PTEN and Hypoxia Regulate Tissue Factor Expression and Plasma Coagulation by Glioblastoma

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Abstract

We have previously proposed that intravascular thrombosis and subsequent vasooobclusion contribute to the development of pseudopalisading necrosis, a pathologic hallmark that distinguishes glioblastoma (WHO grade 4) from lower grade astrocytomas. To better understand the potential prothrombotic mechanisms underlying the formation of these structures that drive tumor angiogenesis, we investigated tissue factor (TF), a potent procoagulant protein known to be overexpressed in astrocytomas. We hypothesized that PTEN loss and tumor hypoxia, which characterize glioblastoma but not lower grade astrocytomas, could up-regulate TF expression and cause intravascular thrombotic occlusion. We examined the effect of PTEN restoration and hypoxia on TF expression and plasma coagulation using a human glioma cell line containing an inducible wt-PTEN cDNA. Cell exposure to hypoxia (1% O2) markedly increased TF expression, whereas restoration of wt-PTEN caused decreased cellular TF. The latter effect was at least partially dependent on PTEN’s protein phosphatase activity. Hypoxic cells accelerated plasma clotting in tilt tube assays and this effect was prevented by both inhibitory antibodies to TF and plasma lacking factor VII, implicating TF-dependent mechanisms. To further examine the genetic events leading to TF up-regulation during progression of astrocytomas, we investigated its expression in a series of human astrocytes sequentially infected with E6/E7/human telomerase, Ras, and Akt. Cells transformed with Akt showed the greatest incremental increase in hypoxia-induced TF expression and secretion. Together, our results show that PTEN loss and hypoxia up-regulate TF expression and promote plasma clotting by glioma cells, suggesting that these mechanisms may underlie intravascular thrombosis and pseudopalisading necrosis in glioblastoma.

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Introduction

Glioblastoma (WHO grade 4) is the most common and the highest grade astrocytoma (1). Currently incurable, it has a mean survival of only 50 weeks following standard surgical and adjuvant therapies (2). More telling of its explosive natural behavior, survival after surgical resection alone averages 14 weeks (3). Compared to lower grade astrocytomas (grades 2 and 3), glioblastomas have radial growth patterns, present in >90% of well-sampled cases. Deep venous thrombosis of the lower extremities occurs in 20% to 30% of glioblastoma patients, indicating a profound systemic disturbance in coagulation. In contrast, lower grade astrocytomas are not associated with the same degree of coagulopathy.

Tissue factor (TF), one of the body’s most potent procoagulants, is up-regulated in a variety of neoplasms including astrocytomas, and its levels correlate with tumor grade (9–11). TF is usually a 47 kDa transmembrane receptor expressed by perivascular stromal cells but also exists as a soluble form. Upon disruption of vascular integrity, TF comes in contact with its activating ligand from the plasma, factor VII/VIIa, which in turn causes thrombin activation, platelet aggregation, fibrin deposition, and local hemostasis. TF is expressed in >90% of malignant astrocytomas, but only in 10% of grades 1 and 2 astrocytomas. Its expression has been localized to neoplastic cells by in situ hybridization and levels correlate with the extent of necrosis (12). This led us to hypothesize that thrombosis in glioblastomas could be promoted by increased TF expression. Precise mechanisms that up-regulate TF in astrocytomas are not known, but triggering events would be expected to be specific to glioblastoma, where intravascular thrombosis and necrosis are apparent, and not present in lower grade astrocytomas.

PTEN is a tumor suppressor with dual lipid and protein phosphatase activity (13). The lipid phosphatase activity of PTEN antagonizes phosphoinositide 3 (PI 3)-kinase by its conversion of phosphatidylinositol 3,4,5-trisphosphate (PIP3) to phosphatidylinositol 3,4-bisphosphate (PIP2). Loss of PTEN leads to Akt activation, a frequent finding in glioblastomas (14–16). PTEN lipid phosphatase activity also normally suppresses the Ras/MEK/ERK signaling cascade that is up-regulated in the majority of glioblastomas (17). PTEN protein phosphatase activity is less well characterized, but likely has critical tumor suppressive functions of its own (18). PTEN is inactivated by mutation in 20% to 40% of
giblastomas (19). Inactivation through other mechanisms, such as PTEN promotor methylation, leads to a loss of PTEN expression in 70% to 80% of glioblastomas (20). Importantly, PTEN loss is a genetic signature of glioblastoma that is rarely noted in lower grade astrocytomas, making it a prime candidate for TF regulation.

