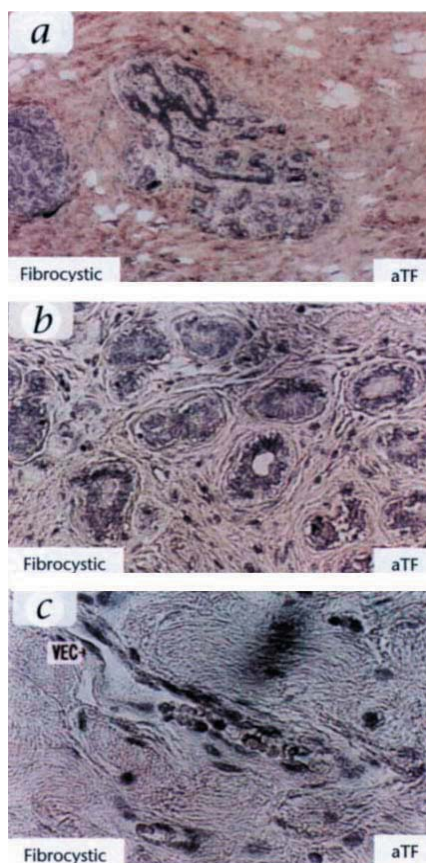
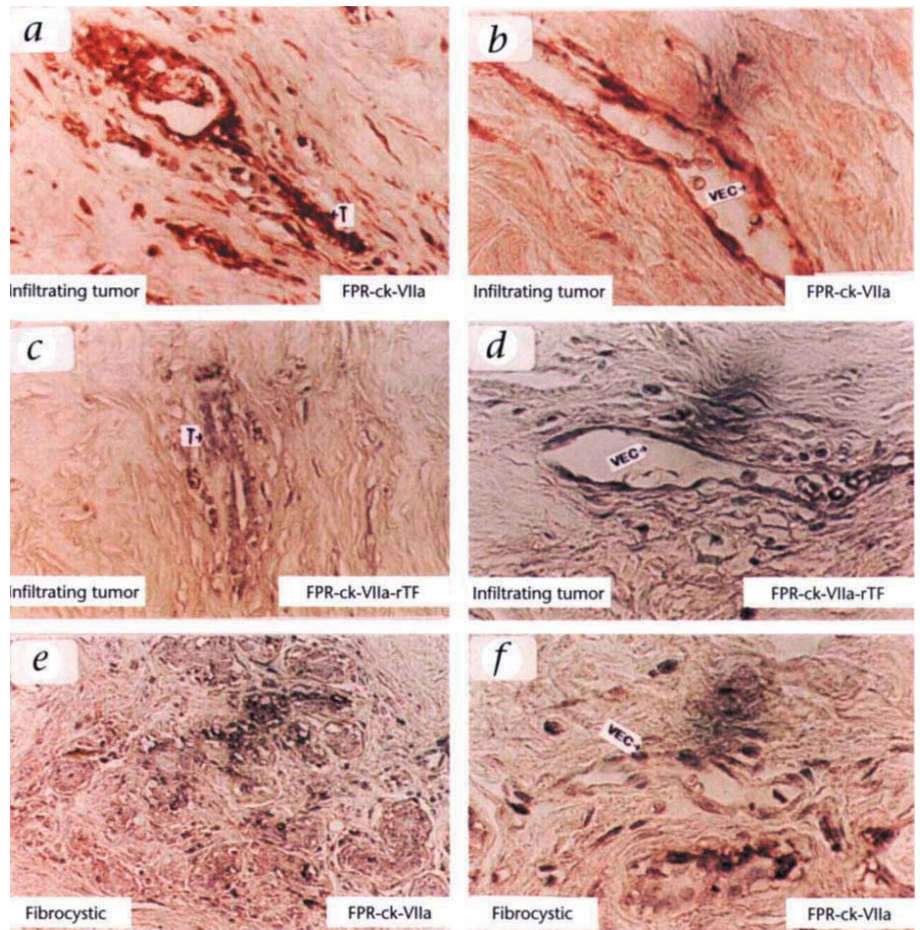


Fig. 2 *In situ* detection of tissue factor antigen in benign fibrocystic disease of the breast. Tissue sections were processed as in Fig. 1, using the same TF mAb (aTF). No TF antigen was observed in either the proliferating glandular epithelium (*a*, $\times 20$; *b*, $\times 40$), or in the VECs of the breast tissue (*c*, $\times 40$).

