

Pentoxifylline Inhibits Hypoxia-induced Upregulation of Tumor Cell Tissue Factor and Vascular Endothelial Growth Factor

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Summary

Tissue factor (TF), the membrane glycoprotein that initiates blood coagulation, is constitutively expressed by many tumor cells and is implicated in peri-tumor fibrin deposition and hypercoagulability in cancer. Upregulation of tumor TF correlates with enhanced metastatic potential. Furthermore, TF has been colocalized with VEGF in breast cancer, specially at sites of early angiogenesis. There are no data on the effect of hypoxia on tumor cell TF expression. Since hypoxia is known to stimulate VEGF production, we studied whether this also induces tumor cell TF expression. Confluent monolayers of A375 melanoma, MCF-7 breast carcinoma and A549 lung carcinoma were cultured in either 95% air, 5% CO_2 (normoxic) or 95% N2, 5% CO_2 (hypoxic; 25-30 mmHg) for 24 h. Procoagulant activity (PCA) was measured by amidolytic and clotting assays, surface TF antigen by flow cytometry, early apoptosis by annexin V binding and VEGF levels in culture supernatants by ELISA. Hypoxia significantly increased tumor cell PCA in all three cell lines tested and TF antigen on A375 cells was increased four-fold ($P < 0.05$). Pentoxifylline (PTX), a methylxanthine derivative, significantly inhibited the hypoxia-induced increase in PCA as well as VEGF release in all three cell lines tested. In A375 cells, PTX significantly inhibited TF antigen expression by both normoxic and hypoxic cells. Hypoxia induced a slight (5%) but not significant, increase in early apoptosis. Intravenous injection of hypoxic A375 cells into nude rats produced more pronounced thrombocytopenia ($n = 5$, $P < 0.01$) and more lung metastases ($n = 3$, $P < 0.05$) compared to normoxic cells. We conclude that hypoxia increases TF expression by malignant cells which enhances tumor cell-platelet binding and hematogenous metastasis. Hypoxia-induced upregulation of TF appears to parallel that of VEGF, although the mechanism remains unclear.

Introduction

Tissue factor (TF) is a membrane-associated glycoprotein that initiates blood coagulation by binding to factor VII (1). TF is constitutively expressed on the surface of many tumor cells (2) and can be pathologically induced on endothelial cells and monocytes (3). The expression of TF by tumor cells has been implicated in tumor-associated fibrin deposition and the systemic hypercoagulable state associated with malignancy. The capacity of tumor cells to activate coagulation appears to be important in several aspects of tumor biology. For example, thrombin is mitogenic for both tumor cells and tumor-associated vascular endothelial cells (4) while subsequent peri-tumor fibrin deposition is central to the formation of tumor stroma (5). In addition, both thrombin and tumor cell-associated fibrin may be important in tumor angiogenesis (6). Angiogenesis is regulated by many molecules released by tumor and host cells, including vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), platelet-derived growth factor (PDGF) and transforming growth factor (7).

Recent evidence indicates that tumor-derived TF plays a role in angiogenesis and metastasis (8-10) by mechanisms independent of clotting activation. Of particular interest in this regard is the apparent relationship between TF and VEGF. Transfection of low TF-producing tumor cells with TF cDNA sense construct (creating tumor cells that overexpressed TF) increased VEGF production by 1000-fold (11). In another study, TF and VEGF appeared to be colocalized in breast tumors (10) and their activity in human breast cancer and melanoma cells were strongly correlated (11). These findings raise questions about possible mechanisms of TF upregulation at sites of tumor neovascularization.

Hypoxic areas commonly occur in solid tumors and induce both angiogenesis (12) and cell apoptosis (13). One important mechanism for hypoxia-initiated angiogenesis is the upregulation of VEGF mRNA expression by both tumor and vascular endothelial cells (14). Although hypoxia primes endotoxin-induced TF expression in human monocytes and endothelial cells by a mechanism dependent on platelet activating factor (15), there are no data on the effect of hypoxia on tumor cell TF expression and activity. The purpose of this study was therefore to investigate the effect of mild hypoxia on the upregulation of TF expression. Furthermore, since the methylxanthine derivative pentoxifylline (PTX, TrentalTM) is known to downregulate TF expression (16, 17), we also investigated whether this agent could prevent hypoxia-induced modulation of TF and VEGF production.

