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-κB (NF-κB), 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% O_2) 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-κB-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% O_2), 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-κB (NF-κB), 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 overexpress 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-κB 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

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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 muristerone-inducible 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% O_2 (normoxia) or 1% O_2 (hypoxia). For hypoxic treatment, cells were placed in Modular Incubator Chambers (Billups-Rothenberg, Del Mar, CA), which were flushed with 94% N_2, 5% CO_2, and 1% O_2 for 5 minutes, sealed, and then kept in a regular tissue culture incubator. Cell pellets and conditioned medium were collected at indicated time points and immediately stored at −70°C. To determine the effect of human recombinant VEGF-A (Sigma-Aldrich, St. Louis, MO) on tissue factor expression, 23.11 glioma cells were incubated with 25 or 50 ng/mL VEGF-A for 24 hours under normoxia. After incubation, cell lysates were collected and analyzed for tissue factor expression by Western blot.

Quantitative real-time one-step reverse transcription-PCR. Total RNA was extracted from glioma cells with Trizol reagent (Invitrogen, Carlsbad, CA). Real-time PCR assay was carried out with the iCyclerIQ System (Bio-Rad Laboratories, Hercules, CA) using iScript one-step reverse transcription-PCR kit with SYBR Green (Bio-Rad) according to the manufacturer's instruction. All PCRs were done in triplicate. The sequences of Egr-1 primers were designed based on human Egr-1 mRNA sequence (Genbank accession no. NM_001964) as follows: Egr-1 forward 5′-TGACCGCAGAGTCTTTTCCT-3′ and reverse 5′-TGGGTTGGTCATGCTCACTA-3′. The primers of tissue factor and β-actin were as follows: tissue factor forward 5′-GCCAGGAGAAAGGGGAAT-3′ and reverse 5′-CAGTGCAATATAGCATTTGCAGTAGC-3′ and β-actin forward 5′-TCACCCACACTGTGCCCATCTACGA-3′ and reverse 5′-CAGCGGAACCGCTCATTGCCAATGG-3′ (19). Hypoxic up-regulation of Egr-1 and tissue factor mRNA was normalized to β-actin levels and reported as relative fold increase compared with normoxia determined by the $2^{-\Delta\Delta Ct}$ method as described before (20, 21).

Plasmids and transient transfection. The Egr-1 expression vector (pEgr-1) used to induce expression of Egr-1 protein contains a copy of the *Egr-1* cDNA under the control of a cytomegalovirus promoter. The corresponding empty vector (pCtrl) was used as a control as described previously (22). A firefly luciferase reporter plasmid (pEgr-1-Luc) containing four Egr-1-binding sites upstream of a prolactin minimal promoter and the control reporter plasmid without Egr-1-binding inserts (pCtrl-Luc) were kindly provided by John Svaren (University of Wisconsin, Madison, WI). AP-1 and NF-κB luciferase reporter plasmids (pAP-1-Luc and pNF-κB-Luc) and the control plasmid (pLuc-MCS) were purchased from Stratagene (Cedar Creek, TX). The Sp1 and Sp3 expression vectors (pSp1 and pSp3) were used as reported previously (23). The tissue factor promoter-luciferase reporter vectors, pTF(−111/+14)-Luc [shown as pTF(wt)-Luc] and pTF(Egr-1m/Sp1m)-Luc [shown as pTF(mu)-Luc], were derived from the luciferase reporter vector pGL2 (Promega Corp., Madison, WI) as described previously (11). The pTF(Egr-1m/Sp1m)-Luc construct contains the oligonucleotide (−111/−56 bp) with a 3-bp mutation (from GCG to AAT) within each of the overlapping binding sequences between the Egr-1/Sp1 sites (11). All plasmid DNA for transfections were isolated using a Qiagen endotoxin-free kit (Qiagen, Valencia, CA). Transient transfection of plasmids was carried out using the lipid reagent Gene Porter (Gene Therapy Systems, Inc., San Diego, CA) according to the manufacturer's instructions. Briefly, 24 hours before transfection, 3×10^5 23.11 glioma cells were seeded in six-well plates and reached 70% to 90% confluency the next day. pEgr-1 or pCtrl (2.5 μg) and Sp1 or Sp3 (2.0 μg) plasmids were transfected under normoxia (21% O_2) in serum-free medium. Cells were collected 24 hours after transfection and proteins were collected for Egr-1 and tissue factor protein expression by Western blot. For Egr-1 luciferase reporter plasmids, 1 μg pEgr-1-Luc or pCtrl-Luc was 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% O_2) or hypoxia (1% O_2) 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 (LUMIstar 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% O_2) 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 (Qiagen) 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 mmol/L Tris (pH 7.0), 150 mmol/L NaCl, 2 mmol/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, Suwannee, GA). Blocking 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, 1:5,000) 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 PharMingen (San Diego, CA). Rabbit polyclonal antibodies (1:1,000) against c-Jun, phospho-c-Jun, NF-κBp65, phospho-NF-κBp65, IκBα, and NF-κBp105/p50 and mouse monoclonal antibody against phospho-IκBα were purchased from Cell Signaling Technology (Beverly, MA). Blots were washed and incubated with horseradish 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′-CCCGGC*GCGGGGGCG*ATTTCGAGTCA-3′) was used as reported previously (25). The Sp1 consensus oligonucleotide (5′-ATTCGATC*GGGGCGGGGC*GAGC-3′) spanning the tissue factor promoter region was purchased from Santa Cruz Biotechnology. Both Egr-1 and Sp1 consensus