Fig. 3 *In situ* detection of tissue factor functional sites in invasive breast cancer and benign fibrocystic disease of the breast. Tissue sections were processed as in Figs 1 and 2, modified for the binding of the biotin-labeled, FPR-ck-rVIIa probe, as described in Methods. *a*, FPR-ck-rVIIa binding to the infiltrating tumor cells (T), $\times 40$. *b*, FPR-ck-rVIIa binding to the VECs within the infiltrating tumor, $\times 40$. *c* and *d*, Specificity controls for the infiltrating tumor: FPR-ck-rVIIa preabsorbed with $10\times$ molar excess of rTF, $40\times$ of tumor cells (T) and VECs, respectively; no significant binding of the FPR-ck-rVIIa was observed to either the glandular epithelium (*e*) or the VECs (*f*) of tissue involved by benign fibrocystic disease of the breast, $\times 40$.



Localization of functional tissue factor in breast tumors

To determine whether the TF expressed by both the tumor cells and VECs in invasive breast cancer was functional, that is, capable of promoting fibrin formation, we utilized our recently described, novel probe, Phe-Pro-Arg-chloromethyl-ketone-labeled recombinant factor VIIa (FPR-ck-rVIIa)²⁶. This probe was designed to take advantage of the property of blood coagulation

factor VIIa (fVIIa) as a high-affinity, specific ligand for cellular TF (ref. 27). We reasoned that demonstration of binding of this probe to cells *in situ* confirmed the functional activity of the TF; the results of specificity controls for this probe are described in detail elsewhere²⁶. As demonstrated in Fig. 3, the distribution of FPR-ck-rVIIa staining in the infiltrating breast cancer was virtually identical to that of antigenic TF observed with

Fig. 4 Immunohistochemical localization of cross-linked fibrin (XLF) in invasive breast cancer. All three of the fibrin-specific mAbs, ADI 352, T2G1 and GC4 gave similar results; only the samples probed with ADI 352 are illustrated. *a*, Distribution of XLF within the tumor nodules (T) is illustrated ($\times 40$). *b*, XLF is seen distributed throughout the wall of tumor-associated blood vessel (V)($\times 40$); specificity controls with normal mouse serum (NMS) for binding of the XLF mAbs to (*c*) tumor nodules (T) and (*d*) to the wall of the tumor-associated blood vessels (V), both $\times 40$.

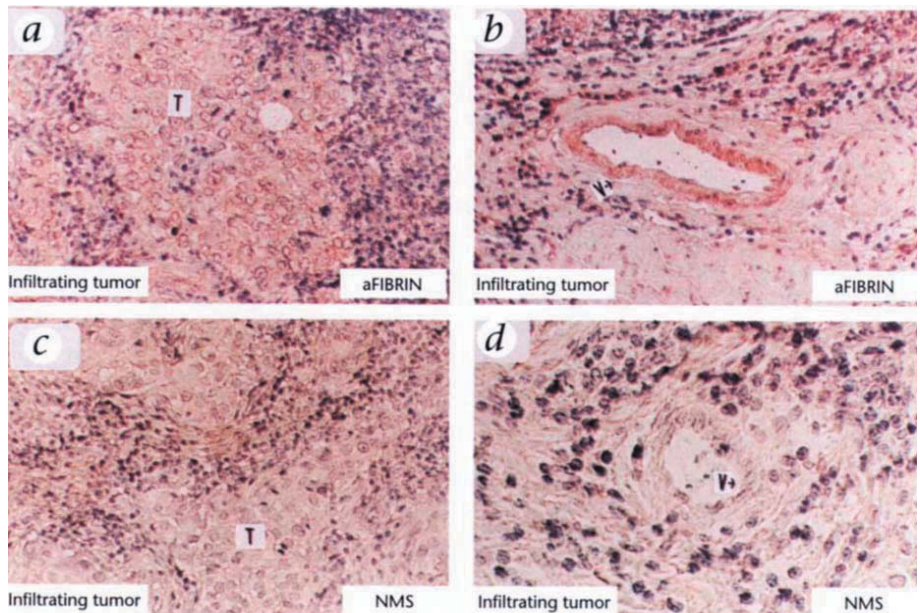
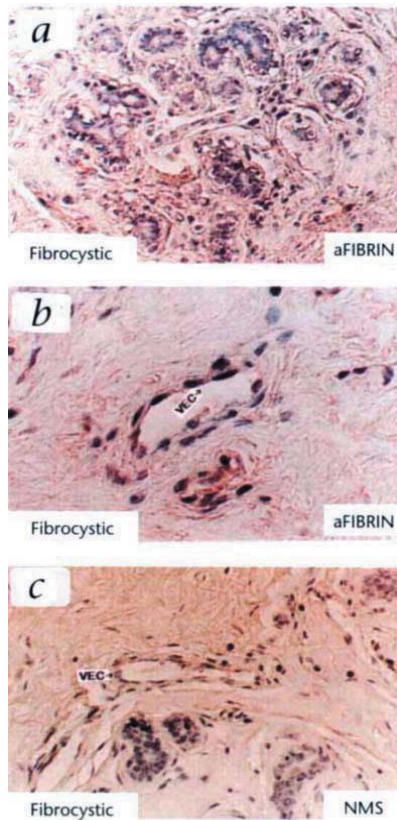