Materials and Methods

Tumor Cell Lines

A375 human melanoma, MCF-7 breast adenocarcinoma cells and A549 lung carcinoma (ATCC, Rockville, MD) were cultured in RPMI-1640 with Glutamax™ and 25 mM HEPES buffer, supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (Sigma Chemical Co., St. Louis, MO).

Induction of Hypoxia

Confluent monolayers (~10^7 cells/flask) were maintained either in 95% air, 5% CO_2 (normoxic conditions) or in a humidified sealed chamber gassed with 95% N2, 5% CO_2 mix (30 l/h, hypoxic conditions) for 24 h. At the end of this period the partial pressure of oxygen (PO_2) in the culture medium was measured using a pH/blood gas analyzer and was in the range 25-30 mmHg and 120 to

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130 mmHg for hypoxic and normoxic cells respectively. The pH of medium maintained under normoxic and hypoxic conditions was 7.0 and 6.85 respectively.

Cells cultured in normoxic and hypoxic conditions were harvested by trypsin-EDTA (Gibco, Grand Island, NY), washed twice with phosphate-buffered saline (PBS, w/o Ca^{2+} and Mg^{2+}) and finally resuspended in 50 mM Tris-buffered saline containing 5 mM $CaCl_2$ (pH 7.8). The concentration was adjusted to 1×10^6 cells /ml. For flow cytometric analysis and plasma clotting assays, the cells were suspended in PBS (w/o Ca^{2+}).

Procoagulant Activity (PCA)

To determine tumor cell PCA, 1×10^5 tumor cells were added to 0.3 ml citrated (3.2%, 1:9 v/v), pooled normal human plasma and the clot time recorded in a Sonoclot™ Analyzer (Sienco Inc., Morrison, CO) as previously described (18). The PCA of all three cell lines in this system was due to TF expression since it was completely inhibited by a blocking monoclonal antibody to human TF (data not shown).

Chromogenic Assay for Factor X Activating Activity

Factor X-activating activity (FXAA) on intact tumor cells was determined with a chromogenic assay performed in triplicate in 96-well plates. Briefly, 100 µl of cell suspension (1×10^5 cells in 50 mM Tris-NaCl buffer with 5 mM $CaCl_2$) were incubated with 20 µl of purified factor X (2 units/ml, Sigma Chemical Co.) followed by 40 µl of the FXa-specific chromogenic substrate S-2765 (Chromogenix, Molndal, Sweden). Factor Xa generation was determined by measuring the reaction rate in a THERMOmax™ microplate reader (Molecular Devices, Menlopark, CA) at 405 nm. Procoagulant activity was expressed as mA^{405}/min. FXAA was due to cell-bound TF-VIIa complex and was completely inhibited by a blocking antibody to human TF (data not shown).

Measurement of Surface TF Antigen

Tumor cells grown in normoxic and hypoxic conditions were labeled with 10 µl of FITC-conjugated anti-human tissue factor (#4508CJ, American Diagnostica Inc., Greenwich, CT) and TF antigen expression determined directly by flow cytometry. Ten µg of mouse IgG (FITC-conjugated, Dako, Carpinteria, CA) was used as negative control. Percent positive fluorescence was determined in comparison to an isotype control for TF antigen. The number of antigen binding capacity (ABC) units of TF per tumor cell population was calculated by incubating saturating amounts of conjugated monoclonal antibody with "Quantum Simply Cellular™" beads (Sigma Chemical Co.) for 1 h at room temperature. Beads were then washed by centrifugation at 350 g for 10 min and analyzed at the same instrument settings used for tumor cells. Specific software (Sigma Chemical Co.) was used to calibrate the PMT parameter and to convert the fluorescent label's channel number into ABC units of TF. The percent positivity was multiplied by ABC units for each sample to give total ABC (TABC) units for the positive population.

Measurement of Vascular Endothelial Growth Factor (VEGF)

VEGF was measured by an immunoassay designed for the quantitative determination of human VEGF in cell culture supernatants, serum and plasma (R&D Systems, Minneapolis, MN). Culture supernatants from cells (grown in normoxic and hypoxic conditions) were centrifuged (1000 rpm, 5 min) to eliminate non-adherent cells, diluted 1:3 in assay diluent RDW1 and assayed according to the manufacturer's instructions.