oligonucleotides were 5′-end-labeled with [γ-^{32}P]ATP (3,000 Ci/mmol) by using T4 polynucleotide kinase as described previously (9). Gel shift assays were done as follows: nuclear extracts (8 μg) were incubated with radiolabeled DNA probes for 20 minutes at room temperature in a 20 μL binding reaction mixture using Gel Shift Assay Core System (Promega). For competition and supershift assays, binding reactions were incubated with unlabeled oligonucleotides (100-fold molar excess) or the rabbit polyclonal anti-Egr-1 antibody (2 μL in 20 μL reaction mixture), respectively, for 10 minutes at room temperature before adding the radiolabeled oligonucleotides. Protein-DNA complexes were separated from free probes by electrophoresis through 5% nondenaturing polyacrylamide/bisacrylamide gels prepared in 0.5× Tris-borate EDTA. Gels were pre-run for 15 minutes before samples were loaded. After electrophoresis for 1.5 hours at 200 V, the gel was dried at 80°C for 1.5 hours and protein-DNA binding was visualized by autoradiography.

Immunofluorescent staining of Egr-1. 23.11 glioma cells were cultured on chamber slides (Nalge Nunc International, Naperville, IL) until 70% to 80% confluent. Cells were changed to serum-free DMEM for another 24 hours before hypoxia treatment (1 hour). For Egr-1 siRNA, cells were transfected with Egr-1 siRNA (Ambion, Genbank accession no. NM_001964) the day before staining. Cells were washed with PBS briefly and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Permeabilization was done in PBS with 0.2% Triton X-100 for 10 minutes at room temperature. After blocking for 60 minutes with 10% goat serum, cells were incubated with anti-Egr-1 antibody (1:200; Santa Cruz Biotechnology) overnight at 4°C. After washing, Alexa Fluor 594 goat anti-rabbit secondary antibody (1:500; Molecular Probes, Eugene, OR) was added for 1 hour. Slides were mounted with Prolong gold antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) for nuclei staining. Cells were visualized on an inverted fluorescence microscope (Olympus IX71, Olympus America, Melville, NY) with FITC and TRITC filters coupled to a CCD camera (Olympus DP70, Olympus America).

ELISA. Concentrations of VEGF in conditioned serum-free medium from 23.11 and U251MG glioma cells treated under hypoxia or normoxia were measured by ELISA kit according to the manufacturer's instructions (Quantikine, R&D Systems, Minneapolis, MN).

Statistical analysis. Quantitative data are expressed as mean ± SD. Comparisons were analyzed by the Student's *t* test and ANOVA. Significance was defined as $P < 0.05$.

