Epidermal Growth Factor Receptor and PTEN Modulate Tissue Factor Expression in Glioblastoma through JunD/Activator Protein-1 Transcriptional Activity

Yuan Rong,1,2 Vladimir E. Belozerov,2,5 Carol Tucker-Burden,1,5 Gang Chen,1,5 Donald L. Durden,3,4,5 Jeffrey J. Olson,2,3 Erwin G. Van Meir,2,3,5 Nigel Mackman,6 and Daniel J. Brat1,5

Departments of Pathology and Laboratory Medicine,1 Neurosurgery,2 and Hematology and Medical Oncology,3 Department of Pediatrics,4 Department of Pathology and Laboratory Medicine, Atlanta, and Department of Hematology/Oncology, University of North Carolina, Chapel Hill, North Carolina

Abstract

Hypoxia and necrosis are fundamental features of glioblastoma (GBM) and their emergence is critical for the rapid biological progression of this fatal tumor; yet, underlying mechanisms are poorly understood. We have suggested that vaso-occlusion following intravascular thrombosis could initiate or propagate hypoxia and necrosis in GBM. Tissue factor (TF), the main cellular initiator of coagulation, is overexpressed in GBMs and likely favors a thrombotic microenvironment. Epidermal growth factor receptor (EGFR) amplification and PTEN loss are two common genetic alterations seen in GBM but not in lower-grade astrocytomas that could be responsible for TF up-regulation. The most frequent EGFR mutation in GBM involves deletion of exons 2 to 7, resulting in the expression of a constitutively active receptor, EGFRvIII. Here, we show that overexpression of EGFR or EGFRvIII in human glioma cells causes increased basal TF expression and that stimulation of EGFR by its ligand, EGF, leads to a marked dose-dependent up-regulation of TF. In all cases, increased TF expression led to accelerated plasma coagulation in vitro. EGFR-mediated TF expression depended most strongly on activator protein-1 (AP-1) transcriptional activity and was associated with c-Jun NH2-terminal kinase (JNK) and JunD activation. Restoration of PTEN expression in PTEN-deficient GBM cells diminished EGFR-induced TF expression by inhibiting JunD/AP-1 transcriptional activity. PTEN mediated this effect by antagonizing phosphatidylinositol 3-kinase (PI3K) activity, which in turn attenuated both Akt and JNK activities. These mechanisms are likely at work in vivo, as EGFR expression was highly correlated with TF expression in human high-grade astrocytoma specimens. [Cancer Res 2009;69(6):2540–9]

Introduction

Glioblastoma (GBM; WHO grade 4) is the most common primary brain tumor in adults and also the most malignant (1). It is distinguished pathologically from lower-grade astrocytomas (grades 2 and 3) by necrosis and microvascular hyperplasia, a florid form of angiogenesis. Although it is recognized that hypoxia associated with necrosis causes microvascular hyperplasia in the periphery of the tumor, compelling explanations for the development of hypoxia and necrosis in GBM have not been established. We have suggested that vascular pathology may initiate this cascade (2). Nearly all GBMs display microscopic evidence of intravascular thrombosis, whereas lower-grade astrocytomas rarely show this finding (3, 4). Both pathologic observations and experimental evidence suggest that hypoxia and necrosis within GBMs could be initiated or propagated by vaso-occlusion secondary to intravascular thrombosis in a manner that drives tumor progression (2, 4, 5). The development of thrombosis may be due in part to the deregulated expression of procoagulant molecules.

One of the most highly up-regulated and potent prothrombotic factors in human cancer is tissue factor (TF), a 47-kDa transmembrane glycoprotein receptor that is a critical regulator of tissue homeostasis (6). A direct correlation between TF levels and tumor grade has been noted for multiple tumor types (7), including gliomas (8). In addition to its prothrombotic function, TF directly promotes tumor invasiveness and angiogenesis by intracellular signals through its cytoplasmic tail, and indirectly through the activation of protease-activated receptor (PAR) I and PAR2 (2, 9).