The Effect of Pentoxifylline on Tumor Cell TF Expression and VEGF Production

Pentoxifylline was dissolved in RPMI-1640 medium (with 10% FBS) and added to tumor cell suspensions at a final concentration of 200 µg/ml. Tumor cells were grown in normoxic and hypoxic conditions with and without PTX. For these experiments, cells were incubated in hypoxic conditions for 24 h. In order to exclude possible non-specific effect of PTX on tumor cells protein expression, A375 melanoma cells were incubated with and without the protein synthesis inhibitor cyclohexamide (10 (g/ml, 24 h) or PTX (200 (g/ml) under normoxic and hypoxic conditions. Total protein in 2×10^6 cells was determined after Triton X-100 solubilization according to the method of Bradford (19) using bovine serum albumin as the standard.

Measurement of Apoptosis

Apoptosis in cells grown in hypoxic conditions was evaluated by flow cytometry using the Annexin V-FITC Kit (TREVIGEN, Gaithersburg, MD) according to the manufacturer's instructions. This method is based on exposure of the negatively charged phospholipid, phosphatidylserine (PS) on the outer leaflet of the plasma membrane that is one of the early changes during apoptosis.

The Effect of Hypoxia on Tumor Cell-induced Thrombocytopenia

Hypoxic and normoxic tumor cell (A375) suspensions were adjusted to 4×10^6 cells/ml. 0.5 ml was injected intravenously (via the tail vein) in the following groups of female athymic nude rats weighing 75-100 g (Harlan, Indianapolis, IN):

Group 1: Rats receiving normoxic cells (n = 5)
Group 2: Rats receiving hypoxic cells (n = 5)
Group 3: Rats receiving 0.5 ml of saline intravenously as controls (n = 5)

Thirty minutes after tumor cell injection, 0.5 ml of blood was collected from the tail vein into EDTA-coated containers. The platelet count was determined electronically using a Coulter MD16 counter (Coulter Electronics, Miami, FL).

The Effect of Hypoxia on Experimental Lung Seeding

Twenty eight days after tumor cell injection, three animals from each group were euthanized by an overdose of halothane inhalation. Approximately 7 ml of Bouin's solution (Sigma, St. Louis, MO) were injected via the trachea thus insufflating the lungs, until the lungs are fully stained. The lungs were then dissected en block from the thoracic cage and rinsed in a beaker of water to remove the excess stain. Finally, the lungs were placed in Bouin's solution and surface metastases counted on the entire lung tissue. Tumor nodules appear as pale spots against a darker yellow background.

Statistical Analysis

Data were analyzed using Statistica™ for Windows (StatSoft, Inc., Tulsa, OK). All data were normally distributed and therefore Student's t-test was used to determine statistical significance between various data sets. Results were expressed as mean ± standard deviation. Statistical significance was assumed when $P < 0.05$.

Results

The Effect of Hypoxia on Tumor Cell Procoagulant Activity and TF Antigen Expression

A moderate decrease in PO_2 (40-50 mmHg for 24 h) markedly increased the PCA of all three cell lines as demonstrated by a shortening of the plasma recalcification time ($P < 0.01$; Fig. 1) and their ability to directly activate factor X in a chromogenic substrate assay ($P < 0.01$; Fig. 2). Hypoxia significantly increased TF antigen expression in all three tumor cell lines ($P < 0.05$; Fig. 3).

The Effect of Pentoxifylline on Hypoxia-induced Procoagulant Activity and Antigen

PTX markedly inhibited the hypoxia-induced shortening of the recalcification time by A375, MCF-7 and A549 cells (Fig. 1) and

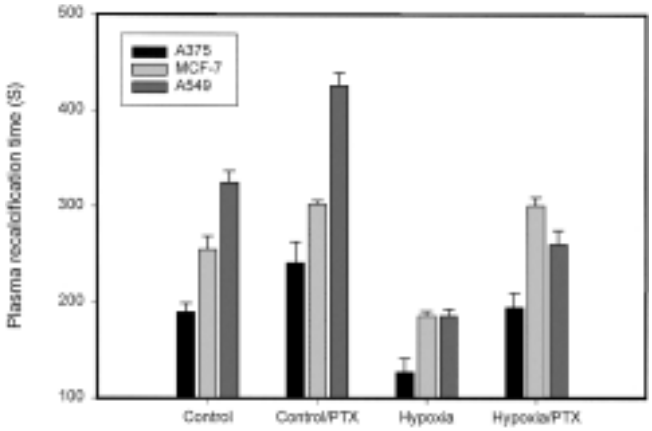

Fig. 1 Effect of hypoxia and pentoxifylline (PTX) on the procoagulant activity of A375 melanoma, MCF-7 breast cancer and A549 lung cancer cells as determined by the recalcification time (s) of normal plasma. The results are expressed as mean ± SD

