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Early Growth Response Gene-1 Regulates Hypoxia-Induced Expression of Tissue Factor in Glioblastoma Multiforme through Hypoxia-Inducible Factor-1–Independent Mechanisms

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Abstract
Hypoxia strongly up-regulates tissue factor and promotes plasma clotting by glioblastoma multiforme, but transcriptional mechanisms remain undefined. Here, we investigated the potential roles of early growth response gene-1 (Egr-1), Sp1, nuclear factor-kB (NF-kB), activator protein-1 (AP-1), and hypoxia-inducible factor-1 (HIF-1) in the hypoxic regulation of tissue factor by glioblastoma multiforme cells in vitro. Hypoxia (1% O2) strongly induced Egr-1 mRNA within 1 hour and led to nuclear localization of Egr-1 protein. Using luciferase reporter plasmids in glioma cells, we found that hypoxia dramatically increased luciferase activity in cells with constructs containing Egr-1-binding sites but not in cells with constructs containing AP-1- or NF-kB-binding sites. Electrophoretic mobility shift assays revealed hypoxia-induced Egr-1, but not Sp1, binding to oligonucleotides containing the Egr-1/Sp1 motif of tissue factor gene promoter. Using an expression vector containing the minimal tissue factor promoter (−111 to +14 bp) and small interfering RNA (siRNA) directed at Egr-1 and Sp1 mRNAs, we found that Egr-1 was required for maximal hypoxic induction of promoter activity. Forced overexpression of Egr-1 but not Sp1 by cDNA transfection caused up-regulation of tissue factor in glioma cells under normoxia (21% O2), whereas siRNA directed at Egr-1 strongly attenuated hypoxia-induced tissue factor expression. To examine the effects of HIF-1α on tissue factor expression, we used glioma cells stably transfected with a HIF-1α siRNA expression vector and found that HIF-1α mRNA silencing did not affect tissue factor expression under hypoxia. We conclude that hypoxic up-regulation of tissue factor in glioblastoma multiforme cells depends largely on Egr-1 and is independent of HIF-1. (Cancer Res 2006; 66(14): 7067-74)

Introduction
Glioblastoma multiforme is the most common form of malignant glioma and is characterized by widespread invasiveness, tumor necrosis, and angiogenesis (1). Microscopic intravascular thrombosis can be identified in >90% of glioblastoma multiforme specimens and vaso-occlusive mechanisms due to thrombosis have been proposed to initiate a cycle of hypoxia, necrosis, and the hypoxia-induced angiogenesis that leads to the rapid growth of these fatal tumors (2–5). Mechanisms that underlie the development of thrombosis within glioblastoma multiforme are not completely understood but may be related to the deregulated expression of procoagulant molecules.

Tissue factor, a 47-kDa transmembrane protein, is the primary initiator of blood coagulation in vivo. It normally triggers clotting by binding to and activating plasma-borne factor VII/VIIa in the setting of compromised vasculature (6). High levels of tissue factor are expressed in human gliomas and its expression correlates with both histologic grade and the extent of necrosis (7). Thus, up-regulated tissue factor expression by glioma cells may be critical for the development of intratumoral thrombosis, especially once vessels become leaky during glioma progression.

We have shown that tumor hypoxia strongly up-regulates tissue factor and promotes plasma clotting by glioblastoma multiforme cells in vitro (8). Previous studies indicate that tissue factor gene expression is regulated by several transcriptional factors that may be sensitive to hypoxia or anoxia, including activator protein (AP-1), nuclear factor-kB (NF-kB), Sp1, and early growth response gene-1 (Egr-1; refs. 9–11). Moreover, hypoxia-inducible factor-1 (HIF-1) is highly expressed in glioblastoma multiforme, especially in the hypoxic, perinecrotic “pseudopalisading” cells that over-express tissue factor, raising the possibility that it, too, may up-regulate tissue factor (3). Hypoxia-responsive elements, which are the primary binding sites for HIF-1, have not been reported within the tissue factor promoter (12). However, vascular endothelial growth factor (VEGF) up-regulates tissue factor expression in endothelial cells through activation of the KDR receptor (VEGFR2), which could suggest an indirect role of HIF-1 in the regulation of tissue factor, as VEGF is a direct HIF-1 target gene. Other HIF-1-dependent mechanisms could be relevant as well.