Tumor hypoxia is another dominant pathophysiological process that develops as gliomas undergo high-grade progression and become more densely cellular (21, 22). A recent model of experimental gliomas suggests that hypoxia may also be initiated by vascular regressive events following the initial co-option of native vessels by neoplastic cells (23). Here we have investigated whether PTEN loss and hypoxia regulate TF expression by glioblastoma in a manner that could promote intravascular thrombosis, necrosis, and biological progression.

Materials and Methods

Glioblastoma Cell Lines and Culture Conditions. Human glioblasto-

ma cell lines (LN229, 23.11, and U87MG) and their cell culture conditions have been previously described (24). 23.11 is a clone derived from PTEN-null U87MG cells after stable transfection with an expression vector containing a murinestable-ducible wt-PTEN cDNA. LN229 cells contain wild-type PTEN. Two glioblastoma cell lines derived from U87MG containing inducible mutant PTEN, 23.24GE (lipid phosphatase inactive due to a G129E mutation) and 23.44GR (both lipid and protein phosphatase inactive due to a G129R mutation) were cultured as previously described (25). Human astrocytes that have been sequentially infected with human papillomavirus oncogenes E6/E7 and the catalytic component of human telomerase (hTERT) in combination with Ras and/or Akt were maintained in culture as previously described (26). Cells used in experiments were grown to 80% confluence in 100 mm culture dishes, placed in serum-free media in conditions of 21% O2 (normoxia) or 1% O2 (hypoxia) for 23.11, 23.24GE, and 23.44GR cells, PTEN was induced using 0.5 μmol/L muristerone (Invitrogen, Carlsbad, CA). For experiments in 1% O2 conditions, cells were placed in small Modular Incubator Chambers (Billups-Rothenberg, Del Mar, CA) which were flushed with 94% N2, 5% CO2, and 1% O2, and then stored in 37°C incubators. After 24 hours, cell pellets and conditioned media were collected and immediately frozen at –70°C.

Reagents. Culture media and other cell culture reagents were obtained from Mediatech, Inc. (Herdon, VA). 129/4002 and U9216 were obtained from EMD Biosciences, Inc. (San Diego, CA); 10 μmol/L of each compound in serum-free media was used in experiments. Rapamycin, purchased from LC Laboratories (Woburn, MA), was used at 5 μmol/L in serum-free media.

Tilt Tube Plasma Clotting Assay. Plasma clotting times induced by tumor cells or conditioned media were measured in triplicate using a tilt-tube assay with all reagents maintained at 37°C. Tumor cells (10²) grown in serum-free media under normoxic or hypoxic conditions for 24 hours were rinsed thrice in PBS, scraped from the dish, and resuspended in 1.0 mL PBS. The conditioned medium was collected and briefly centrifuged (1,500 rpm for 5 minutes) to remove any cell debris before its use in clotting assays. Two hundred microliters of cell suspension or conditioned media was added to 200 μL of citrated human plasma (Precision Biologic, Dartmouth, Nova Scotia) and 200 μL of 25 mmol CaCl₂ was added to the tube to initiate the clotting process. Clotting time was assayed visually by noting when the liquid formed a semisolid gel that did not flow during tube tilting. Positive controls for each experiment included Neoplastine clotting reagent (Diagnostica Stago, Parsippany, NJ) in place of tumor cell suspension (clot time = 17-18 seconds in tilted tube assays). Negative controls omitted tumor cell suspension. To establish whether TF was important for in vitro clotting, an inhibitory antibody to TF (monoclonal, 1:100; cat. no. 4509, American Diagnostica, Stamford, CT) was incubated for 20 minutes at 37°C with the cell suspension or conditioned media prior to assessing clotting time. Nonspecific IgG served as a negative control. Because TF requires factor VII to initiate coagulation, the importance of TF was also addressed by performing clotting assays using human plasma depleted of factor VII (R² Diagnostics, South Bend, IN).

Western Blot Analysis. Immunoblots were done on proteins from cell lysates of the indicated cell lines. Cells were lysed immediately before use in 50 mmol Tris-base (pH 7.0), 150 mmol NaCl, 2 mmol EDTA, 1% NP-40, and 1% protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentrations were determined by a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (30 μg) were resolved on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated in blocking solution (PBS containing 0.2% Tween 20 and 5% nonfat milk) and incubated overnight at 4°C with antibodies specific for TF (mouse monoclonal, 1:1,000; cat. no. 4503, American Diagnostica), PTEN (rabbit polyclonal, 1:100; Cell Signaling, Beverly, MA), Akt and phospho-Akt (rabbit polyclonal, 1:1,000; Cell Signaling), ERK (rabbit, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) and phospho-ERK (mouse monoclonal, 1:200; Santa Cruz Biotechnology). Blots were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (1:2,000, Bio-Rad) for 1 hour at room temperature and developed by enhanced chemiluminescence reagents (Pierce Biotechnol-
yogy, Rockford, IL). β-Actin was probed by goat anti-human actin antibody (1:1,000, Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated swine anti-goat antibody (1:1,000, Roche Molecular Biochemicals, Indianapolis, IN).