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% O_2) 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. 1*A*). 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. 1*B*). siRNA directed at *Egr-1* markedly inhibited both Egr-1 mRNA ($P < 0.001$) and protein expressions under hypoxia (Fig. 1*A* 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

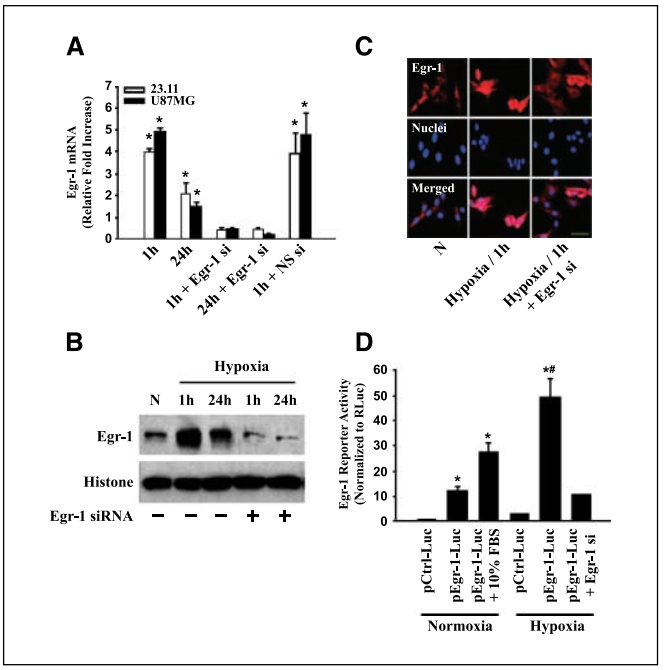

Figure 1. *A* to *D,* hypoxia up-regulates Egr-1 expression in glioblastoma multiforme cells. *A,* real-time PCR for Egr-1 mRNA expression. U87MG and 23.11 cells transfected with siRNA directed against Egr-1 (*Egr-1 si*) or nonspecific siRNA (*NS si*) for 24 hours were subjected to hypoxic (1% O_2) for 1 and 24 hours. Egr-1 mRNA expression was analyzed by real-time PCR and expression was recorded as the fold increase normalized to normoxia (21% O_2). Egr-1 mRNA levels were increased by 4.93 ± 0.2–fold in U87MG and 4.02 ± 0.2–fold in 23.11 after 1 hour and by 1.49 ± 0.2– and 2.1 ± 0.5–fold, respectively, at 24 hours. Egr-1 siRNA but not nonspecific siRNA significantly inhibited hypoxia-induced Egr-1 mRNA expression at both 1 and 24 hours (*, $P < 0.001$). *Columns,* mean of experiments done in triplicate; *bars,* SD. *B,* Western blot for Egr-1. Nuclear proteins from 23.11 cells under similar 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. *Histone,* loading controls for nuclear protein. *C,* immunofluorescent staining of Egr-1. 23.11 cells were grown on chamber slides transfected with or without Egr-1 siRNA for 24 hours before hypoxia (1 hour). After hypoxia, the cells were incubated with an Egr-1 antibody and signal was detected by immunofluorescence for Alexa Fluor 594 (*red*) secondary antibody and DAPI for nuclear stain (*blue*). Hypoxia increased nuclear localization of Egr-1 compared with normoxia and Egr-1 siRNA inhibited Egr-1 nuclear accumulation. Bar, 50 μm. *D,* Egr-1 luciferase reporter assay. Egr-1 activity in 23.11 cells was assessed using a firefly luciferase reporter plasmid containing four Egr-1-binding sites upstream of a prolactin minimal promoter (pEgr-1-Luc) along with a control reporter plasmid without Egr-1-binding inserts (pCtrl-Luc). RLuc plasmids were cotransfected as the internal control. Firefly luciferase activities were normalized to RLuc. pEgr-1-Luc-transfected cells showed significantly increased luciferase activity under both normoxia and hypoxia compared with pCtrl-Luc-transfected cells (*, $P < 0.001$). Hypoxia strongly increased luciferase signals in pEgr-1-Luc-transfected cells compared with pEgr-1-Luc-transfected cells under normoxia and Egr-1 siRNA attenuated this effect (#, $P < 0.001$). FBS (10%) caused increased luciferase activity as well but not as much as hypoxia. *Columns,* mean of experiments done in triplicate; *bars,* SD.