The tissue factor promoter contains two AP-1 sites and a NF-kB site within a distal enhancer region (−227 to −172 bp) and three overlapping Egr-1/Sp1-binding sites within a proximal enhancer region (−111 to +14 bp; ref. 12). The regulation of the tissue factor by each of these transcription factors strongly depends on the stimulus and on the cell type (13). Egr-1 is a zinc finger transcription factor that belongs to a family of early growth response genes (14). This phosphoprotein rapidly accumulates in the nucleus on stimulation by mitogens, a variety of cytokines, and cellular stress, including hypoxia (9, 15–17). Egr-1 has been reported to regulate tissue factor expression by displacing Sp1 from its binding site following external stimuli, whereas Sp1 is believed to mediate basal tissue factor expression (11). In the present study, we have investigated the transcriptional regulation...
of tissue factor expression in glioblastoma multiforme cells under hypoxia and show that Egr-1 is critical to this function, whereas HIF-1 is not.

Materials and Methods

Cell lines and cell culture. Human glioblastoma multiforme cell lines U87MG, 23.11, and U251MG cells were cultured in DMEM with 10% fetal bovine serum (FBS) as described previously (8, 18). 23.11, a clone derived from PTEN-null U87MG cells, has been stably transfected with an expression vector containing a murine-realisducible wt-PTEN cDNA (8).

In this study, it was used only under noninduced conditions. U251MG HIF-1α glioma cells were established by stable transfection with a HIF-1α small interfering RNA (siRNA) expression vector, and U251MG HIF-1α glioma cells were transfected with a nonsense siRNA expression vector. Cells used in experiments were grown to 70% to 90% confluence in 60-mm culture dishes or six-well plates and maintained in serum-free medium in conditions of 21% O2 (normoxia) or 1% O2 (hypoxia). For hypoxic treatment, cells were placed in Modular Incubator Chambers (Billups-Rothenberg, Del Mar, CA), which were flushed with 94% N2, 5% CO2, and 1% O2 for 5 minutes, sealed, and then kept in a regular tissue culture incubator. Cell pellets and conditioned medium were collected at indicated time points after transfection with plasmid. For hypoxic treatment, 23.11 glioma cells transfected with pEgr-1-Luc and pCtrl-Luc were cotransfected with 0.01 μg Renilla luciferase (RLuc) plasmid (Promega) as an internal control. For AP-1 and NF-κB luciferase reporter plasmids, 2 μg pAP-1-Luc or pNF-κB-Luc was cotransfected with 0.01 μg RLuc plasmid, pLuc-mCS plasmid (2 μg) was used as the control. For tissue factor minimal promoter plasmids, 2 μg pTF(wt)-Luc or pTF(mu)-Luc was cotransfected with the same amount of RLuc plasmid. Transfections were done for 5 hours in serum-free medium before normoxia (21% O2) or hypoxia (1% O2) treatment (16 hours). In a separate experiment, 23.11 glioma cells transfected with pEgr-1-Luc and pCtrl-Luc plasmids were treated with 10% FBS for 16 hours under normoxia. After harvesting, cell extracts were assayed for luciferase activity using a dual-luciferase reporter system (Promega). Light emission was quantified in a microplate luminescence reader (LU8lstar Galaxy, BMG Labtechnologies, GmbH, Germany). Transfections were done in triplicate and the results were calculated as the activity of firefly luciferase relative to that of the RLuc.

siRNA transfection. Egr-1- and Sp1-specific siRNA sequences (21 nucleotides) were purchased from Ambion (Austin, TX; Genbank accession nos. NM_001964 and NM_138473). Transfections (50 nmol/L Egr-1 or Sp1 siRNA) were accomplished by using Silencer siRNA Transfection II kit according to the supplier’s instruction (Ambion). Twenty-four to 48 hours after transfection, 23.11 glioma cells were placed in hypoxia (1% O2) for 1 or 24 hours, respectively. For cotransfection with plasmids, the cells were transfected with Egr-1, Sp1, or scrambled siRNA 24 hours before transfection with plasmids. Rhodamine-labeled nonsilencing siRNA (Qiana) was used to monitor the efficiency of transfection.