Fig. 5 Immunohistochemical localization of cross-linked fibrin (XLF) in benign fibrocystic disease of the breast. As in Fig. 4, all three mAbs for XLF were tested, and they produced identical results but only the ADI 352 mAb results are illustrated. The absence of XLF in the glandular portions of the fibrocystic tumor is demonstrated in (a) ($\times 40$). b, The absence of XLF in the vascular endothelial cells (VEC) of the tumor-associated blood vessels ($\times 40$). c, A specificity control with normal mouse serum (NMS) ($\times 40$).



immunohistochemical staining using anti-human-TF monoclonal antibodies (Fig. 1). As with the use of TF monoclonal antibodies as probes, both the malignant tumor cells (Fig. 3a) and the VECs (Fig. 3b) stained with the FPR-ck-rVIIa probe, indicating that functional TF was accessible to its ligand. The distribution of functional TF was similar in all seven malignant tumors, and preabsorption of the probe with $10\times$ molar excess of rTF (specificity control) eliminated all reactivity in both cell types (Fig. 3, c and d).

Examination of multiple sections of tissue from patients with fibrocystic disease of the breast failed to reveal any significant staining by the FPR-ck-rVIIa probe of either the hyperplastic ductal epithelium or the VECs located in the stroma of this benign tumor (Fig. 3, e and f). These studies demonstrate that the antigenic TF detected in invasive breast cancer is functional.

Localization of cross-linked fibrin in breast tumors

In an effort to determine whether a relationship existed between the expression of TF and the local activation of coagulation, we stained these same tumors for XLF, using the specific monoclonal antibodies T2G1, GC4, which do not cross-react with fibrinogen^{28,29} or the monoclonal antibody, ADI352. As noted in Fig. 4, the distribution of XLF is virtually identical to that of TF in the invasive breast cancer tissue, with the tumor nodules expressing the highest density of the antigen. Cross-linked fibrin was also observed in the stroma with localization to tumor-associated macrophages and tumor vessels. Definitive localization of XLF to the lumen of the VECs was not possible at this microscopic resolution. No significant deposition of XLF was seen in the benign tumors (Fig. 5). The staining pattern was identical with all 3 monoclonal antibodies for XLF (data not shown).

Discussion

Endothelial cell expression of tissue factor *in vivo*. In spite of the substantial evidence that VECs grown in tissue culture can be stimulated readily by LPS, cytokines and other growth factors to develop procoagulant activity¹¹⁻¹⁹, significant controversy has existed as to the likelihood that VEC tissue factor is generated *in vivo*. Indeed, tissue factor mRNA and TF antigen were not observed in the intima of human blood vessels in two important previous studies, respectively, of atherosclerotic large arteries²⁰ and of normal vessels obtained from post-mortem tissues²¹. However, because tumor cells, VECs and TAMs, under suitable culture conditions, are each capable of synthesizing, binding and/or assembling and activating a series of competent procoagulant molecules *in vitro* (including TF, and factors VII, V, X and so on)³⁰, we reasoned that the appropriate handling of breast tumor tissue might reveal the presence of TF antigen. Furthermore, because we had observed previously that in occasional lung tumors VECs appeared to stain with a monoclonal antibody to TF (ref. 25), we wondered whether other types of tumors with a more intense angiogenic response (such as breast cancer) might prove more revealing in terms of VEC tissue factor expression.

In the present study, we have examined the ability of tumor cells and VECs to express antigenic and functional TF in tissue obtained from patients with invasive breast cancer or benign fibrocystic breast disease. We utilized monoclonal antibodies to various non-cross-reacting epitopes on human TF and a new functional probe for TF, a biotin-labeled tripeptide, Phe-Pro-Arg, linked to chloromethyl ketone and reacted with the active site of recombinant factor VIIa (FPR-ck-rVIIa). Using these reagents, we have demonstrated that antigenic and functional TF are expressed by both the tumor cells and the VECs of malignant infiltrating intraductal breast cancer, but not in benign fibrocystic disease of the breast. These studies support the hypothesis that TF in the tumor cell and in "activated" VECs of the tumor angiogenic response may be responsible for the generation of thrombin and the subsequent cleavage of fibrinogen to fibrin within the tumor and may contribute to the pathogenesis of the thrombotic complications in patients with breast cancer.