Two common and highly specific genetic events associated with the GBM histology are epidermal growth factor receptor (EGFR) amplification and PTEN loss. The EGFR gene is amplified in 40% to 50% of GBMs and this event is often accompanied by genetic alterations (10). The most common mutant, EGFRvIII, is formed following the deletion of exons 2 to 7, resulting in a constitutively active transmembrane receptor that lacks a functional ligand-binding domain (11). EGFR activation causes a variety of downstream biological processes associated with tumor growth, invasion, and angiogenesis (12). PTEN, a tumor suppressor gene implicated in multiple human cancers, is inactivated by mutation in 20% to 40% of GBMs and lost through other mechanisms in a much larger percentage (13–15). In the current study, we investigated whether EGFR or EGFRvIII caused increased TF expression by GBM cells and explored the relationship between EGFR and PTEN signaling in this regulation. We found that EGFR strongly induced TF expression by up-regulating JunD/activator protein-1 (AP-1) transcriptional activity on the TF promoter and was regulated by c-Jun NH2-terminal kinase (JNK). Reconstitution of PTEN in PTEN-deficient GBM cells attenuated EGFR-induced TF expression by antagonizing phosphatidylinositol 3-kinase (PI3K) activity, which in turn reduced both Akt and JNK activities.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Daniel J. Brat, Department of Pathology and Laboratory Medicine, Emory University Hospital, G-169, 1364 Clifton Road Northeast, Atlanta, GA 30322. Phone: 404-712-1266; Fax: 404-727-3133; E-mail: dbrat@emory.edu.

©2009 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-08-1547
Materials and Methods

**Cell lines.** The human GBM cell line U87MG was cultured in DMEM with 10% fetal bovine serum. U87MG-wt-EGFR and U87MG-EGFRvIII cell lines (kindly provided by Frank Furnari and Webster Cavenee, Ludwig Institute, San Diego, CA) were cultured in the same conditions. The lentiviral vector expressing PTEN was constructed using the FUW vector as previously described (16). The full-length COOH terminally HA-tagged human PTEN cDNA (17) was subcloned into the EcoRI site of FUW. The resulting PTEN-HA/FUW plasmid was used to produce lentiviral particles using ViralPower Lentiviral Expression System (Invitrogen). Both cell lines were infected with either the PTEN-HA or green fluorescent protein (GFP) lentiviruses and propagated as stable cell lines.

**Reagents and chemicals.** PD188780, SP600125, LY294002, and U0126 were from EMD Biosciences, Inc.; rapanycin was from LC Laboratories; EGFR was from Sigma-Aldrich; and the citrated human plasma was from Precision Biologic. Neoplastine (Diagnostica Stago) and human plasma depleted of factor VII (R2 Diagnostics) were used as positive and negative controls, respectively.

**Immunohistochemistry.** Archived surgically resected GBM (23 cases) and anaplastic astrocytoma (AA; 7 cases) specimens were obtained from Emory University Hospital Department of Pathology. GBMs were selected from patients with no previous radiation or chemotherapy. Paraffin-embedded sections were deparaffinized and subjected to heat-induced epitope retrieval by steaming for 15 min. For TF, slides were incubated with primary antibody (1:100; American Diagnostica) at 4°C for overnight. Avidin-biotin-peroxidase complex was used to detect the antibodies using 3,3′-diaminobenzidine (DAB) as the chromogen. For EGFR, slides were incubated with primary antibody (1:25; ZYMED Laboratories) at room temperature for 1 h and detected with goat anti-mouse IgG-alkaline phosphatase conjugate (1:100; Molecular Probes) using Fast Red (ZYMED Laboratories) as the substrate. Staining intensity of EGFR and TF in neoplastic cells was graded on a scale of 0 (no staining) to 3+ (strong staining) without knowing EGFR gene status or diagnosis.

**Fluorescence in situ hybridization analysis of EGFR gene amplification.** On the same set of histologic sections, we used the commercially available LSI EGFR SpectrumOrange/CEP7 SpectrumGreen dual-color probe set (Vysis, Inc., Abbott Laboratories), which included directly labeled DNA fluorescence in situ hybridization (FISH) probes for the EGFR gene (SpectrumOrange) and the centromere of chromosome 7 (SpectrumGreen). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes). Detection of an average of >10 EGFR signals per nucleus was defined as amplification and detection of ≤6 EGFR signals per nucleus was defined as nonamplified.