Western blot analysis. For total protein extraction, cells were lysed immediately before use in cold lysis buffer containing 50 mM/L Tris (pH 7.0), 150 mM/L NaCl, 2 mM/L EDTA, 1% NP40, and 1% protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). For separation of nuclear and cytoplasmic protein fractions before Western blot, we used the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined by Bio-Rad detergent-compatible (DC) protein assay. Equal amounts of protein (30–40 μg) were separated on a 10% SDS-PAGE and transferred to nitrocellulose membranes (Fisher Scientific, Suwanee, GA). Blotting was done with 5% nonfat dry milk (Bio-Rad) in PBS containing 0.01% Tween 20. Membranes were then incubated overnight at 4°C with antibodies specific for tissue factor (mouse monoclonal, 1:4,000; American Diagnostica, Stamford, CT) and Egr-1 (rabbit polyclonal, 1:3,000) as described previously (8, 24). HIF-1α (rabbit polyclonal, 1:200) and histone H1 (mouse monoclonal, 1:4,000) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-Sp1 antibody (mouse monoclonal, 1:2,000) was from BD Biosciences PharsMingen (San Diego, CA). Rabbit polyclonal antibodies (1:1,000) against c-Jun, phospho-c-Jun, NF-κB, phospho-NF-κB, Erk, and NF-κB/p50 and mouse monoclonal antibody against phospho-Erk were purchased from Cell Signaling Technology (Beverly, MA). Blots were washed and incubated with horseshad peroxidase (HRP) conjugated to goat anti-mouse or goat anti-rabbit antibodies (1:5,000; Bio-Rad) for 1 hour at room temperature and developed using enhanced chemiluminescence reagents (Pierce Biotechnology). β-Actin was detected by goat anti-human actin antibody (1:2,000; Santa Cruz Biotechnology) followed by HRP-conjugated swine anti-goat antibody (1:5,000; Roche Molecular Biochemicals, Indianapolis, IN).

Electrophoretic mobility shift assay. The nuclear protein fractions for electrophoretic mobility shift assay (EMSA) were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents following the manufacturer’s protocol. Protein concentrations were determined using the Bio-Rad DC protein assay. The oligonucleotide containing the Egr-1 consensus binding site (5’-CCCCGGGCCCCGGATCTTGAGTACA-3’) was used as reported previously (25). The Sp1 consensus oligonucleotide (5’-ATTCGATCCGGGGGGCCGAG-3’) spanning the tissue factor promoter region was purchased from Santa Cruz Biotechnology. Both Egr-1 and Sp1 consensus...
Egr-1 Regulates Tissue Factor in Glioblastoma

Hypoxia up-regulates Egr-1 expression in glioblastoma multiforme cells. Previous studies of monocytes and macrophages have indicated that Egr-1 is critical to the hypoxic regulation of tissue factor in nonneoplastic cells (9), but its role in neoplastic diseases and the contributions of other transcription factors have not been fully considered. We therefore determined whether hypoxia was able to up-regulate Egr-1 expression in glioma cells at a time that precedes up-regulation of tissue factor. We have shown previously that hypoxia (1% O₂) induces tissue factor expression after 6 to 8 hours in 23.11 and U87MG glioma cells (8). By real-time PCR, we found hypoxia strongly induced Egr-1 mRNA levels in both U87MG and 23.11 cells within 1 hour (4.93 ± 0.2– and 4.02 ± 0.2-fold induction, respectively) and maintained a moderate increase at 24 hours (1.49 ± 0.2– and 2.1 ± 0.5-fold induction, respectively) compared with normoxia (Fig. 1A). Similarly, we found increased Egr-1 protein levels detectable by Western blot at 1 and 24 hours in 23.11 cells that mirrored the increases in mRNA (Fig. 1B). siRNA directed at Egr-1 markedly inhibited both Egr-1 mRNA (P < 0.001) and protein expressions under hypoxia (Fig. 1A and B). Using immunofluorescence with antibodies directed against Egr-1, we showed that Egr-1 accumulated in the nuclei of 23.11 glioma cells at 1 hour following hypoxia and that siRNA directed against Egr-1 mRNA could inhibit this hypoxia-induced accumulation (Fig. 1C). 23.11 cells transfected with a luciferase reporter plasmid containing four Egr-1-binding sites (pEgr-1-Luc) along with a control reporter plasmid without Egr-1-binding sites (pCtrl-Luc) showed significantly increased luciferase activity under hypoxic conditions as in (A) were collected and Western blot showed a marked increase in Egr-1 protein expression at both 1 and 24 hours of hypoxia compared with normoxia (N). Egr-1 siRNA significantly inhibited the hypoxic up-regulation of Egr-1 protein. Luciferase activity was measured by ELISA. Columns, mean of experiments done in triplicate; bars, SD.