Tissue factor antigen in breast cancer. In previously published studies TF antigen has not been observed in human breast cancer using specific monoclonal antibodies³¹. Our experiments suggest that matching tissue fixation techniques with appropriate TF monoclonal antibodies can circumvent this problem (see Methods). Callander and colleagues reported that both normal and malignant breast tissues were positive for TF antigen in three out of eight tumors examined with a polyclonal antiserum³². The origin of the "normal breast tissue" in the study by Callander and her colleagues was not identified, and no benign tumors were included. Although we were unable to confirm the finding of TF in normal breast tissue and our negative results in benign fibrocystic disease of the breast are in contrast to those of Callander and colleagues, we have observed TF antigen in the glandular epithelium of "normal-appearing" breast tissue immediately adjacent to invasive carcinoma. If the activation of TF in the VECs is a result of secretion of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) or other vasoactive cytokines, from the tumor cells (and/or the TAMs), an effect on VECs in adjacent "normal breast tissue" would be entirely possible (*vide infra*). A larger study of normal breast tissue, as well as breast tumors of varying histologic grade of malignancy, will be necessary to determine whether TF and VPF/VEGF

are useful markers for the conversion of the VEC to the "angiogenic phenotype" in human breast disease.

Tissue factor, hypercoagulability, tumor growth and angiogenesis. The relationship between TF expression in the VECs in breast cancer and the process of angiogenesis deserve further scrutiny. Several angiogenic peptides have been isolated from human and guinea pig tumors³³⁻³⁵, some of which exhibit both chemotactic activity for VECs and the ability to stimulate the expression of endothelial cell TF. Furthermore, regulation of TF gene expression in tumors may have an indirect or even a direct relationship to angiogenesis *in vivo*²⁴. The preliminary data of Zhang and colleagues suggests the possibility that TF expression in tumors may have an important regulatory role in the neovascular response²⁴. Because tumor microvessel density appears to be an independent prognostic marker in human breast cancer⁵, it may be useful to examine more closely the potential interactive relationship of these two biologic variables.

The association between malignant disease and the activation of blood coagulation has been known for more than 100 years³⁶. The underlying pathogenesis of this hypercoagulable state in cancer must be extraordinarily complex and is still incompletely understood. The potential importance of tumor cell-mediated adhesive interactions with platelets, immune cells and vascular endothelial cells (VECs) has been reviewed recently^{9,25,30,37}. A number of autocrine, juxtacrine and paracrine loops are possible in view of the variety of mediators generated by tumor cells that can activate the cellular participants, and lead ultimately to local fibrin deposition^{25,30,37}. In addition to its primary role in the cleavage of fibrinogen to form fibrin, thrombin generated in the tumor environment, along with cytokines like IL-1 β (ref. 15, 38), VPF/VEGF (ref. 34, 35, 37) and TNF- α (ref. 17, 39) may also activate cell-surface integrins and selectins to amplify interactions between tumor cells and VECs⁴⁰.

Dissecting out the role of each of the participants is quite difficult, although as suggested by earlier studies and supported by the data presented in the current study, it seems reasonable to focus attention on direct and/or indirect injury of the endothelium of the tumor neovasculature by tumor-derived mediators^{17,24,33-35,37-39}. The determination of the relative contribution of the products of the tumor cell itself versus those of the host inflammatory and vascular cells may prove impossible, however, because many of the vasoactive peptides under consideration are produced readily by both tumor cells and host cells. Nevertheless, some potential for differential expression is possible⁴¹. It may ultimately prove useful to develop a classification schema for tumors with both therapeutic and prognostic import, based on their capability to express a variety of factors, including proclotting and/or fibrinolytic proteins^{25,30-32,42}, oncogenes^{4,5,43} and angiogenic factors^{4-6,37,44}.

It is likely that activation of clotting can occur on the surface of the tumor cell or the TAM and/or on the surface of VECs in response to one or more of these mediators. Depending on the tumor type, therefore, the elaboration of TF in the VECs of the new vessels may influence primary tumor growth, tumor cell migration into and out of blood vessels and both intra- and extravascular clot formation. In breast cancer, large-scale clinical/pathologic correlative studies are needed to determine the potential utility of VEC TF expression as a marker for the "angiogenic phenotype" and as a predictor of thromboembolic complications.