accumulation (Fig. 1*C*). 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. 1*D*). 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. 1*D*). 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 ($P < 0.001$; Fig. 2*A*). Similarly, hypoxic up-regulation of tissue factor protein in 23.11 cells was significantly inhibited by siRNA directed against *Egr-1* (Fig. 2*B*). 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 (pEgr-1, 2.5 μg) and found increased tissue factor expression compared with the cells transfected with control plasmid (pCtrl, 2.5 μg) and untransfected cells (mock; Fig. 2*C*). Combined, these results show that the increased

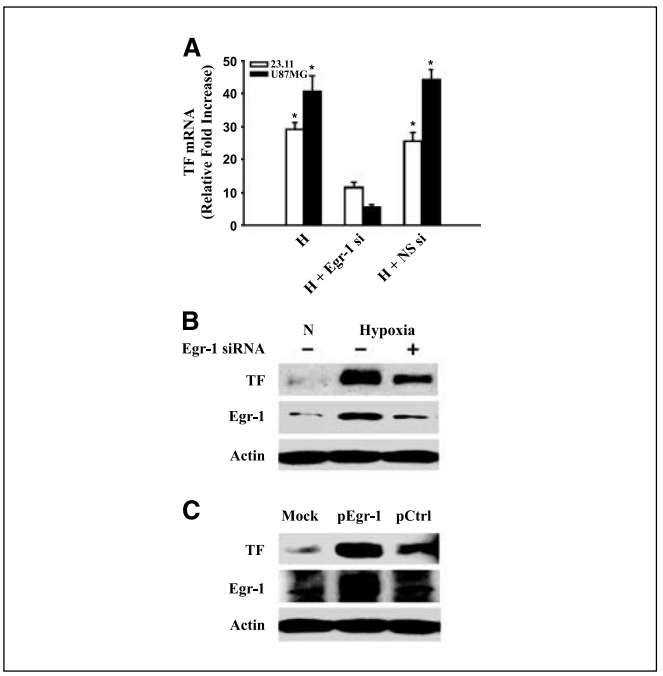

Figure 2. *A* to *C,* Egr-1 is responsible for hypoxia-induced tissue factor up-regulation in glioblastoma multiforme cells. *A,* real-time PCR for tissue factor mRNA expression. U87MG and 23.11 cells were transfected with Egr-1 siRNA or nonspecific siRNA the day before hypoxic treatment (24 hours). Tissue factor (*TF*) mRNA levels were analyzed by real-time PCR. Hypoxia (*H*; 24 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 (*, $P < 0.001$, 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 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 (pEgr-1) or a control plasmid (pCtrl) under normoxia for 24 hours. Overexpression of Egr-1 caused increased tissue factor expression by Western blot. β-Actin was used as loading control.

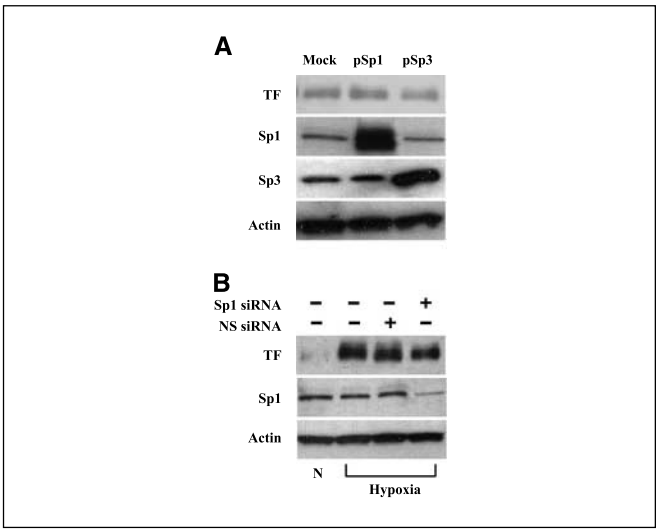

Figure 3. *A* and *B,* Sp1 is not involved in the hypoxic up-regulation of tissue factor expression in glioma cells. *A,* 23.11 cells were transfected with either Sp1 or Sp3 expression plasmids under normoxia for 24 hours and cell lysates were collected for tissue factor and Sp1 protein analysis by Western 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. β-Actin was used as loading control.