**Protein extraction from human GBM specimens.** Eleven frozen human GBM specimens (100 mg) were obtained from the brain tumor bank at the Winship Cancer Institute. Proteins were extracted using Trizol LS reagent (Invitrogen) and expression of EGFR and TF was determined by Western blot.

**Plasmids and transfections.** The human TF promoter-luciferase reporter plasmids were as described previously (18). The constructs containing mutations in each of the Egr-1 [pTF(EGr-1m)] or Sp1 sites [pTF(Sp1m)] have been reported previously (19). The AP-1 luciferase plasmid (pAP-1-Luc) and the control plasmid (pLuc-MCS) were from Stratagene. The dominant-negative mutant c-Jun expression plasmid (c-JunDN) and the control vector were provided by Michael J. Birrer (National Cancer Institute, Bethesda, MD). The dominant-negative mutant JunD expression plasmid (JunDDN) and the control vector were provided by Lester Lau (University of Illinois, Chicago, IL). Transient transfection of plasmids was carried out using Gene Porter (Gene Therapy Systems, Inc.) and performed in triplicate. The results were calculated as the activity of firefly luciferase relative to that of the Renilla luciferase.

**RNA interference.** Small interfering RNAs (siRNA) against JNK1, JNK2, c-Jun, JunD, and rhodamine-labeled nonsilencing siRNA were purchased from Qiagen. Seventy-two hours after transfection, glioma cells were harvested for Western blot analysis.

**Western blot.** Cells were lysed in radioimmunoprecipitation assay buffer supplemented with 1 mmol/L phenylmethylsulfonyl and the protease inhibitor cocktail (Santa Cruz Biotechnology). Lysates were centrifuged at 10,000 × g for 10 min at 4°C. The NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) were used to separate nuclear and cytoplasmic protein fractions. Equal amounts of protein were separated on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk for 1 h and incubated with primary antibodies in 5% bovine serum albumin overnight at 4°C. The antibodies were used against Akt, phosphorylated Akt (p-Akt Ser473), extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated ERK1/2, PTEN, c-Jun, phosphorylated c-Jun (p-c-Jun; Ser 73), phosphorylated s6rp, and nuclear factor κB1 (NF-κB1; Cell Signaling Technology); TF (American Diagnostica); HA.11 (Covance); phosphorylated JNK1/2 (p-JNK1/2), JNK1/2, and JunD (ZYMED Laboratories); and JNK2, histone H1, and β-actin (Santa Cruz Biotechnology).

**ELISA for interleukin-8.** Interleukin-8 (IL-8) levels in serum-free medium of U87MG-EGFRvIII and U87MG-wt-EGFR cells treated with NF-κB1 siRNA were measured by ELISA (R&D Systems). A complete description of the IL-8 analysis is found within Supplementary Materials and Methods.

**Plasma clotting assay.** Plasma clotting times induced by GBM cells were measured in triplicate using a MLA Electra 750 Coagulation Timer (Diamond Diagnostics, Inc.). Tumor cells grown in serum-free medium with or without EGF stimulation for 24 h were collected and resuspended in PBS to 1 × 10^6/mL. The clotting reaction was performed as previously described (6).

**Statistical analysis.** ANOVA was used to compare group differences. The Student’s t test was used to assess whether differences in the values of two groups were statistically significant, with the Bonferroni correction used for multiple simultaneous comparisons. Differences were considered to be significant with P < 0.05. Spearman’s rank test was used to evaluate the strength of correlation between EGFR and TF expression in human high-grade astrocytoma samples.

**Results**

**wt-EGFR and EGFRvIII up-regulate TF expression in human GBM cells and cause accelerated plasma coagulation.** To investigate whether wt-EGFR or EGFRvIII regulates TF expression, we used a human glioma cell line (U87MG) stably transfected to overexpress either wt-EGFR (U87MG-wt-EGFR) or EGFRvIII (U87MG-EGFRvIII). Under basal conditions, TF was strongly up-regulated by U87MG-EGFRvIII cells compared with parental U87MG or U87MG-wt-EGFR cells (Fig. IA, lane 3 versus lanes 1 and 5) based on comparison of the TF:α-actin ratios determined by densitometry. Stimulation of wt-EGFR with EGF led to its activation within 10 minutes and also caused a marked up-regulation of TF expression in a dose-dependent manner within 24 hours (Fig. IB). The EGR inhibitor PD158780 strongly attenuated EGFRvIII-induced (Fig. IA, lane 4) and partially reduced EGF-stimulated TF expression (Fig. IA, lane 6 versus lane 7), indicating that the tyrosine kinase activity of EGFR was necessary for this effect. PD158780 did not inhibit TF expression by U87MG, which expressed little, if any, EGFR protein (Fig. IA, lane 2).