Northern Blot Analysis. Total cellular RNA was collected from confluent cell cultures by scraping cells in TRIzol reagent (Invitrogen). Ten micrograms of total RNA sample was subjected to electrophoresis in 1% agarose-formaldehyde gels. RNA was transferred overnight in 12× SSC buffer to nylon membranes (Amersham Pharmacia, Piscataway, NJ) and then UV cross-linked. A TF cDNA probe (1.35 kb fragment) was isolated from plasmid pSP64TFHS containing human TF cDNA (kindly provided by Dr. Josiah Wilcox, Department of Hematology/Oncology, Emory University, Atlanta, GA) with the HinDIII and SacI restriction enzymes (New England Biolabs, Beverly, MA). The probe was then labeled with 32P-dCTP (DuPont NEN, Boston, MA) using random priming labeling kit (Stratagene, Cedar Creek, TX) and ProbeQuant G-50 Micro Columns (Amersham Pharmacia) to separate the probe from free nucleotides. Hybridization was done in QuikHyb solution (Stratagene, La Jolla, CA) using 40 μL of Salmon Sperm DNA (Ambion Inc., Austin, TX) at 42°C overnight. Blots were washed twice with 2× SSC containing 0.1% SDS at 37°C, and then exposed to film. Membranes were stripped and rehybridized with human glyceraldehyde-3-phosphate dehydrogenase cDNA probe as a control for RNA loading.

ELISA. Concentrations of TF in conditioned serum-free media from 23.11, LN229, U87MG and sequentially transformed astrocytes (E6/E7/ TERT, Akt, Ras, and Ras + Akt) grown in hypoxia or normoxia were measured by ELISA per the manufacturer’s instructions (Immunobind TF ELISA kit, American Diagnostica).

Immunohistochemistry. Archived surgically resected glioblastoma specimens were retrieved from the Department of Pathology, Emory University Hospital. Glioblastomas were selected from patients with no prior treatment with radiation or chemotherapy. Cell pellets were collected from 23.11 cells grown in serum-free media in hypoxia or normoxia for 24 hours. Both cell pellets and glioblastoma tissue were fixed in 10% buffered formalin, routinely processed, and paraffin-embedded. Immunohistochemical studies were done on 6 μm sections. Sections were deparaffinized and subjected to heat-induced epitope retrieval by steaming for 15 minutes. Slides were then incubated with antibodies directed toward TF (1:1,000, cat. no. 4503, American Diagnostica) at 4°C overnight. Antibodies were detected using the avidin-biotin-peroxidase complex method using 3,3’-diaminobenzidine as the chromogen. Standard positive controls were used throughout. Normal sera served as the negative control. Sections were counterstained with hematoxylin.

Statistical Analysis. All quantitative analyses were repeated thrice. Quantitative data are expressed as mean ± SE. Comparisons were analyzed by Student’s t test and ANOVA. Significance was defined as P < 0.05.
Results

Hypoxia and PTEN Loss Promote Plasma Clotting by Glioblastoma Cells and Conditioned Media. To test whether glioma cells or conditioned media derived from them could promote plasma coagulation, we used a tilt tube assay where citrated human plasma, CaCl₂, and a procoagulant (thromboplastin, glioma cells, or conditioned media) are mixed and gently rocked at 37°C until a gel clot is formed. We observed that plasma clotting occurred in 17 to 18 seconds with the positive control thromboplastin in our assay (Neoplastine; mean ± SE = 17.3 ± 0.3 seconds), comparable to values of automated prothrombin times in coagulation laboratories (11-15 seconds, depending on the source of thromboplastin; Fig. 1A). Without thromboplastin (PBS, negative control), plasma clotting occurred after 15 minutes. Addition of 10⁷ normaloxic 23.11 glioma cells (−PTEN) resulted in plasma clotting at 136 ± 4 seconds (Fig. 1A).

We next determined whether hypoxia and PTEN restoration could influence the ability of glioma cells to promote plasma coagulation. Plasma clotting was significantly accelerated (65 ± 2 seconds; *P < 0.05) in the presence of 23.11 glioma cells grown in hypoxia (Fig. 1A). Restoration of wt-PTEN in hypoxic 23.11 glioma cells by exposure to muristerone (see Materials and Methods) resulted in a significant increase in plasma clotting time (83 ± 2 seconds; **P < 0.05).