Results

Hypoxia up-regulates Egr-1 expression in glioblastoma multiforme cells. Previous studies of monocytes and macrophages have indicated that Egr-1 is critical to the hypoxic regulation of tissue factor in nonneoplastic cells (9), but its role in neoplastic diseases and the contributions of other transcription factors have not been fully considered. We therefore determined whether hypoxia was able to up-regulate Egr-1 expression in glioma cells at a time that precedes up-regulation of tissue factor. We have shown previously that hypoxia (1% O₂) induces tissue factor expression after 6 to 8 hours in 23.11 and U87MG glioma cells (8). By real-time PCR, we found hypoxia strongly induced Egr-1 mRNA levels in both U87MG and 23.11 cells within 1 hour (4.93 ± 0.2– and 4.02 ± 0.2-fold induction, respectively) and maintained a moderate increase at 24 hours (1.49 ± 0.2– and 2.1 ± 0.5-fold induction, respectively) compared with normoxia (Fig. 1A). Similarly, we found increased Egr-1 protein levels detectable by Western blot at 1 and 24 hours in 23.11 cells that mirrored the increases in mRNA (Fig. 1B). siRNA directed at Egr-1 markedly inhibited both Egr-1 mRNA (P < 0.001) and protein expressions under hypoxia (Fig. 1A and B). Using immunofluorescence with antibodies directed against Egr-1, we showed that Egr-1 accumulated in the nuclei of 23.11 glioma cells at 1 hour following hypoxia and that siRNA directed against Egr-1 mRNA could inhibit this hypoxia-induced accumulation (Fig. 1C). 23.11 cells transfected with a luciferase reporter plasmid containing four Egr-1-binding sites (pEgr-1-Luc) under serum-free conditions showed significantly increased (4-fold) luciferase activity under hypoxia (16 hours) compared with normoxia (P < 0.001), whereas the cells transfected with the plasmid lacking Egr-1-binding sites (pCtrl-Luc) showed only mild increase in luciferase activity (Fig. 1D). The cells transfected with pEgr-1-Luc showed significantly increased luciferase activities compared with the cells transfected with pCtrl-Luc both under normoxia and hypoxia (P < 0.001). Under normoxia, the cells transfected with pEgr-1-Luc and stimulated with 10% FBS, an
established activator for Egr-1, showed a 2-fold increased luciferase activity compared with those in serum-free medium. Hypoxia-induced luciferase activity could be significantly attenuated by siRNA directed against Egr-1 (Fig. 1D). We concluded that hypoxia strongly up-regulates Egr-1 expression and transcriptional activity in malignant glioma cells in vitro.

**Role of Egr-1 in hypoxia-induced tissue factor up-regulation in glioblastoma multiforme cells.** We next considered whether the overexpression of tissue factor that is consistently seen under hypoxia in gliomas depended on this increased Egr-1 activity. First, we found that tissue factor mRNA expression was strongly up-regulated by hypoxia (24 hours) in both U87MG and 23.11 glioma cells and that siRNA directed at Egr-1 dramatically attenuated this hypoxic up-regulation of tissue factor mRNA \((P < 0.001; \text{Fig. 2A})\). Similarly, hypoxic up-regulation of tissue factor protein in 23.11 cells was significantly inhibited by siRNA directed against Egr-1 (Fig. 2B). We next examined whether forced expression of an exogenous Egr-1 cDNA would induce increased tissue factor expression under normoxia. We transfected 23.11 cells with an Egr-1 expression plasmid \((p\text{Egr-1}, 2.5 \mu g)\) and found increased tissue factor expression compared with the cells transfected with control plasmid \((p\text{Ctrl}, 2.5 \mu g)\) and untransfected cells (mock; Fig. 2C). Combined, these results show that the increased expression of Egr-1 that occurs in hypoxic gliomas is able to up-regulate tissue factor expression.