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 (pSp1, 2.0 μg) 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 (pSp3, 2.0 μg) under the same conditions did not lead to increased tissue factor expression either (Fig. 3*A*). Conversely, we found that siRNA directed at *Sp1* in hypoxic 23.11 cells was able to significantly reduce the amount of Sp1 protein expression but did not substantially affect the hypoxia-induced tissue factor expression (Fig. 3*B*). Moreover, Sp1 is a constitutively expressed protein that is not up-regulated by hypoxia in 23.11 cells (Fig. 3*B*). We conclude that Sp1 is not up-regulated by hypoxia in glioma cells and does not directly up-regulate tissue factor under these conditions.

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% 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. 4*A*). 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. 4*B*). 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. 4*B*). 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. 4*C*). 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% O_2) 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.

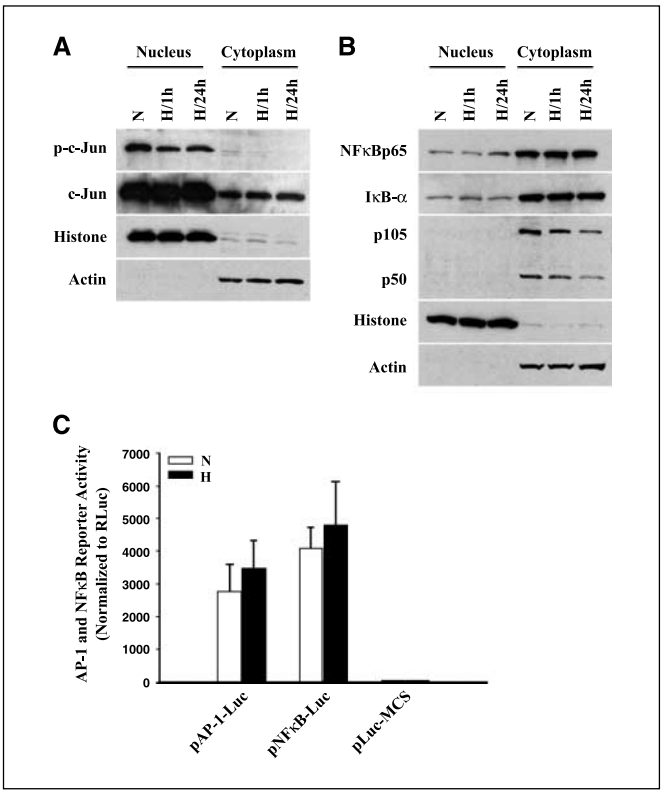

Figure 4. *A* to *C,* hypoxia does not cause up-regulation or increased transcriptional activity of c-Jun/AP-1 or NF-κB in 23.11 gliomas. *A,* Western blot for c-Jun/AP-1. Nuclear and cytoplasmic proteins from 23.11 gliomas under normoxia or hypoxia (1% O_2) were collected at 1 and 24 hours. c-Jun is present in both the cytoplasm and the nucleus, whereas phospho-c-Jun is present exclusively in the nucleus. The levels of total c-Jun and phospho-c-Jun are similar under normoxia and hypoxia and there is no increased nuclear accumulation of c-Jun or phospho-c-Jun under hypoxia. *B,* Western blot for NF-κB-related proteins. Hypoxia does not cause degradation of IκBα or increased levels of NF-κBp65 compared with normoxia. A mild decrease in p105/p50 levels was seen under hypoxia at 24 hours. No nuclear accumulation NF-κBp65 or p50 was noted under hypoxic conditions. *A* and *B,* histone H1 is loading control for nuclear protein. β-Actin is loading control for cytoplasmic protein. *C,* AP-1 and NF-κB luciferase reporter assay. AP-1 and NF-κB activities in 23.11 glioma cells were analyzed using a firefly luciferase reporter plasmid containing either seven AP-1-binding (pAP-1-Luc) or five NF-κB-binding (pNF-κB-Luc) sites. A control reporter plasmid (pLuc-MCS) lacked the AP-1- and NF-κB-binding inserts. RLuc was cotransfected as the internal control and firefly luciferase was normalized to RLuc. Hypoxia did not significantly increase luciferase activity in cells containing the plasmids with specific AP-1 or NF-κB enhancer elements compared with normoxia. *Columns,* mean of experiments done in triplicate; *bars,* SD.