To determine whether glioma cells have extracellular procoagulant factors, we examined the ability of conditioned media from 23.11 cells to induce plasma clotting. We found that conditioned media from normoxic 23.11 glioma cells (−PTEN) caused plasma clotting at 104 ± 5.5 seconds. Clotting occurred more rapidly in the presence of conditioned media from hypoxic glioma cells (82 ± 7.1 seconds; *P < 0.05; Fig. 1B). Restoration of wt-PTEN modestly prolonged clotting times from conditioned media under both normoxic and hypoxic conditions. Serum-free media that had not been exposed to glioma cells induced plasma clotting after 15 minutes and was not affected by muristerone (data not shown). Hypoxia also led to shortened plasma clotting induced by parental U87MG cells and conditioned media, but plasma clotting times were not influenced by muristerone in these cells (data not shown). These results showed that both cell-bound and extracellular factors expressed by glioma cells can dramatically accelerate plasma clotting. The factors responsible are up-regulated by hypoxia and somewhat restrained by PTEN.

Hypoxia and PTEN Loss Cause Increased Tissue Factor Levels in Cultured Glioblastoma Cells and in Conditioned Media. To determine whether the procoagulant TF could potentially contribute to shortened plasma clotting times caused by glioma cells under hypoxia or following PTEN loss, we examined the influence of these factors on TF expression in U87MG and 23.11 cells. Exposure to hypoxia caused dramatic increases in TF protein and mRNA expression as shown on Western and Northern blots (Fig. 2A and B). Restoration of wt-PTEN in 23.11 cells by exposure to muristerone led to a decrease in TF protein and mRNA levels. Muristerone had no effect on TF levels in U87MG cells. Immunohistochemical staining of formalin-fixed 23.11 cell pellets confirmed the increased TF expression under hypoxia and revealed a cell membrane staining pattern, typical of a transmembrane receptor (Fig. 2C).

TF concentrations in conditioned media from 23.11 glioma cells under normoxia and hypoxia were determined by ELISA. Under normoxia, TF levels were 2097 ± 11 and 2059 ± 72 pg/mg protein without and with wt-PTEN, respectively (Fig. 2D). Under hypoxia, TF protein concentrations were 3649 ± 20 and 2878 ± 35 pg/mg protein without and with wt-PTEN, respectively, significantly higher than normoxia (*P < 0.001). Restoration of wt-PTEN significantly reduced the concentration of TF in conditioned media under hypoxic conditions (**P < 0.05) but not under normoxia. Combined the above results show that hypoxia as well as signaling pathways activated by PTEN loss augmented TF expression by glioma cells in a manner that could account for accelerated plasma clotting.

Shortened Plasma Clotting Times Are Dependent on Tissue Factor. Two experimental approaches were undertaken to determine whether the TF expression by glioma cells were at

Figure 1. Hypoxia and PTEN loss promote plasma clotting by glioblastoma cells and conditioned media. A, 23.11 cells (10⁷) grown in serum-free media under hypoxic (H, 1% O₂) conditions for 24 hours and suspended in PBS induced plasma clotting at shorter times than those grown under normoxia (N, 21% O₂; *P < 0.05). Restoration of wt-PTEN significantly prolonged the plasma clotting time under hypoxia (*P < 0.05) but only slightly under normoxia. The positive control (Neoplastine) caused clotting at 17.3 ± 0.3 seconds, whereas negative control containing no tumor cells (PBS) caused clotting only after 15 minutes (broken bar in graph). Points, mean of experiments done in triplicate; bars, SE. B, hypoxia and PTEN loss promote plasma clotting by conditioned media. Conditioned serum-free media collected from 23.11 cells following 24 hours of hypoxia (H) induced plasma clotting at shorter times than media collected following normoxia (N, *P < 0.05) Restoration of wt-PTEN slightly increased the clotting time under both normoxia and hypoxia (points, mean; bars, SE; n = 3).
least in part responsible for promoting plasma clotting. First, we used an inhibitory TF monoclonal antibody to counteract the effects of TF on plasma coagulation. Second, because TF depends on binding and activation of factor VII to initiate clotting, we examined tumor-induced plasma clotting times using factor VII–deficient human plasma. We found that plasma clotting times were significantly prolonged by both the addition of the inhibitory antibody to TF and by the use of plasma lacking factor VII ($P < 0.001$; Fig. 3), whereas the use of nonspecific IgG had no effect. Hypoxia alone caused significant shortened clotting time ($P < 0.05$).