Methods

Materials. Monoclonal antibodies specific for TF were obtained from American Diagnostica, Inc. (ADI 4504, ADI 4507 (Greenwich, Connecticut)). The specificities of several of these mAbs have been described in detail elsewhere⁴⁵⁻⁴⁷. A rabbit anti-human vWF antibody (Dako, Carpenter, California) was used as a marker for VECs. The mAbs specific for XLF, T2G1 and GC4 were obtained from Accurate Chemicals and Scientific Corporation, Westbury, New York. A mAb (352), specific for the fibrin neotope of the alpha chain, was purchased from ADI. The fibrin-specific mAbs were used as described by Kudryk and colleagues^{28,29}. Human recombinant TF (rTF), generously supplied by Karen Fisher and Gordon Vehar (Genentech, San Francisco, California), was utilized for verification of the specificity of the anti-TF mAbs in immunohistochemical and FPR-ck-rVIIA binding reactions. Other reagents and fine chemicals used in these studies were obtained from Sigma Chemical Co.

Formalin-fixed and Bouin's-fixed human breast tumors were obtained from the Cooperative Human Tissue Network (NDRI Division, Philadelphia, Pennsylvania) and the Departments of Pathology of the University of Connecticut Health Center (Farmington) and Emory University (Atlanta, Georgia). Tumors were prepared as paraffin-embedded, 8- μ m sections, and light microscopy was performed on hematoxylin and eosin-stained tissue. In selected studies, performed for the purpose of comparing fixation techniques for the preservation of TF-specific antigens, breast tumors were fixed in acetone-methylbenzoate-xylene (AMeX) in the laboratory of Leo Zacharski (Dartmouth Medical School and the Veterans Administration Medical Center, White River Junction, Vermont). In addition to the slides kindly provided by Zacharski, a total of seven samples from seven patients with breast cancer and ten samples from ten patients with fibrocystic disease of the breast were studied. Independent confirmation of the histologic diagnosis in each case was provided by Melinda Sanders (Department of Pathology, University of Connecticut School of Medicine, Farmington) and Cynthia Cohen (Department of Pathology, Emory University School of Medicine, Atlanta, Georgia).

Preparation of biotinyl-FPR-ck-rVIIa. To detect functional TF *in situ*, we utilized our recently developed novel probe, biotinylated Phe-Pro-Arg-chloromethyl ketone (Biocap-FPR-ck), reacted with recombinant human factor VIIa (FPR-ck-rVIIa). Chloromethyl ketone-labeling of serine proteases has been described previously⁴⁸. We have modified the technique to develop a probe specific for functional TF, defined operationally as TF capable of binding factor VII or VIIa. In brief, 250 μ g of rVIIa (a generous gift from Ulla Hedner, NOVO Pharmaceuticals, Uppsala, Sweden) was resuspended in 1.0 ml of HEPES/saline, pH 7.4 (5.0 μ M), Biocap-FPR-ck (Haematologic Technologies, Inc., Essex Junction, Vermont) was added to a final concentration of 200 μ M, and the mixture incubated at room temperature (RT) for 2 h. A further aliquot of Biocap-FPR-ck was added to the solution to bring the final concentration to 400 μ M, the solution was incubated overnight at RT, and the labeled rVIIa separated from residual Biocap-FPR-ck with a 0.5 \times 20 cm G-25 Sephadex column in HEPES/saline. Fractions containing protein and testing positive for biotin (using avidin-HRP) were recovered. The characteristics and specificity of this reagent for TF have been evaluated and are reported in detail elsewhere²⁶.

Immunohistochemical studies. Indirect immunoperoxidase staining⁴⁹ of fixed tissue was performed using anti-human rTF mAbs (ADI 4504, 4507 and 4509), mAbs to XLF (T2G1 and GC4,

Accurate Chemicals) and a rabbit, anti-human-vWF (Dako). Biotinylated horse, anti-mouse-IgG and biotinylated goat, anti-rabbit-IgG (Vector, Burlington, California) were used as secondary antibodies. The specificity of each of the mouse, anti-human TF mAbs was confirmed by blocking the staining reaction with preabsorption of the mAb with rTF (10× molar excess for 8 h at 4 °C).

Paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol (100%, 95%, 75% and 50%). Samples were immersed in methanol containing 0.01% hydrogen peroxide (H₂O₂) at 4 °C for 15 min, for inhibition of endogenous peroxidases, allowed to air-dry, and then blocked with normal horse serum or normal goat serum (Vector) at RT for 1 h. The sections were then washed 3 times with PBS (pH 7.4) and reacted with the mAbs (1/100 dilution in PBS/0.5% BSA) overnight at 4 °C. Mouse anti-rTF preabsorbed with rTF or nonimmune rabbit serum, served as the control antisera for these reactions. Samples were washed 3 times with PBS between each of the steps. Following overnight incubation, biotinylated horse, anti-mouse-IgG or biotinylated goat, anti-rabbit IgG (1/100 dilution in PBS/0.5% BSA) was applied to the samples and allowed to incubate for 1 h at RT. HRP-streptavidin (Zymed, San Francisco, California) at a 1/250 dilution in PBS was applied to the sections and incubated at RT for 1 h. The samples were incubated sequentially with 3-amino-9-ethylcarbazole (AEC) in 0.1 M sodium acetate buffer (pH 5.0) and 0.03% H₂O₂ for 30 min at RT. Samples were then counterstained in Mayer's hematoxylin (Sigma) for 10 min, washed extensively in dH₂O, and dipped in dilute ammonium hydroxide. Samples were then mounted in Crystal mounting solution (Biomed, Foster City, California) and analyzed by light microscopy.

Other investigators have reported that breast cancer is not among those tumors that express procoagulant proteins such as tissue factor³¹. To investigate the basis of this possible discrepancy with the current work, we obtained breast tumor samples from L. Zacharski, which had been fixed in AMeX, according to published methods⁵⁰, and were said to be negative for TF antigen by conventional immunohistochemistry. We demonstrated that those TF mAbs that worked well with formalin-fixed tissue in our own study, reacted poorly or not at all with AMeX-fixed breast cancer tissue (data not shown). In contrast, other TF mAbs not used in Zacharski's studies (for example, ADI 4507, 4509) stained TF in the breast cancer cells in the tissue fixed with AMeX (data not shown). We conclude that the failure of other investigators to find TF in malignant breast tumors may simply be a technical issue related to choice of tissue fixatives compatible with the mAbs utilized for *in situ* immunohistochemistry.

FPR-ck-rVIIA histochemical studies. The procedure for identification of functional TF *in situ* utilizing this reagent has been described in detail²⁶, and is virtually identical to the procedure used for the immunohistochemical studies (*vide supra*), including the same specificity controls. It should be reemphasized that complete inhibition of labeling with FPR-ck-rVIIA was achieved by preincubation of the probe with rTF (50-fold molar excess), preincubation with the tissue with saturating concentrations of blocking mAbs to TF (detailed above) or unlabeled rVIIa (ref. 26). Although others have suggested that factor VIIa may bind with high affinity to human cells in tissue culture by TF-independent mechanisms^{51,52}, we and others have been unable to confirm their observations (unpublished observations and Y. Nemerson *et al.*, personal communication).