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. 5*A*, *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. 5*A, lanes 4* and *6*). Nuclear protein binding was not affected either by nonspecific siRNA or by competition with excess unlabeled Sp1 consensus oligonucleotides (Fig. 5*A, 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. 5*A, lane 8*). In separate EMSA experiments, we found that labeled Sp1 oligonucleotides showed a protein-DNA-binding pattern distinct from Egr-1 (Fig. 5*B*). Most importantly, there was slightly decreased binding activity of nuclear proteins to the labeled Sp1 oligonucleotides under hypoxia compared with normoxia (Fig. 5*B, 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 unlabeled Sp1 oligonucleotide, which both attenuated the Sp1 protein-DNA binding (Fig. 5*B, lanes 4* and *5*). Excess unlabeled Egr-1 probe did not decrease Sp1 oligonucleotide binding (Fig. 5*B, 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. 6*A*). We found that the cells transfected with pTF(wt)-Luc plasmid showed significantly increased luciferase activities under hypoxia compared with normoxia ($P < 0.001$), whereas pTF(mu)-Luc-transfected cells showed virtually no luciferase activity under normoxia or hypoxia (Fig. 6*A*). To determine if Egr-1 and/or Sp1 contributed to the hypoxia-induced *tissue factor* promoter activity, 23.11 cells were first transfected with *Egr-1* and/or *Sp1* siRNA 24 hours before plasmid transfection. The *tissue factor* promoter activity under hypoxia was significantly inhibited by *Egr-1* siRNA or Egr-1 and Sp1 siRNA combined but not by *Sp1* siRNA alone or nonspecific siRNA ($P < 0.001$; Fig. 6*C*). 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.