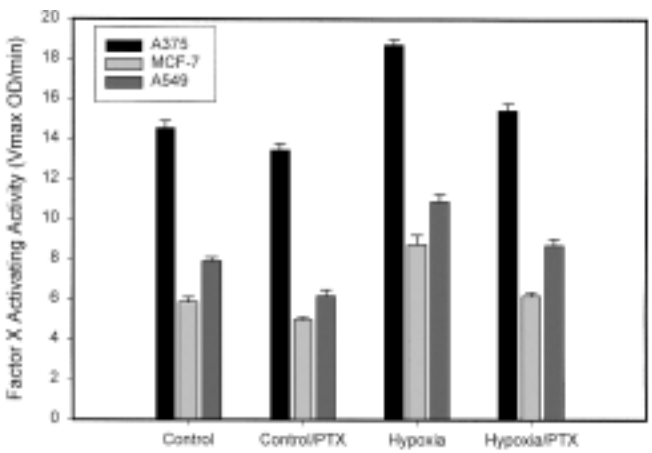

Fig. 2 Effect of hypoxia and pentoxifylline (PTX) on the procoagulant activity of A375 melanoma, MCF-7 breast cancer and A549 lung cancer cells as determined by their ability to activate human factor X (mOD/min). The results are expressed as mean ± SD

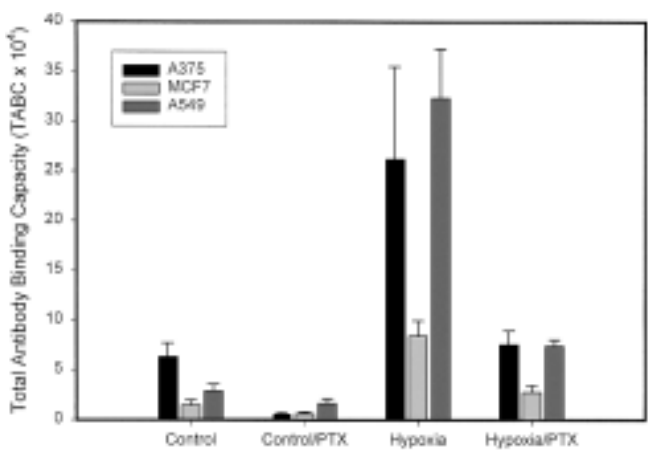

Fig. 3 Effect of hypoxia and pentoxifylline (PTX) on TF antigen expression of A375 melanoma, MCF-7 breast cancer and A549 lung cancer cells as determined by flow cytometry. The results are expressed as mean ± SD

inhibited the resultant increase in FXAA by 72%, 83% and 65% respectively (Fig. 2). Pentoxifylline inhibited the hypoxia-induced increase in TF antigen in all three cell lines ($P < 0.05$, Fig. 3)

The Effect of Pentoxifylline on Hypoxia-induced VEGF Release

In all the cell lines tested, hypoxia markedly increased VEGF release into the culture supernatant. The largest change was observed in the A375 melanoma cells which produced a mean of 15.5 pg/ml of VEGF (n = 3) under normoxic conditions compared to 2270 pg/ml (n = 3, $P < 0.001$) in hypoxic conditions (Fig. 4). PTX reduced VEGF release by hypoxic cells by 60% (901 pg/ml, n = 3, $P < 0.001$). Although normoxic MCF-7 and A549 cells produced higher levels of VEGF (865 ± 345 and 3125 ± 135 pg/ml respectively) than A375 cells, these were nevertheless further increased by hypoxia (2436 ± 484 and 6611 ± 1324 pg/ml respectively) and this increase was completely prevented by PTX (856 ± 91 and 3076 ± 108 pg/ml respectively; n = 3, $P < 0.001$). Cyclohexamide (10 μg/ml, 24 h) inhibited protein synthesis by 44%. However, only 5% inhibition was detected by PTX (200 μg/ml, 24 h) under both normoxic and hypoxic conditions.