We further showed that clotting caused by conditioned media was TF-dependent using the inhibitory monoclonal antibody to TF. Plasma clotting time induced by conditioned media from hypoxic 23.11 cells was prolonged from 82 ± 7.2 to 540 ± 26 seconds in the presence of the inhibitory TF antibody ($P < 0.05$; data not shown). Together, these data strongly suggest that a large component of accelerated coagulation induced by glioma cells and conditioned media is due to TF.

PTEN Suppresses Tissue Factor Expression Independently of Its Lipid Phosphatase Activity. To better understand which of the signaling pathways activated by PTEN loss induce TF expression, we used two mutant PTEN cell lines to investigate the relative contribution of lipid and protein phosphatase activity. 23.24GE is a cell line with a muristerone-inducible PTEN mutant (G129E) that lacks lipid phosphatase activity. 23.44GR contains an inducible PTEN mutant (G129R) that lacks both lipid and protein phosphatase activity. We found that cells lacking lipid and protein phosphatase activity (G129R) did not show any appreciable suppression of hypoxia-induced TF expression (Fig. 4A). Cells lacking only lipid phosphatase activity (G129E; noted by their inability to reduce Akt phosphorylation) retained the ability to suppress hypoxia-induced TF levels. Thus, the protein phosphatase activity of PTEN seems to substantially contribute to TF regulation (Fig. 4A). Phosphorylation of Akt was not appreciably

**Figure 2.** Hypoxia and PTEN loss cause increased TF levels in glioblastoma cells and in conditioned media. A. 23.11 cells grown in serum-free media for 24 hours under hypoxia (H, 1% O2) show increased TF protein by Western blot, but very little TF protein by Western blot, but very little TF protein expression under normoxia (N, 21% O2). Restoration of wt-PTEN in these cells following 0.5 pmol/L muristerone (M) exposure led to decreased TF expression. B. TF mRNA was increased by hypoxia in both 23.11 and parental U87 MG cells on Northern blots. Down-regulation of TF mRNA following exposure to muristerone (M) was noted for 23.11 cells (inducible PTEN) but not for parental U87MG (PTEN null). C. 23.11 cells grown under normoxia (top) and hypoxia (bottom) for 24 hours were pelleted and formalin-fixed for immunohistochemical staining for TF. Cells grown under hypoxia showed substantially higher TF expression, which was present in the highest levels near the cell membrane (arrow). Cells are counterstained with hematoxylin. D. conditioned media collected from hypoxic 23.11 cells showed increased TF concentrations compared with normoxia as determined by ELISA ($P < 0.05$). Restoration of wt-PTEN by exposure to muristerone (M) decreased TF levels under hypoxia ($P < 0.05$), but not under normoxia (points, mean; bars, SE; n = 3).

**Figure 3.** Shortened plasma clotting times are dependent on TF. 23.11 cells (~PTEN, 106) grown in serum-free media under hypoxia (H, 1% O2) or normoxia (N, 21% O2) for 24 hours were tested for their ability to induce plasma clotting in the presence of an inhibitory anti-TF monoclonal antibody (Anti-TF mAb) or nonspecific IgG. Hypoxia caused significantly shortened plasma clotting times compared with normoxia ($P < 0.05$). The addition of TF mAb significantly prolonged the plasma clotting times both under normoxia and hypoxia ($P < 0.001$). Points, mean of experiments done in triplicate; bars, SE.
due to direct activity on phospho-Akt. be down-regulated by the protein phosphatase activity of PTEN, but was not of expression of phospho-Akt and TF. These results suggested that TF could be regulated by PTEN lipid phosphatase–inactive (G129R) forms of PTEN caused reduced levels of phospho-ERK1/2. The MEK/ERK inhibitor (U0126, 10 μm) led to reduced levels of phospho-ERK1/2. These results suggested that regulation of TF by the protein phosphatase activity of PTEN was not due to its activity on pERK1/2.

Reduced in G129E cells in a manner that might suggest that phospho-Akt was a direct target of PTEN protein phosphatase activity and was responsible for reduced TF levels (Fig. 4A). As a positive control, we found that glioma cells with wt-PTEN (LN229) had low levels of both phosphorylated Akt and TF. We also found that the levels of phosphorylated ERK 1/2 (ERK 44/42) were not reduced by lipid phosphatase inactive PTEN (G129E; Fig. 4B), suggesting that phospho-ERK 1/2 is an unlikely candidate for the suppressive effects PTEN protein phosphatase on TF. Cell treatment with U0126, a known inhibitor of ERK 1/2 phosphorylation, was used as a positive control. Thus, although a component of TF regulation depends on the protein phosphatase activity of PTEN, the precise substrate(s) of this activity remains to be identified.