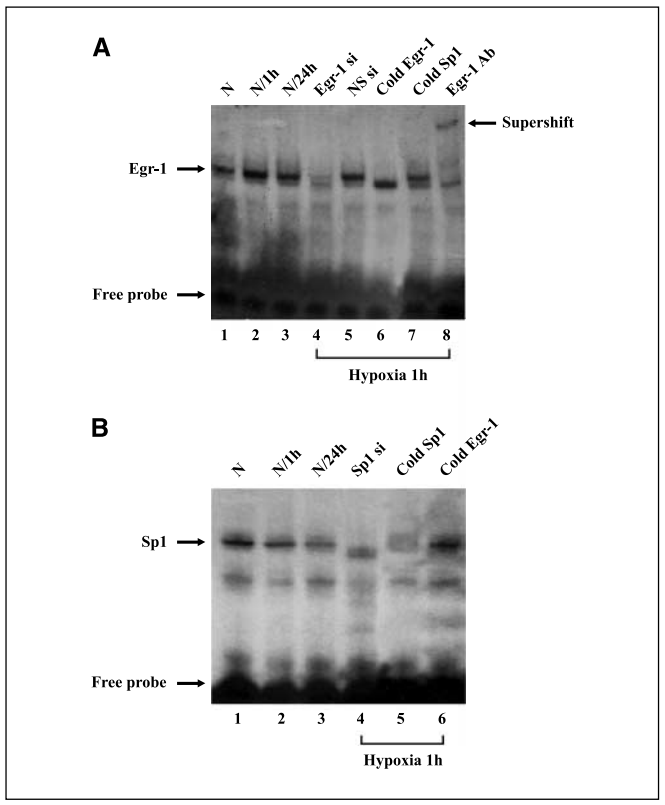

Figure 5. *A* and *B,* EMSA. *A,* EMSA for Egr-1 binding to tissue factor promoter. 23.11 cells were subjected to normoxia or hypoxia for 1 or 24 hours. Egr-1 siRNA or nonspecific siRNA was transfected with the cells 24 hours before hypoxia treatment at 1 hour. Nuclear proteins were collected and incubated with ^{32}P-labeled Egr-1 consensus oligonucleotides. Hypoxia (1 and 24 hours) significantly enhanced nuclear protein binding (*lanes 2* and *3*) compared with normoxia (*lane 1*). Increased binding was inhibited by Egr-1 siRNA (*lane 4*) but not nonspecific siRNA (*lane 5*). The addition of excess unlabeled consensus Egr-1 oligonucleotide significantly reduced the level of protein binding to the labeled Egr-1 probe (*Cold Egr-1*, 100-fold; *lane 6*), but excess unlabeled Sp1 oligonucleotide did not (*Cold Sp1*, 100-fold; *lane 7*). The specific protein-DNA complex was supershifted following the addition of an Egr-1 antibody (*Supershift*; *lane 8*). *B,* EMSA for Sp1 DNA binding. Nuclear extracts of 23.11 cells treated similarly as Egr-1 EMSA assay were incubated with ^{32}P-labeled Sp1 oligonucleotides. Hypoxia caused decreased protein-DNA binding to the Sp1 oligonucleotide (*lanes 2* and *3*) compared with normoxia (*lane 1*). Sp1 siRNA (*lane 4*) and unlabeled Sp1 consensus oligonucleotide (*lane 5*) could inhibit or compete the specific protein-DNA complexes respectively. Unlabeled Egr-1 oligonucleotide (*lane 6*) did not reduce protein binding to labeled Sp1 oligonucleotide.

Although the *tissue factor* gene promoter does not contain a binding site for HIF-1, an indirect role of HIF-1 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. 7*A*). 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. 7*B*). 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. 7*C*). 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. 7*D*). 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

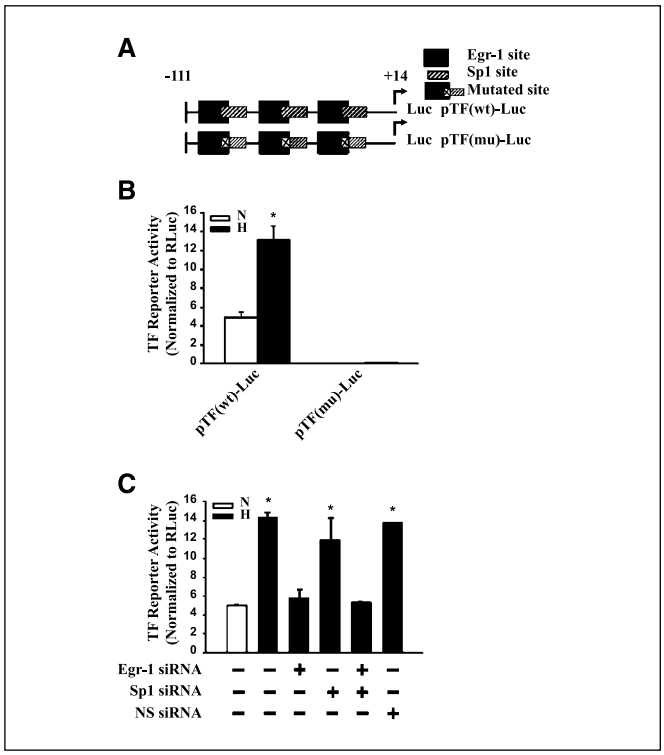

Figure 6. *A* to *C,* functional analysis of the human tissue factor promoter in glioblastoma multiforme cells. *A,* schematic of pTF(wt)-Luc and pTF(Egr-1m/Sp1m)-Luc reporter constructs showing Egr-1 and Sp1 sites. *Black box,* Egr-1 sites; *hatched box,* Sp1 sites; *white box with X,* a 3-bp mutation (from GCG to AAT) in the overlapping Egr-1/Sp1-binding sites. *B,* plasmids (2 μg) containing the minimal wild-type tissue factor promoter [pTF(wt)-Luc] or tissue factor promoter with all six Egr-1/Sp1 sites mutated [pTF(mu)-Luc] were transfected into 23.11 cells for 5 to 6 hours and cells were subjected to normoxia or hypoxia for another 16 hours. Hypoxia significantly increased luciferase activity in pTF(wt)-Luc-transfected cells compared with normoxia (*, $P < 0.001$). Luciferase signals were minimal in pTF(mu)-Luc-transfected cells. *C,* 23.11 cells were transfected with Egr-1 siRNA, Sp1 siRNA, or nonspecific siRNA 24 hours before the transfection of pTF(wt)-Luc (2 μg) and then subjected to normoxia or hypoxia for 16 hours. Egr-1 siRNA or combined with Sp1 siRNA but not Sp1 siRNA alone or nonspecific siRNA significantly inhibited hypoxia-induced luciferase activity (*, $P < 0.001$). Luciferase activity expressed by each plasmid was normalized to RLuc signals. *Columns,* mean of experiments done in triplicate; *bars,* SD.