**Sp1 is not up-regulated by hypoxia in glioblastoma multiforme cells.** Because Sp1 is also a critical regulator of tissue factor gene expression, we examined whether it contributed to the increased tissue factor found under hypoxia in human gliomas. Transfection of glioma cells with a Sp1 expression plasmid \((p\text{Sp1}, 2.0 \mu g)\) for 24 hours under normoxia led to a large increase in both nonphosphorylated (bottom band) and phosphorylated (top band) Sp1 protein expression without a concomitant tissue factor expression. Transfection with the related transcription factor Sp3 \((p\text{Sp3}, 2.0 \mu g)\) under the same conditions did not lead to increased tissue factor expression (Fig. 3A). Conversely, we found that siRNA directed at Sp1 in 23.11 cells significantly inhibited Sp1 protein level but did not affect the marked hypoxic up-regulation of tissue factor compared with normoxia. Nonspecific siRNA also did not affect hypoxic up-regulation of tissue factor. \(\alpha\)-Actin was used as loading control.

![Figure 2](image2.png)

**Figure 2.** A to C, Egr-1 is responsible for hypoxia-induced tissue factor up-regulation in glioblastoma multiforme cells. A, real-time PCR for tissue factor mRNA expression. U87MG and 23.11 cells were transfected with Egr-1 siRNA or nonspecific siRNA the day before hypoxic treatment (24 hours). Tissue factor (TF) mRNA levels were analyzed by real-time PCR. Hypoxia \((H, 24 \text{ hours})\) caused a large increase in tissue factor mRNA and Egr-1 siRNA treatment significantly inhibited this effect. Nonspecific siRNA had no effect on the hypoxic up-regulation of tissue factor \((* \text{ or } \times, P < 0.001, \text{ fold increase normalized to normoxia). Columns, mean of experiments done in triplicate; bars, SD. B, Western blot. Cell lysates of 23.11 cells treated similarly as tissue factor mRNA analysis \((A)\) were collected for tissue factor and Egr-1 protein analysis by Western blot. Hypoxia \((24 \text{ hours})\) strongly increased tissue factor protein expression compared with normoxia and this hypoxia-induced tissue factor up-regulation was inhibited greatly by Egr-1 siRNA. C, Egr-1 expression plasmid transfection. 23.11 cells were transfected with an Egr-1 expression plasmid \((p\text{Egr-1})\) or a control plasmid \((p\text{Ctrl})\) under normoxia for 24 hours. Overexpression of Egr-1 caused increased tissue factor expression by Western blot. \(\alpha\)-Actin was used as loading control.

![Figure 3](image3.png)

**Figure 3.** A and B, Sp1 is not involved in the hypoxic up-regulation of tissue factor expression in glioma cells. A, 23.11 cells were transfected with either Sp1 or Sp3 expression plasmids under normoxia for 24 hours and cell lysates were collected for tissue factor and Sp1 protein analysis by Western blot. Although forced expression of Sp1 caused increased both nonphosphorylated (bottom band) and phosphorylated (top band) Sp1 protein levels, this did not lead to increased tissue factor expression. Forced expression of the related Sp3 had no effect on tissue factor expression. B, siRNA targeted at Sp1 in 23.11 cells significantly inhibited Sp1 protein level but did not affect the marked hypoxic up-regulation of tissue factor compared with normoxia. Nonspecific siRNA also did not affect hypoxic up-regulation of tissue factor. \(\alpha\)-Actin was used as loading control.