To determine if EGFR-induced TF was biologically active as a procoagulant at the cell surface (20), we measured the ability of glioma cell lines to induce plasma clotting in vitro. Neoplastic served as the positive control and consistently caused clot formation within 11 to 12 seconds. The negative control (PBS instead of tumor cells) did not cause plasma clotting within 90 seconds (data not shown). When added to plasma, cell suspensions of U87MG-EGFRvIII caused a significantly shortened plasma clotting time (19.3 ± 1.4 seconds) compared with parental U87MG (23.6 ± 0.5 seconds) and U87MG-wt-EGFR (23.8 ± 0.1 seconds); *, P < 0.01, one-way ANOVA and confirmed by Student’s t test with the
Bonferroni correction). When this assay was performed using human plasma lacking factor VII, the natural ligand for TF, the plasma clotting time after adding U87MG-EGFRvIII cells was significantly prolonged (> 90 seconds), strongly implicating TF-dependent mechanisms (Fig. 1C). Similarly, the addition of EGF to wt-EGFR glioma cells caused a dose-dependent acceleration of plasma clotting that could be reversed by the use of plasma lacking factor VII (*, \( P < 0.001 \), one-way ANOVA; Fig. 1C; data not shown). Thus, EGFRvIII-expressing gliomas show increased basal TF expression and wt-EGFR gliomas show a dose-dependent increase in TF on receptor activation. In both instances, EGFR-induced TF resulted in accelerated plasma coagulation by tumor cells in vitro.

**AP-1 is required for both basal and EGF-stimulated TF promoter activity.** To determine whether EGFR signaling augmented TF expression through transcriptional mechanisms, we examined TF gene promoter activity using luciferase reporter constructs. The basal human TF promoter (−250 to +1) consists of a distal lipopolysaccharide response element, which contains two AP-1 and one NFκB binding sites, and a more proximal serum response region, which contains three overlapping Egr-1/Sp1 binding sites [21]. We used 5′-deletion constructs of the wild-type TF promoter [pTF(wt)] to determine which transcription factors were most relevant to basal and EGF-stimulated TF expression by EGFR (Fig. 2A, left). U87MG-EGFRvIII showed a greater basal TF promoter activity (\( \hat{z}, P < 0.001 \), Student’s \( t \) test) than parental U87MG, whereas the basal promoter activity in U87MG-wt-EGFR was similar to U87MG (Fig. 2A, right). On EGF stimulation (50 ng/mL, 24 hours), TF promoter activity was significantly increased in U87MG-wt-EGFR cells and similar to levels observed in U87MG-EGFRvIII (*, \( P < 0.001 \)). The deletion of the distal AP-1 binding sites [pTF(AP-1 del)] was associated with a markedly reduced promoter activity in all three cell lines, including the EGF-stimulated conditions [***, \( P < 0.001 \) versus pTF(wt)]. Further deletion of the NFκB binding site [pTF(NFκB del)] caused additional, albeit much smaller, reductions in basal TF promoter activity. Deletion of the promoter to −67 [pTF(vector)] resulted in the complete loss of detectable transcriptional activity, suggesting that the proximal Sp1 and Egr-1 sites are required. These data indicated that AP-1 is necessary for maximal basal and EGF-stimulated TF promoter activity in GBM cells.