Both Akt and Ras Pathways Modulate Tissue Factor Expression. The above experiments showed a significant regulation of TF by the protein phosphatase activity of PTEN, but could not adequately establish the effects of PTEN lipid phosphatase on TF. Because we do not possess a glioma cell line with a mutant PTEN lacking only protein phosphatase activity, we chose to investigate intracellular signaling pathways under the regulation of PTEN lipid phosphatase. PTEN regulates both the PI 3-kinase/Akt/mTOR and Ras/MK/ERK signaling pathways. We investigated the relative contribution of Akt and Ras pathway activation in the regulation of TF in a cell culture model that recapitulates astrocytoma progression, which uses human astrocytes that have been sequentially infected with E6/E7/hTERT, Akt, and/or Ras. We found that TF expression was consistently up-regulated by hypoxia at each stage of astrocyte transformation (Fig. 5A). Although Ras and Akt both caused up-regulation of TF, the normoxic and hypoxic increases in TF protein were substantially greater following Akt compared with Ras transformation. The combination of Ras and Akt showed the highest level of TF expression by Western blot (Fig. 5A).

TF concentrations in conditioned media of these sequentially transformed astrocytes was measured by ELISA and showed a similar pattern to TF levels in cell lysates (Fig. 5B). The largest incremental increase in TF concentrations was noted following Akt transformation and the highest level overall was present in conditioned media from cells transformed with both Ras and Akt. In addition to the up-regulation of TF at each stage of astrocyte

**Figure 4.** PTEN suppresses TF expression independently of its lipid phosphatase activity. A, Western blotting shows increased TF expression by 23.11 cells under hypoxia (H) that is suppressed by wt-PTEN (M, + muristerone). Hypoxia-induced TF was also suppressed following muristerone exposure in 23.24G cells, which have an inducible mutant form of PTEN that lacks lipid phosphatase activity (G129E). Hypoxia-induced TF was not suppressed in 23.44GR, which contains a muristerone-inducible form of PTEN that lacks both lipid and protein phosphatase activity (G129R). The level of Akt phosphorylation was reduced following the restoration of wt-PTEN, but not following the restoration of mutant forms of PTEN in G129E or G129R cells. LN229 is a glioma cell line with endogenous wt-PTEN and shows low levels of expression of phospho-Akt and TF. These results suggested that TF could be regulated by PTEN lipid phosphatase activity of PTEN, but was not due to direct activity on phospho-Akt. B, to investigate whether the protein phosphatase activity of PTEN might regulate the Ras/MK/ERK pathway, the phosphorylation of ERK1/2 (p44/42) was studied by Western blot. Neither induction of lipid phosphatase inactive (G129E) or lipid and protein phosphatase–inactive (G129R) forms of PTEN caused reduced phospho-ERK1/2. The MEK/ERK inhibitor (U0126, 10 μm) led to reduced levels of phospho-ERK1/2. These results suggested that regulation of TF by the protein phosphatase activity of PTEN was not due to its activity on pERK1/2.

**Figure 5.** Both Akt and Ras pathways modulate TF expression by sequentially transformed astrocytes. A, Western blots done on cell lysates show that TF was up-regulated by hypoxia (H) at each transformation step in sequentially transformed human astrocytes as compared with normoxia (N). Both E6/E7/hTERT/Akt- and E6/E7/hTERT/Ras-transformed astrocytes showed up-regulated TF expression compared with E6/E7/hTERT-transformed astrocytes, with transformation by Akt causing the largest incremental increase. The combination of Ras and Akt showed the highest overall level of TF. B, both Akt and Ras pathways modulate TF concentrations in conditioned media by sequentially transformed astrocytes. TF concentrations from conditioned media of sequentially transformed human astrocytes were measured by ELISA. Both E6/E7/hTERT/Akt and E6/E7/hTERT/Ras transformed astrocytes showed increased TF concentrations in conditioned media compared with E6/E7/hTERT-transformed astrocytes, with transformation by Akt causing the largest incremental increase (*P < 0.05). The combination of Ras and Akt showed the highest overall level of TF. TF concentrations were also significantly higher in conditioned media following hypoxia compared with normoxia at each step of transformation (*P < 0.001; points, mean; bars, SE; n = 3).
Increased Tissue Factor Expression is Present in Hypoxic Pseudopalisades in Human Glioblastoma Specimens. TF expression in astrocytomas increases with tumor grade and is highest in glioblastoma (9). To investigate whether the expression pattern of TF in human glioblastoma could be explained by its hypoxic regulation, we did an immunohistochemical analysis in seven formalin-fixed paraffin-embedded surgical resection specimens. We found that the highest TF expression was present within cells that form the hypoxic pseudopalisades surrounding necrosis, supporting the contention that hypoxia could cause TF up-regulation in human disease. Cells at the inner aspect of pseudopalisades showed the greatest expression. We also noted a moderate expression of TF in a perivascular distribution, which is most likely not due to hypoxia (Fig. 7). TF expression in neoplastic cells that were infiltrating brain parenchyma was mildly elevated. No TF expression was identified in vascular endothelial cells, nonneoplastic astrocytes or neurons in adjacent normal brain (data not shown).