**Hypoxia does not up-regulate c-Jun/AP-1 or NF-κB in 23.11 glioma cells.** The tissue factor promoter contains a distal enhancer region with AP-1- and NF-κB-binding sites, so we also investigated the expression and activity of these transcription factors under hypoxia in 23.11 glioma cells. We found that hypoxia \((1% \text{ O}_2)\) did not cause an increase in either the total or phosphorylated form of c-Jun at time points of 1 and 24 hours (Fig. 4A). Phospho-c-Jun was located exclusively in the nucleus under both normoxic and hypoxic conditions and we did not find any differences in the nuclear or cytoplasmic localization of phospho-c-Jun or c-Jun under hypoxia. NF-κB nuclear translocation is regulated primarily by the phosphorylation and degradation of IκBα (26). Once IκBα is degraded...
following its phosphorylation, NF-κB is released as a p50/p65 heterodimer, which is the most common and active complex that translocates into the nucleus and regulates target gene expression (26). We did not identify any degradation of IκBα under hypoxia at 1 and 24 hours (Fig. 4B). Moreover, exposure of glioma cells to hypoxia for either 1 or 24 hours did not lead to increased expression of NF-κBp65 or p105/p50. The levels of nuclear p50 and p65 were similar under normoxia and hypoxia (Fig. 4B). Hypoxia did not cause phosphorylation of IκBα or NF-κBp65 (data not shown).

To study transcriptional activity of AP-1 and NF-κB under hypoxia in gliomas, we transiently transfected 23.11 cells with luciferase reporter plasmids that contained either seven AP-1 or five NF-κB enhancer elements and compared them with a control reporter plasmid under both normoxia and hypoxia (Fig. 4C). We found that hypoxia did not significantly increase the transcriptional activities of either AP-1 or NF-κB compared with normoxia. Combined, our data indicate that hypoxia (1% O₂) does not lead to up-regulation or increased transcriptional activity of AP-1 or NF-κB in 23.11 glioma cells and suggests that these factors are not primarily involved in the hypoxic regulation of tissue factor.

Analysis of Egr-1 and Sp1 binding to tissue factor promoter oligonucleotides by EMSA. To determine if there was increased binding of specific nuclear transcription factors to the tissue factor promoter under hypoxia, we performed EMSA. We showed that both 1 and 24 hours of hypoxia caused increased binding of nuclear proteins to oligonucleotides corresponding to the tissue factor promoter region that contains the Egr-1/Sp1-binding sites (Fig. 5A, lanes 2 and 3 versus lane 1). This increased protein binding was blocked by siRNA directed at Egr-1 and by the addition of excess unlabeled Egr-1 consensus oligonucleotide, strongly implicating Egr-1 as the responsible nuclear protein (Fig. 5A, lanes 4 and 6). Nuclear protein binding was not affected either by nonspecific siRNA or by competition with excess unlabeled Sp1 consensus oligonucleotides (Fig. 5A, lanes 5 and 7). The specific binding complex of protein and labeled Egr-1 oligonucleotides was shifted by the addition of an anti-Egr-1 antibody (Fig. 5A, lane 8). In separate EMSA experiments, we found that labeled Sp1 oligonucleotides showed a protein-DNA-binding pattern distinct from Egr-1 (Fig. 5B). Most importantly, there was slightly decreased binding activity of nuclear proteins to the labeled Sp1 oligonucleotides under hypoxia compared with normoxia (Fig. 5B, lanes 2 and 3 versus lane 1). This decreased Sp1 binding under hypoxia could allow Egr-1 access to the overlapping binding sites. The specificity of Sp1-binding activity was confirmed by using Sp1 siRNA and competition with excess unlabeled Sp1 oligonucleotide, which both attenuated the Sp1 protein-DNA binding (Fig. 5B, lanes 4 and 5). Excess unlabeled Egr-1 probe did not decrease Sp1 oligonucleotide binding (Fig. 5B, lane 6). We concluded that hypoxia causes increased binding of Egr-1 but not Sp1 to the region of the tissue factor promoter containing the Egr-1/Sp1-binding sites.