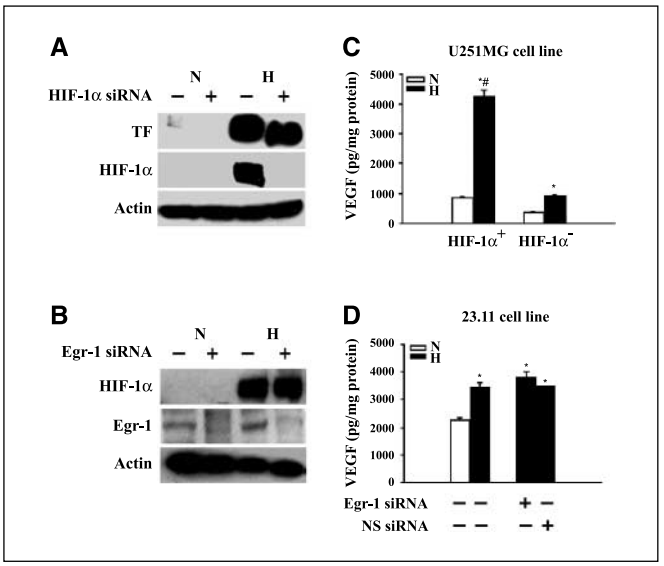

Figure 7. *A* to *D,* hypoxic up-regulation of tissue factor is independent on HIF-1α and VEGF. *A,* knockdown of HIF-1α expression did not affect tissue factor expression under hypoxia. U251MG *HIF-1α*$^-$ (stably transfected with HIF-1α siRNA) and *HIF-1α*$^+$ (without HIF-1α siRNA transfection) cells were subjected to normoxia or hypoxia, and cell lysates were analyzed by Western blot. Hypoxia caused marked increase in HIF-1α and tissue factor in U251MG *HIF-1α*$^+$ cells. The knockdown of hypoxia-induced HIF-1α in U251MG *HIF-1α*$^-$ cells did not reduce hypoxia-induced tissue factor expression. *B,* 23.11 cells were transfected with Egr-1 siRNA 24 hours before normoxia or hypoxia and analyzed for HIF-1α expression by Western blot. siRNA directed at Egr-1 did not affect HIF-1α expression. *C,* knockdown of HIF-1α reduces hypoxia induced VEGF secretion. Conditioned medium was collected from normoxic or hypoxic U251MG *HIF-1α*$^-$ and *HIF-1α*$^+$ cells cultured in serum-free DMEM and VEGF was measured by ELISA. VEGF levels in conditioned medium from U251MG *HIF-1α*$^+$ cells were significantly higher than that from U251MG *HIF-1α*$^-$ cells (#, $P < 0.01$). Hypoxia strongly increased VEGF secretion from both U251MG *HIF-1α*$^-$ and *HIF-1α*$^+$ cells compared with normoxia (*, $P < 0.05$). *D,* Egr-1 siRNA does not reduce hypoxia-induced VEGF secretion. Egr-1 siRNA was transfected into 23.11 cells before treatment by hypoxia. Hypoxia significantly increased VEGF secretion compared with normoxia (*, $P < 0.001$), and inhibition of Egr-1 by Egr-1 siRNA did not affect VEGF secretion under hypoxia. VEGF levels in conditioned medium were normalized to protein concentrations. *Columns,* mean of experiments done in triplicate; *bars,* SD.

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 in astrocytomas and other tumors correlate with increasing tumor grade, extent of necrosis, tumor progression, and an unfavorable prognosis mostly due to its prothrombotic and proangiogenic effects (31–33). Mechanisms responsible for the increased expression of tissue factor in hypoxic gliomas are not known.

Transcriptional regulation of the human *tissue factor* gene is complex. Two AP-1 sites and a NF-κB site are present within a distal enhancer region (−227 to −172 bp) of the *tissue factor* promoter and have been shown to direct tissue factor expression in monocytic and endothelial cells exposed to bacterial lipopolysaccharide or cytokines (13). Although hypoxic and anoxic conditions 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% O_2. 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 stimulants, 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 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 Egr-1 is critical to the hypoxic up-regulation of 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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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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