The Effect of Hypoxia on Tumor Cell Apoptosis

Early apoptosis was determined in normoxic and hypoxic A375 melanoma cells by measuring the exposure of phosphatidylserine on the outer leaflet of the plasma membrane and late apoptosis with the DNA probe 7-Actinomycin D. In confluent normoxic cultures, 21 ± 9% and 10 ± 7% of the cells were in early and late apoptosis respectively, compared to values of 25 ± 7% and 11 ± 9% respectively in confluent hypoxic cultures (n = 4, P = NS).

The Effect of Hypoxia on Tumor Cell-induced Thrombocytopenia and Lung Seeding

Intravenous injection of normoxic A375 tumor cells reduced the mean (± SD) platelet count by 25% ($416 \pm 39 \times 10^9$/l vs. $559 \pm 55 \times 10^9$/l in controls; $P < 0.01$). In contrast, injection of hypoxic cells reduced the platelet count by almost 49% ($287 \pm 26 \times 10^9$/l; $P < 0.01$). Animals receiving normoxic tumor cells (n = 3) subsequently developed a mean

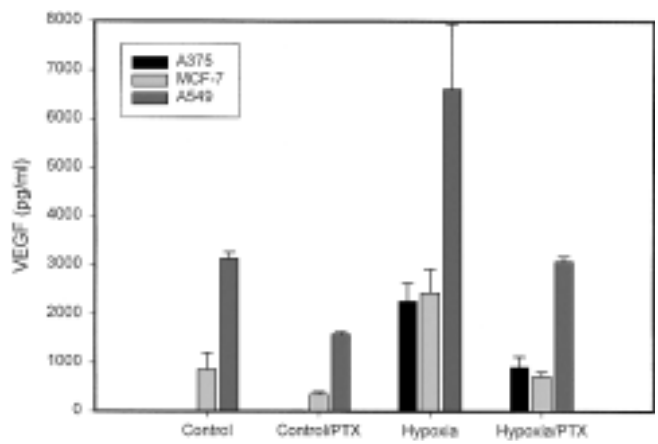

Fig. 4 Effect of hypoxia and pentoxifylline (PTX) on the release of vascular endothelial growth factor (VEGF; pg/ml) from A375 melanoma, MCF-7 breast cancer and A549 lung cancer cells. The results are expressed as mean ± SD

(± SD) of 3.0 ± 1.7 tumor nodules in the lungs. Rats receiving a similar number of hypoxic cells, in contrast, developed 10.6 ± 3.0 lung nodules ($n = 3$; $P < 0.05$).

Discussion

Although tumor-associated procoagulant activity (PCA) particularly TF (2, 24, 25) may be important in tumor growth and spread (20-23) TF expression correlated with the malignant phenotype of human breast disease and appeared to be a marker of early angiogenesis (24). The colocalization of TF and VEGF in malignant lung and breast tumors and the strong correlation between TF and VEGF expression produced by human breast cancer and melanoma cell lines (10) suggest a related role for these proteins in tumor biology.

In the present study, we demonstrated that relatively minor hypoxia significantly increased TF expression by three tumor cell lines, suggesting that hypoxia could be one mechanism by which tumor cells become more procoagulant than their normal counterparts. As expected from the work of others (13), hypoxia also significantly increased VEGF production, which is consistent with the correlation between TF and VEGF previously reported in human tumor cells (10). The hypoxic conditions used in the present study are considerably milder than those that probably occur within solid tumors *in vivo* (0-5 mmHg). At these extreme hypoxic conditions, however, apoptosis and even necrosis may occur. In the present study, we aimed to lower oxygen tension *in vitro* without causing apoptosis, since this may, by itself, induce TF expression (Amirkhosravi et al., unpublished data). Since these mild hypoxic conditions did not induce significant apoptosis, the observed upregulation of TF expression was presumably independent of this process. It is possible that more significant upregulation of TF could occur at lower PO_2. Indeed, while this work was in progress, Yan et al., (25) reported a time-dependent increase in TF mRNA (maximizing at 2 h) by HELA cultures exposed to hypoxia (12-14 mmHg). Whether apoptosis also resulted from these conditions was not reported.