Egr-1 is responsible for the increased tissue factor promoter activity under hypoxia. To further define the role of Egr-1 and Sp1 in the regulation of the tissue factor promoter, we analyzed 23.11 cells transfected with pTF(wt)-Luc, a minimal wild-type tissue factor promoter plasmid containing three overlapping Egr-1/Sp1-binding sites, and pTF(mu)-Luc, a plasmid in which all three Egr-1/Sp1 sites were mutated (Fig. 6A). Therefore, we concluded that the increased tissue factor promoter activity noted under hypoxia was mainly due to increased binding and transcriptional activity of Egr-1 and not Sp1.

HIF-1α and VEGF are not involved in hypoxia-up-regulated tissue factor expression. Because the transcription factor HIF-1 is up-regulated by hypoxia in many tumors, including glioblastoma multiforme (4, 5), we investigated the role of HIF-1 in the hypoxic regulation of tissue factor in glioblastoma multiforme cells.
Although the tissue factor gene promoter does not contain a binding site for HIF-1α, an indirect role of HIF-1α has been suggested by the ability of VEGF, the product of a HIF-1 target gene, to cause up-regulation of tissue factor in endothelial cells (27, 28). U251MG HIF-1α+/− glioma cells, which contain a stably transfected siRNA directed at HIF-1α, showed no increased HIF-1α expression under hypoxia, whereas control U251MG HIF-1α−/− glioma cells expressing a nonsense siRNA showed strong up-regulation of HIF-1α.

Although HIF-1α was not up-regulated by hypoxia in HIF-1α−/− cells, tissue factor showed the same degree of hypoxic up-regulation as in HIF-1α+/− cells (Fig. 7A). Thus, HIF-1α does not seem to be directly related to tissue factor expression under hypoxia. In addition, Egr-1 siRNA did not affect HIF-1α expression under hypoxia in 23.11 glioma cells, indicating that the effects of Egr-1 on tissue factor expression are not due to an indirect effect on HIF-1 (Fig. 7B). In contrast to the lack of direct hypoxic regulation of tissue factor by HIF-1α, the level of hypoxia-induced VEGF secretion was significantly reduced in U251MG HIF-1α−/− conditioned medium compared with that of U251MG HIF-1α+/− as determined by ELISA (P < 0.01), whereas hypoxia significantly increased VEGF levels in conditioned medium from both U251MG HIF-1α−/− and HIF-1α+/− cells compared with that under normoxia (P < 0.05; Fig. 7C). Hypoxia also caused a marked increase in the levels of VEGF in conditioned medium from 23.11 cells compared with normoxia (P < 0.001) and inhibition of Egr-1 by siRNA did not affect VEGF levels in conditioned medium under hypoxia (Fig. 7D). Incubating 23.11 glioma cells with human recombinant VEGF-A (25-50 ng/ml) for 24 hours under normoxia did not increase tissue factor protein expression (data not shown). Thus, HIF-1α does not seem to be a key regulator of the hypoxic expression of tissue factor either directly or through the indirect effects of VEGF.

Discussion

As human astrocytomas progress to their most malignant grade, glioblastoma multiforme (WHO grade IV), two biologically critical features develop: necrosis and microvascular hyperplasia. "Pseudopalisading" cells surrounding the necrotic foci secrete...
proangiogenic factors, such as VEGF and interleukin-8 (29, 30), which promote vascular proliferation and further tumor growth. We have shown that intravascular thrombosis emerges during the progression of astrocytomas to glioblastoma multiforme and have proposed that it initiates or propagates this cascade (5). Intravascular thrombosis can be shown histologically within >90% of surgically resected glioblastoma multiforme specimens and is noted within a substantial subset of pseudopalisades surrounding necrosis (4, 8).

Previous studies have shown that both hypoxia and PTEN loss cause a marked up-regulation of tissue factor and accelerated plasma coagulation by glioblastoma multiforme cells in vitro, suggesting that tissue factor contributes to prothrombotic mechanisms of tumor progression (8). Indeed, tissue factor levels have been associated with increased AP-1 and NF-κB activities in other investigations (34), we found no significant up-regulation of these factors under hypoxia, and the transcriptional activities associated with AP-1 and NF-κB were not appreciably increased in gliomas by 1% O₂. Also present in the tissue factor promoter are three overlapping Egr-1/Sp1-binding sites within a proximal enhancer region (−111 to +14 bp) that are responsible for tissue factor expression in human epithelial cells stimulated by serum and phorbol ester (11).