We next examined whether EGFR-induced expression of TF might be due to activation of JNK, a critical upstream kinase that regulates AP-1 activity. EGFRvIII and EGF stimulation of wt-EGFR caused increased levels of p-JNK1 expression, but not p-JNK2, indicating specific activation of JNK1 (Fig. 2B, left). Reduced TF expression was seen by the specific JNK inhibitor SP600125, which blocks the kinase activity of JNK and results in reduced p-JNK1 levels. Both basal TF levels (EGFRvIII) and EGF-stimulated TF levels in U87MG-EGFRvIII cells were significantly reduced by the selective JNK inhibitor SP600125, which blocks the kinase activity of JNK and results in reduced p-JNK1 levels. Both basal TF levels (EGFRvIII) and EGF-stimulated TF levels in U87MG-EGFRvIII cells were significantly reduced by the selective JNK inhibitor SP600125, which blocks the kinase activity of JNK and results in reduced p-JNK1 levels. Both basal TF levels (EGFRvIII) and EGF-stimulated TF levels in U87MG-EGFRvIII cells were significantly reduced by the selective JNK inhibitor SP600125, which blocks the kinase activity of JNK and results in reduced p-JNK1 levels.
expression were greatly reduced by SP600125, implicating JNK1 as a necessary kinase for EGFR-induced TF expression.

The inhibitory effect of SP600125 on JNK activity was confirmed by its ability to reduce the level of its substrate, p-JunD, on Western blots (Fig. 2C). For these experiments, we used the p-c-JunSer73 antibody, which detects both p-c-Jun at Ser73 and p-JunD at Ser100. To determine whether the solitary p-Jun band detected in Fig. 2C represented p-c-Jun or p-JunD, we used specific siRNA directed against c-Jun or JunD. We found that siRNA directed at JunD decreased the intensity of the p-Jun band, but siRNA directed at c-Jun did not, indicating that this band represents p-JunD (Supplementary Fig. S1A, left).

Suppression of JunD by siRNA substantially reduced EGFR-induced TF expression (Supplementary Fig. S1A, right), whereas suppression of c-Jun did not (Supplementary Fig. S1B, left), further implicating JunD in TF transcription. These findings were also supported by cotransfecting U87MG-wt-EGFR or U87MG-EGFRvIII cells with the TF promoter-luciferase reporter [pTF(wt)] together with either a dominant-negative mutant c-Jun (c-JunDN) or a dominant-negative mutant JunD (JunDDN) expression plasmid. JunDDN was capable of significantly reducing luciferase signals compared with vector controls (*, P < 0.001, Student’s t test; Supplementary Fig. S1B, right), whereas expression of c-JunDN caused only a modest reduction (data not shown). We also found that knocking down JNK1 but not JNK2 by siRNA significantly reduced EGFR-induced TF expression (Fig. 2D; Supplementary Fig. S1C, left).

Because the TF promoter also contains a NFκB site that could mediate the effects of EGFR activity, we investigated the effect of its siRNA knockdown on TF expression. We found that knockdown of NFκB1 (p105/p50) did not inhibit EGFRvIII-induced or EGF-stimulated TF expression (Supplementary Fig. S1C, right). The effect of targeted siRNA against NFκB1 activity was confirmed by showing reduced expression of IL-8, a traditional target of NFκB-directed transcription (22). IL-8 levels in serum-free medium were greatly reduced by SP600125, implicating JNK1 as a necessary kinase for EGFR-induced TF expression.
PTEN was recently shown to inhibit JNK through its antagonizing effect on PI3K (24). To determine the relative contribution of PI3K and JNK to the inhibition of EGFR-mediated TF promoter activity, we used the specific PI3K inhibitor LY294002 and the specific JNK inhibitor SP600125 in dual-luciferase assays. Both the basal (Fig. 3C, left) and EGF-stimulated (Fig. 3C, right) TF promoter activities were dramatically inhibited by LY294002 and SP600125, suggesting that both PI3K and JNK are important regulators of this response (*, $P < 0.001$, one-way ANOVA, confirmed by Student’s $t$ test with the Bonferroni correction).

**PTEN antagonizes EGFR-induced AP-1 transcriptional activity.** To determine if the effects of PTEN on EGFR-induced TF transcription were due to specific regulation of AP-1 activity, we used lentivirus infection of PTEN-HA–infected U87MG-EGFRvIII cells with a luciferase reporter plasmid that contained AP-1 enhancer elements. PTEN substantially reduced cellular TF expression under both basal and EGF-stimulated conditions (Fig. 3C, right). We then investigated which of the Jun family member(s) of the AP-1 complex were most affected by PTEN. On Western blotting of separated nuclear and cytoplasmic proteins, we found that PTEN decreased the level of nuclear p-JNK1 and p-JunD levels in U87MG-EGFRvIII cells (**, $P < 0.001$) and under EGF stimulation; Fig. 4B, right). These results are consistent with the notion that PTEN antagonizes EGFR-induced AP-1 transcriptional activity in the context of gliomas.