The increase in TF expression induced by hypoxia translated into a more severe coagulopathy (as assessed by fall in platelet count) *in vivo* when previously hypoxic cells were injected intravenously. This, in turn, resulted in an increased efficiency of pulmonary seeding of the malignant cells. These data are entirely compatible with our previous work in which we demonstrated close correlation between tumor cell PCA *in vitro* and subsequent generation of intravascular coagulation and lung seeding *in vivo* (26). The inference is that previously hypoxic tumor cells gaining access to the circulation may not only be more thrombogenic, but may also have a metastatic advantage.

We also demonstrated that hypoxia-induced increases in both TF and VEGF could be blocked by pentoxifylline (PTX), a methylxanthine derivative which is known to downregulate TF synthesis and expression by monocytes and endothelial cells (15, 16, 27, 28). We previously reported that PTX significantly reduces liver metastasis in the mouse Neuro2a neuroblastoma model (29) suggesting that this agent may have anti-tumor properties of its own. This effect was not due to cytotoxicity, but to reduction in the rate of tumor cell proliferation. Our findings suggest that downregulation of VEGF by PTX may have contributed to this effect. In the present study, PTX not only inhibited the hypoxia-induced upregulation of TF antigen and activity, but also downregulated TF expression by normoxic cells. Thus, it appears unlikely that the effect of PTX on TF expression is via a hypoxia-specific mechanism. Of particular note is that PTX significantly reduced VEGF production in two normoxic cell lines (MCF-7 breast carcinoma and A549 lung carcinoma), suggesting that it may have anti-angiogenic properties. In addition, Melnyk et al. (30) showed that inhibition of VEGF alone was sufficient to prevent tumor growth and dissemination *in vivo* and suggested that VEGF promotes tumor dissemination by a mechanism distinct from its effect on primary tumor growth.

The mechanism(s) by which TF and VEGF expression may be linked remains unclear. Hypoxia-Inducible Factor 1-α (HIF-1α) has been shown to regulate VEGF expression under hypoxic conditions (31). However, no hypoxia-specific response element has been identified in the TF promoter.

Clauss et al. (32) showed that TF expression on the surface of endothelial cells could be induced by VEGF. However, a more recent study by Ollivier and colleagues (33) has shown TF-dependent VEGF-production by human fibroblasts in response to activated factor VII. The latter study supports the involvement of TF's coagulant capacity in the process of neovascularization. Furthermore, Mueller and Ruf (34) have recently reported a requirement for binding of catalytically active factor VIIa in tissue factor-dependent experimental metastasis. Collectively these studies indicate that both the extracellular functions of the catalytically active TF-VIIa complex and signaling functions of the TF cytoplasmic domain play an important role in supporting the processes of angiogenesis and metastasis. Our results might support the hypothesis that TF plays a role in VEGF regulation, since inhibition of the hypoxia-induced upregulation of tumor cell procoagulant activity by pentoxifylline (PTX) also inhibited the increase in VEGF synthesis by three different malignant cell lines.

The cellular functions that occur during angiogenesis can be divided into the 'activation' phase that includes initiation and progression, and the 'resolution' phase that encompasses termination and vessel maturation (35). Upregulation of VEGF is clearly an activation event which could arise from stimuli such as hypoxia, hypoglycemia and, perhaps, upregulation of TF expression. Amongst molecules that are active during the 'activation' phase, only VEGF meets most of the criteria for an angiogenic factor (36, 37). TF may play a dual role in promoting angiogenesis. Thus, in the 'activation' phase of neovascularization, the role of TF could be independent of its procoagulant activity possibly by potentiating VEGF production as a result of hypoxia. Conversely, TF expression may be secondary to VEGF synthesis as evidenced by the report that endothelial and monocyte procoagulant activity may be induced by VEGF (38). In the 'resolution' phase of the angiogenic process, however, TF may promote local thrombin generation and intra-tumoral fibrin deposition through its procoagulant activity.

In conclusion, we report for the first time that tumor cell TF activity is increased by hypoxia, a stimulus that is known to upregulate VEGF release and effect angiogenesis. We further demonstrated that PTX (a known downregulator of TF) inhibits both the hypoxia-induced increase in procoagulant activity and VEGF release. Our results do not shed light on the mechanism by which hypoxia renders tumor cells more procoagulant, or by which pentoxifylline inhibits this phenomenon. Such studies are currently ongoing. Nevertheless, they further emphasize an apparent relationship between TF and VEGF, elucidation of which may have important implications for our understanding of angiogenesis and metastasis.

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