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- Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Med.* **1**, 27–31 (1995).
- Weidner, N. *et al.* Tumor angiogenesis: A new significant and independent prognostic indicator in early-stage breast carcinoma. *J. Natl. Cancer Inst.* **84**, 1875–1887 (1992).
- Van Hoef, M.E.H.M., Knox, W.F., Dhesi, S.S., Howell, A. & Schor, A.M. Assessment of tumour vascularity as a prognostic factor in lymph node negative invasive breast cancer. *Eur. J. Cancer* **29A**, 1141–1145 (1993).
- Gasparini, G. *et al.* Tumor microvessel density, p53 expression, tumor size and peritumoral lymphatic vessel invasion are relevant prognostic markers in node-negative breast carcinoma. *J. Clin. Oncol.* **12**, 454–466 (1994).
- Folkman, J. Angiogenesis and breast cancer. *J. Clin. Oncol.* **12**, 441–443 (1994).
- Weidner, N., Carroll, P.R., Flax, J., Blumenfeld, W. & Folkman, J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am. J. Pathol.* **143**, 401–409 (1993).
- Li, V.W. *et al.* Microvessel count and cerebrospinal fluid basic fibroblast growth factor in children with brain tumors. *Lancet* **344**, 82–86 (1994).
- Nemerson, Y. Tissue factor and hemostasis. *Blood* **71**, 1–8 (1988).
- Rickles, F.R., Levine, M.N. & Edwards, R.L. Hemostatic alterations in cancer patients. *Cancer Metastasis Rev.* **11**, 237–248 (1992).
- Dvorak, H.F., Dickersin, G.R., Dvorak, A.M., Manseau, J.E. & Pyne, K. Human breast carcinoma: Fibrin deposits and desmoplasia. *J. Natl. Cancer Inst.* **67**, 335–340 (1981).
- Maynard, J.R., Dreyer, B.E., Stemerman, M.B. & Pitlick, F.A. Tissue factor coagulant activity of cultured human endothelial and smooth muscle cells and fibroblasts. *Blood* **50**, 387–396 (1977).
- Stern, D.M., Nawroth, P.P., Handley, D. & Kisiel, W. An endothelial cell-dependent pathway of coagulation. *Proc. Natl. Acad. Sci. USA* **82**, 2523–2527 (1982).
- Colucci, M. *et al.* Cultured human endothelial cells generate tissue factor in response to endotoxin. *J. Clin. Invest.* **71**, 1893–1896 (1983).
- Lyberg, T., Galdal, K.S., Evensen, S.A. & Prydz, H. Cellular cooperation in endothelial thromboplastin synthesis. *Br. J. Haematol.* **53**, 85–95 (1983).
- Bevilacqua, M.P., Pober, J.S., Majeau, G.R., Cotran, R.S. & Gimbrone, M.A., Jr. Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *J. Exp. Med.* **160**, 618–623 (1984).
- Tannenbaum, S.H., Finko, R. & Cines D.B. Antibody and immune complexes induce tissue factor production by human endothelial cells. *J. Immunol.* **137**, 1532–1537 (1986).
- Conway, E.M., Bach, R., Rosenberg, R.D. & Konigsberg, W.H. Tumor necrosis factor enhances expression of tissue factor mRNA in endothelial cells. *Thromb. Res.* **53**, 231–241 (1989).
- Crossman, D.C., Carr, D.P., Tuddenham, E.G.D., Pearson, J.D. & McVey, J.H. The regulation of tissue factor mRNA in human endothelial cells in response to endotoxin or phorbol ester. *J. Biol. Chem.* **265**, 9782–9787 (1990).
- Kirchofer, D. *et al.* Relationship between tissue factor expression and deposition of fibrin, platelets and leukocytes on cultured endothelial cells under venous blood flow conditions. *Blood* **81**, 2050–2058 (1993).
- Wilcox, J.N., Smith, K.M., Schwartz, S.M. & Gordon, D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc. Natl. Acad. Sci. USA* **86**, 2839–2843 (1989).
- Drake, T.A., Morrissey, J.H. & Edgington, T.S. Selective cellular expression of tissue factor in human tissues. *Am. J. Pathol.* **134**, 1087–1097 (1989).
- Drake, T.A., Cheng, J., Chang, A. & Taylor, F.B., Jr. Expression of tissue factor, thrombomodulin, and E-selectin in baboons with lethal *Escherichia coli* sepsis. *Am. J. Pathol.* **142**, 1458–1470 (1993).
- Faulk, W.P., Labarrere, C. & Carson, S. Tissue factor: Identification and characterization of cell types in human placenta. *Blood* **76**, 86–96 (1990).
- Zhang, Y. *et al.* Tissue factor controls the balance of angiogenic and antiangiogenic properties of tumor cells in mice. *J. Clin. Invest.* **94**, 1320–1327 (1994).
- Rickles, F.R., Hancock, W.W., Edwards, R.L. & Zacharski, L.R. Antimetastatic agents. I. The role of cellular procoagulants in the pathogenesis of fibrin deposition in cancer and the use of anticoagulants and/or antiplatelet drugs in cancer treatment. *Semin. Thromb. Hemost.* **14**, 126–132 (1988).
- Contrino, J., Hair, G.A., Schmeizl, M.A., Rickles, F.R. & Kreutzer, D.L. *In situ* characterization of antigenic and functional tissue factor expression in human tumors utilizing monoclonal antibodies and recombinant factor VIIa as probes. *Am. J. Pathol.* **145**, 1315–1322 (1994).
- Broze, G., Jr. Binding of human factor VII and VIIa to monocytes. *J. Clin. Invest.*