Our studies have shown that the hypoxic induction of tissue factor expression in human malignant gliomas depends on the up-regulation and enhanced transcriptional activity of Egr-1, a transcriptional regulator known to be rapidly induced by several microenvironmental stimuli, including hypoxia, growth factors, and hormones (9, 11, 35, 36). Previous studies have shown that Egr-1 is the major mediator of tissue factor expression in epithelial cells (11), mononuclear phagocytes (9), and endothelial cells (37). However, the role of Egr-1 in the hypoxic regulation of tissue factor in neoplasms has not been explored fully. Indeed, there have been suggestions that Egr-1 is a suppressor gene in malignant gliomas rather than a transcription factor for tumor progression (38). Others have argued convincingly that Egr-1 is required for both angiogenesis and tumor growth (39).

Sp1 and Egr-1 are zinc finger transcription factors that share three GC-rich binding regions within the tissue factor promoter that are critical for maintaining its basal activity (11, 40). We have shown that the proximal Egr-1/Sp1-binding sites in the tissue factor promoter are also necessary for its hypoxia-induced activity in glioblastoma multiforme cells. Mutation of these sites led to a complete abrogation of hypoxia-induced tissue factor promoter activity in our luciferase reporter assays. Dissecting the relative contributions of Sp1 and Egr-1 to the hypoxic up-regulation of tissue factor is challenging given their overlapping binding sites. We found that Sp1 was constitutively expressed in glioma cells and showed no increased expression or binding to the tissue factor promoter under hypoxia. It has been suggested that the phosphorylated form of Sp1 has increased binding and transcriptional activity, which could explain an increased promoter activity in the absence of a large increase in Sp1 protein (41). However, we also found that overexpression of Sp1 in glioma cells using a Sp1 cDNA expression plasmid led to elevated levels of both non-phosphorylated and phosphorylated forms of Sp1 but did not lead to the up-regulation of tissue factor expression. Most importantly, we found that forced overexpression of Egr-1 under normoxia led to increased tissue factor expression and that hypoxia-induced tissue factor expression could be attenuated by siRNA directed at Egr-1, whereas these findings could not be duplicated for Sp1. Combined, our results indicate that Egr-1 is the most critical element that engages Egr-1/Sp1-binding site of the tissue factor promoter in hypoxic glioblastoma multiforme cells and that Sp1 likely plays a role in its basal expression.

In endothelial cells, the regulation of tissue factor has been shown to depend on VEGF and requires activation of the KDR receptor (VEGFR2; ref. 37). Because the VEGF gene is under the transcriptional regulation of HIF-1 (42), which is strongly up-regulated in malignant gliomas, the expression of tissue factor could be under the indirect regulation of HIF-1 in glioblastoma multiforme cells. Instead, we found that HIF-1 was not responsible for the hypoxic up-regulation of tissue factor expression in glioma.
cells either directly or indirectly through VEGF. Silencing of HIF-1α using siRNA in glioma cells had virtually no effect on the hypoxic induction of tissue factor, consistent with the previous reports that HIF-1α is not involved in tissue factor expression by hypoxic mononuclear phagocytes (9). Moreover, VEGF, a major regulator of angiogenesis and a HIF-1α target (43, 44), did not cause up-regulation of tissue factor when added directly to glioma cells. Thus, although HIF-1α is certainly critical for the hypoxic induction of angiogenesis that occurs in glioblastoma multiforme (4), it does not seem to regulate the expression of tissue factor or plasma coagulation by these tumors.

In summary, our study has provided evidence that tissue factor expression in glioma cells. Our previous studies have shown that tissue factor is responsible for promoting plasma coagulation by glioma cells in vitro and could potentially lead to the intravascular thrombosis in glioblastoma multiforme cells that is associated with tumor progression (5, 8). Preventing vaso-occlusive and prothrombotic events caused by tissue factor could have substantial therapeutic benefit.

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