Discussion

A dramatic shift in biological behavior occurs following the transition from anaplastic astrocytomas (grade 3) to glioblastoma (grade 4). The latter is characterized by pseudopalisading necrosis and increased levels of angiogenesis, features that are pathophysiologically linked and mechanistically instrumental to disease progression (8, 27). Recent studies indicate that intravascular thrombosis may initiate these events by causing vasoocclusion, thereby sparking a cascade that includes tumor cell migration away from hypoxia (pseudopalisading), hypoxia-inducible factor–mediated vascular endothelial growth factor secretion, adjacent angiogenesis (microvascular hyperplasia), and rapid peripheral tumor expansion (8, 22, 23).

A strong relationship between abnormal blood clotting and human malignancy is well established (23, 28). In particular, patients with glioblastoma have a high frequency of deep vein thrombosis and pulmonary thromboembolism (29). Moreover, microscopic thrombotic vascular occlusion is present within in nearly all human glioblastoma specimens (8, 30). The frequencies of intravascular thrombosis in neoplastic tissue and at distant sites are much higher in patients with glioblastomas than anaplastic transformation (\(^*P < 0.05\)), a significant hypoxic up-regulation of TF was noted at each step (\(^*P < 0.001\); Fig. 5B).

We independently examined the relative contribution of Ras/MEK/ERK and PI 3-kinase/Akt/mTOR intracellular signaling pathways on TF expression in human glioma cells using pharmacologic inhibitors. Western blots on 23.11 glioma cells (−PTEN) showed that hypoxia-induced TF expression was attenuated following restoration of wt-PTEN (Fig. 6, compare lanes 5 and 6). We found that hypoxia-induced TF expression was reduced to a similar degree following inhibition of PI 3-kinase (LY294002, 10 μmol/L), mTOR (rapamycin, 5 μmol/L), and Ras/MEK/ERK (U0126, 10 μmol/L) showed decreased expression of TF, with levels similar to those following PTEN restoration. Akt-phosphorylation was reduced by restoration of PTEN and by LY294002 treatment, but not by rapamycin or U0126 treatment.

Figure 6. Inhibition of Ras/MEK/ERK and PI 3-kinase/Akt/mTOR signaling pathways leads to reduced TF expression. 23.11 cells exposed to hypoxia for 24 hours showed increased TF expression by Western blot and exposure to muristerone (M, +PTEN) led to a reduction in hypoxia-induced TF expression (compare lanes 5 and 6). Hypoxic 23.11 cells (−PTEN) incubated with inhibitors of PI 3-kinase (LY, LY294002, 10 μmol/L), mTOR (Rap, rapamycin, 5 μmol/L), and Ras/MEK/ERK (U0126, 10 μmol/L) showed decreased expression of TF, with levels similar to those following PTEN restoration. Akt-phosphorylation was reduced by restoration of PTEN and by LY294002 treatment, but not by rapamycin or U0126 treatment.
astrocytomas, indicating that critical prothrombotic events must occur at this transition.

Here we have shown that PTEN loss and hypoxia—both events occurring at the anaplastic astrocytoma to glioblastoma transition—lead to increased expression of the potent procoagulant TF by neoplastic cells and promote plasma coagulation. We found a consistently strong up-regulation of TF in response to hypoxia (1% O2) in glioma cell lines and sequentially transformed astrocytes. Hypoxia-induced TF expression was most evident within neoplastic cells and at the cell surface, as might be expected for a transmembrane protein that elicits a strong procoagulant response. We also noted a hypoxia up-regulation of TF within conditioned media of 23.11 glioma cells and sequentially transformed astrocytes, suggesting that a cleaved or secreted form of the protein might be released into the extracellular compartment during tumorigenesis and cause thrombotic events at a distance. Soluble extracellular TF has been documented as a full-length protein (31), an alternatively spliced variant (32), and a microparticle associated form (33). Our experiments showed that both TF expressed at the cell surface and in the conditioned media were capable of promoting plasma coagulation in vitro. In addition to its hypoxia-inducible procoagulant function in gliomagenesis, recent studies indicate that TF may also contribute to tumor progression by directly up-regulating vascular endothelial growth factor secretion and angiogenesis (34, 35).