- 70, 526–536 (1982).
28. Kudryk, B., Rohozza, A., Ahadi, M., Chin, J. & Wiebe, M.E. Specificity of a monoclonal antibody for the NH² terminal region of fibrin. *Mol. Immunol.* **21**, 89–94 (1984).
29. Kudryk, B., Rohozza, A., Ahadi, M., Gidlund, M. & Harfenist, E.F. Antibodies specific for neoantigens expressed on chains or degradation products of fibrin(ogen). in *Fibrinogen 3 — Biochemistry, Biological Functions, Gene Regulation and Expression*. (eds. Mosesson, M.W., Armani, D.B., Siebenlist, K.R. & DiOrio, J.P.) 129–132 (Elsevier Science Publishers, Amsterdam, 1988).
30. Rickles, F.R. & Edwards, R. Leukocytes and tumor cells in thrombosis. in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. (eds. Colman, R.W., Hirsh, J., Marder, V.J. & Salzman, E.W.) 1164–1179 (Lippincott, Philadelphia, 1994).
31. Costantini, V. *et al.* Fibrinogen deposition without thrombin generation in primary human breast cancer tissue. *Cancer Res.* **51**, 349–353 (1991).
32. Callander, N.S., Varki, N. & Rao, L.V.M. Immunohistochemical identification of tissue factor in solid tumors. *Cancer* **70**, 1194–1201 (1992).
33. Noguchi, M., Sakai, T. & Kisiel, W. Identification and partial purification of a novel tumor-derived protein that induces tissue factor on cultured human endothelial cells. *Biochem. Biophys. Res. Commun.* **160**, 222–226 (1989).
34. Clauss, M. *et al.* Vascular permeability factor: A tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity and promotes monocyte migration. *J. Exp. Med.* **172**, 1535–1545 (1990).
35. Kao, J. *et al.* Endothelial monocyte-activating polypeptide II. *Biochem. J.* **267**, 20239–20247 (1992).
36. Trouseau, A. Phlegmasia alba dolens. in *Clinique de l'Hôtel-Dieu de Paris*. vol. 3, 654–712 (Balliere et Fils, Paris, 1865).
37. Dvorak, H.F. Abnormalities of hemostasis in malignant disease. in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. (eds. Colman, R.W., Hirsh, J., Marder, V.J. & Salzman, E.W.) 1238–1254 (Lippincott, Philadelphia, 1994).
38. Miyachi, S. *et al.* Malignant tumor cell lines produce interleukin-1-like factor. *In Vitro Cell Dev. Biol.* **24**, 753–755 (1988).
39. Brett, J. *et al.* Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. *J. Exp. Med.* **169**, 1977–1991 (1989).
40. McKeever, R.P. Leukocyte interactions mediated by selectins. *Thromb. Haemost.* **66**, 80–87 (1991).
41. Berse, B., Brown, L.F., Van De Water, L., Dvorak, H.F. & Senger, D.R. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol. Biol. Cell* **3**, 211–220 (1992).
42. Janicke, F. *et al.* Urokinase (uPA) and its inhibitor PAI-1 are strong and independent prognostic factors in node-negative breast cancer. *Breast Cancer Res. Treat.* **24**, 195–208 (1993).
43. Muss, H.B. *et al.* c-erbB-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. *N. Engl. J. Med.* **330**, 1260–1266 (1994).
44. Brown, L.F. *et al.* Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res.* **53**, 4727–4735 (1993).
45. Morrissey, J.H., Fair, D.S. & Edgington, T.S. Monoclonal antibody analysis of purified and cell-associated tissue factor. *Thromb. Res.* **52**, 247–261 (1988).
46. Ruf, W., Rehemtulla, A. & Edgington, T.S. Antibody mapping of tissue factor implicates two different exon-coded regions in function. *Biochem. J.* **278**, 729–733 (1991).
47. Ruf, W. & Edgington, T.S. An anti-tissue factor monoclonal antibody which inhibits TF-VIIa complex is a potent anticoagulant in plasma. *Thromb. Haemost.* **66**, 529–533 (1992).
48. Williams, E.B., Krishnaswamy, S. & Mann, K.G. Zymogen/enzyme discrimination using peptide chloromethyl ketones. *J. Biol. Chem.* **264**, 7536–7545 (1989).
49. Guesdon, J.L., Ternynck, T. & Avrameus, S. The use of avidin-biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem.* **27**, 1131–1139 (1979).
50. Sato, Y., Mukai, K., Watanabe, S., Goto, M. & Shimosato, Y. The AMeX method. *Am. J. Pathol.* **125**, 431. Pathol. Muk.
51. Reuning, U., Preissner, K.T., Muller-Berghaus, G. Two independent binding sites on monolayers of human endothelial cells are responsible for interaction with coagulation factor VII and factor VIIa. *Thromb. Haemost.* **69**, 197–204 (1993).
52. Hoffman, M., Monroe, D.M., Roberts, H.R. Human monocytes support factor X activation by factor VIIa, independent of tissue factor: implications for the therapeutic mechanism of high-dose factor VIIa in hemophilia. *Blood* **83**, 38–42 (1994).