**Restoration of PTEN inhibits both basal and EGF-stimulated TF expression.** PTEN loss is a genetic signature of GBM (23). To examine whether PTEN influences the EGFR-mediated expression of TF, we restored PTEN in PTEN-null U87MG-wt-EGFR and U87MG-EGFRvIII cells using a HA-tagged lentiviral expression vector. Infection with GFP-expressing lentivirus was used as a negative control. Restoration of PTEN inhibited Akt phosphorylation and also caused a marked reduction of basal TF protein expression by U87MG-EGFRvIII (Fig. 3A, left). It also attenuated TF expression following EGF stimulation of U87MG-wt-EGFR cells (Fig. 3A, right). To determine whether the effect of PTEN on TF expression was due to transcriptional activation of the TF gene, we performed dual-luciferase reporter assays. Lenti-PTEN-HA infection of U87MG-EGFRvIII (Fig. 3B, left) and U87MG-wt-EGFR (Fig. 3B, right) glioma cells caused a marked reduction in basal and EGF-stimulated TF promoter activity compared with lenti-GFP–transfected controls (**, $P < 0.001$ versus GFP-infected cells; #, $P < 0.001$ versus GFP-infected cells under EGF stimulation, Student’s $t$ test).

PTEN was recently shown to inhibit JNK through its antagonizing effect on PI3K (24). To determine the relative contribution of PI3K and JNK to the inhibition of EGFR-mediated TF promoter activity, we used the specific PI3K inhibitor LY294002 and the specific JNK inhibitor SP600125 in dual-luciferase assays. Both the basal (Fig. 3C, left) and EGF-stimulated (Fig. 3C, right) TF promoter activities were dramatically inhibited by LY294002 and SP600125, suggesting that both PI3K and JNK are important regulators of this response (**, $P < 0.001$, one-way ANOVA, confirmed by Student’s $t$ test with the Bonferroni correction).

**PTEN attenuates EGFR-induced TF expression.** A, left, Western blot of U87MG-EGFRvIII cell lysates following lentiviral infection of PTEN-HA or GFP shows reduced expression of TF in PTEN-HA–infected cells; right, Western blot of cell lysates from U87MG-wt-EGFR cells following lentiviral infection of PTEN-HA or GFP shows reduced TF expression under both basal and EGF-stimulated (50 ng/mL, 24 h) conditions. B, left, dual-luciferase assay of wild-type TF promoter [pTF(wt)] activity in U87MG-EGFRvIII cells shows that lentiviral infection of PTEN-HA reduces promoter activity compared with GFP-infected cells, **, $P < 0.001$. Right, dual-luciferase assay of wild-type TF promoter [pTF(wt)] activity in U87MG-wt-EGFR cells infected with lenti-PTEN-HA shows reduced TF promoter activity under both basal (**, $P < 0.001$) and EGF-stimulated (#, $P < 0.001$) conditions compared with GFP-infected cells. C, left, dual-luciferase assay of wild-type TF promoter [pTF(wt)] activity in U87MG-EGFRvIII cells shows that inhibitors of PI3K (LY294002, 10 μmol/L) and/or JNK (SP600125, 10 μmol/L) significantly reduce TF promoter activity compared with the controls (DMSO treated), *, $P < 0.001$. Right, dual-luciferase assay of wild-type TF promoter [pTF(wt)] activity in U87MG-wt-EGFR cells shows that inhibitors of PI3K (LY294002, 10 μmol/L) and/or JNK (SP600125, 10 μmol/L) activity significantly reduced TF promoter activity in EGF-stimulated cells (50 ng/mL) compared with the control [DMSO + EGF (50 ng/mL) treated], #, $P < 0.005$.
In EGF-stimulated U87MG-wt-EGFR cells, PTEN reduced the nuclear levels of p-JNK1 and p-JunD levels but did not reduce p-c-Jun levels (Fig. 4D). Thus, PTEN inhibits cellular AP-1 activity mainly through down-regulation of JNK1 and JunD activities.