How might hypoxia arise in astrocytomas to cause increased TF expression? One possibility is that the increased cell density associated with disease progression leads to modest hypoxia due to heightened metabolic demands. Alternatively (or in combination), vascular compromise could cause hypoxia. Recent investigations suggest that regression of native blood vessels may occur prior to hypoxic induction of angiogenesis in gliomas (8, 23). In this model, tumor cells initially access a vascular supply by “co-opting” existing vessels. In response, vascular endothelial cells up-regulate angiopoietin-2, which acts in an autocrine fashion as a Tie-2 receptor antagonist to cause endothelial apoptosis and vascular regression (36–38). Hypoxic up-regulation of TF by gliomas would then follow the initial angiopoietin-2-mediated vascular insult and accentuate tumor hypoxia due to intravascular thrombosis. Transcriptional mechanisms responsible for hypoxic up-regulation of TF expression in gliomas have not been defined. In nonneoplastic tissue, hypoxic up-regulation of TF seems to be due to activation of the early growth response-1 transcription factor (39).

In addition to the strong up-regulation of TF by hypoxia, we found that restoration of wild-type PTEN into a PTEN-null cell line (U87MG) leads to a moderate attenuation of TF expression, both within the cellular compartment and in extracellular conditioned media. These findings suggest that the loss of PTEN expression, which is noted in >80% of glioblastomas and occurs at the anaplastic astrocytoma to glioblastoma transition, might be a genetic trigger that promotes TF expression and intravascular thrombosis during the biological progression of astrocytomas. In our attempt to define downstream signaling cascades relevant to PTEN regulation of TF, we found that both the Ras/MEK/ERK and Pi 3-kinase/Akt/mTOR pathways are capable of modulating TF expression, especially under hypoxic conditions. Inhibitors of the MEK1/MAPK and Pi 3-kinase have also been shown to reduce TF expression in colon cancer cell lines (40). Combined, our studies using pharmacologic inhibitors of Ras and Akt pathways and our investigations using sequentially transformed human astrocytes indicated that both Ras and Akt pathways contribute to the regulation of TF in gliomas. In sequentially transformed astrocytes, activation of the Akt pathway had the largest incremental influence on TF expression.

PTEN normally down-regulates Akt activation due to its lipid phosphatase activity on phosphoinositols and antagonism of PI 3-kinase (14, 16). In light of the previous findings suggesting the relevance of Akt on TF expression, we were somewhat surprised to find that the mutant forms of PTEN that lack lipid phosphatase activity (G129E) retained the ability to attenuate hypoxia-induced TF expression. In contrast, the mutant form of PTEN lacking both lipid and protein phosphatase activity was not able to modulate TF. These experiments strongly suggested that the protein phosphatase activity of PTEN was capable of regulating TF. In these same studies, we also found that the levels of phospho-Akt were not altered by lipid phosphatase–inactive PTEN, suggesting that the protein phosphatase activity of PTEN was not acting directly on phospho-Akt to control TF expression. Similarly, the levels of phospho-ERK1/2 were not diminished by lipid phosphatase–inactive PTEN, indicating that it is not a likely substrate of PTEN protein phosphatase that controls TF. Altogether, the data indicate that the Akt and Ras pathways both have a regulatory impact on TF expression. However, a second component of TF regulation by PTEN depends on protein phosphatase activity and seems to be independent of the phosphorylation status of Akt or ERK1/2.

Lastly, our immunohistochemical studies indicate that TF expression levels vary within glioblastoma tissues in a pattern that is consistent with regulation by hypoxia. Previous studies have shown that TF expression is up-regulated in astrocytomas and that its levels increase with tumor grade (9). Here we showed that the highest level of TF expression in glioblastoma was seen in the hypoxic cells surrounding necrosis. These pseudopalisading cells are thought to represent a wave of cells migrating away from vasoclosure, at least in part related to intravascular thrombosis (8). Another modest yet consistent increase in TF expression was present in a perivascular distribution within glioblastomas and therefore unlikely explained by hypoxic regulation. TF expression is normally highest in the perivascular region in nonneoplastic tissue, perhaps because cells in this distribution receive regulatory signals directly from the blood.

Increased TF expression by glioblastomas may partially explain the strong tendency toward intravascular thrombosis within neoplastic tissue and the high rate of coagulopathy in patients with this disease. Whether TF expression and subsequent intravascular thrombosis are initiating vasoocclusive events that triggers hypoxia in glioblastoma is not currently known. However, these phenomena could at least accentuate and potentially propagate the hypoxic conditions that are thought to drive glioblastoma into its rapid growth phase.

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