EGFR-induced TF expression through AP-1 activation is signaled via the PI3K and mitogen-activated protein kinase/ERK1/2 pathways. Mitogen-activated protein kinase (MAPK) and PI3K are two major downstream signaling pathways of receptor tyrosine kinases, including EGFR (25). To examine the roles of these pathways in the EGFR-mediated up-regulation of TF, we used the PI3K inhibitor LY294002, the MAPK/ERK1/2 inhibitor U0126, and the mammalian target of rapamycin (mTOR) inhibitor rapamycin. We found that LY294002 and rapamycin strongly reduced basal TF expression by U87MG-EGFRvIII and also attenuated EGF-stimulated TF in U87MG-wt-EGFR cells (Fig. 5A and B). U0126 also reduced TF expression but less potently. We also found that LY294002 reduced the levels of p-JNK1/2 in both U87MG-EGFRvIII and U87MG-wt-EGFR cells, consistent with previous reports that PI3K stimulates JNK activity (Fig. 5C and D; ref. 24). Interestingly, mTOR inhibition by rapamycin led to a moderate increase in p-JNK1/2 levels in U87MG-EGFRvIII (Fig. 5C), whereas it reduced p-JNK1/2 in U87MG-wt-EGFR cells under EGF stimulation (Fig. 5D). Mechanisms that account for this difference between EGFRvIII and wt-EGFR require further study. The increased p-JNK observed in U87MG-EGFRvIII cells following rapamycin could be due to the loss of negative feedback regulation of mTOR on EGFR, leading to enhanced JNK activity (24). p-JunD levels were greatly reduced by all three inhibitors indicating both JNK-dependent and JNK-independent regulation of JunD activity (Fig. 5C and D).

Both ERK1/2 and JNK are MAPK family members (26). Inhibition of ERK1/2 activity by U0126 did not greatly affect basal p-JNK1/2 levels in U87MG-EGFRvIII cells (Fig. 5C) and had moderate inhibitory effects on p-JNK1/2 in U87MG-wt-EGFR cells (Fig. 5D). This observation is consistent with the reports that activation of Ras/ERK1/2 directly up-regulates AP-1 transcriptional activity, whereas inhibition of ERK1/2 reduces it (27). Combined, the data suggest that EGFR-induced TF expression occurs predominantly through JNK and regulated by both PI3K and MAPK/ERK1/2.

Human GBMs overexpress EGFR and TF compared with AAs. We investigated the expression of EGFR and TF proteins in human high-grade astrocytomas, including 23 GBMs (WHO grade 4) and 7 AAs (WHO grade 3). The results of this analysis are shown.

Figure 4. PTEN antagonizes EGFR-induced TF expression by down-regulating AP-1 activity. A, dual-luciferase assay of AP-1 activity in U87MG-EGFRvIII cells shows that lentiviral infection of PTEN-HA causes a significant reduction of reporter activity compared with GFP-infected cells. *, $P < 0.005$. B, dual-luciferase assay of AP-1 activity in U87MG-wt-EGFR cells shows that lentiviral infection of PTEN-HA reduces reporter activity under basal and EGF-stimulated conditions compared with GFP-infected cells. *, $P < 0.005$ versus GFP-infected cells; $\#$, $P < 0.001$ versus EGF-stimulated (50 ng/mL) GFP cells. C, Western blot of nuclear and cytoplasmic compartments of U87MG-EGFRvIII cells infected with PTEN-HA or GFP reveals a reduced expression of nuclear p-JNK1 and p-JunD levels in PTEN-HA–infected cells compared with GFP-infected cells. No differences were noted in the expression of total JNK1/2, c-Jun, or JunD. Histone H1, loading control for nuclear protein. D, Western blot of nuclear and cytoplasmic compartments of U87MG-wt-EGFR cells infected with PTEN-HA or GFP under basal (0 h) or EGF-stimulated conditions (50 ng/mL, 1 and 24 h) shows decreased expression of nuclear p-JNK1 and p-JunD levels in PTEN-HA–infected cells compared with GFP-infected cells following EGF stimulation. Total JNK, c-Jun, and JunD levels were not affected by EGF. Histone H1, loading controls for nuclear protein.
in Tables 1 and 2. These specimens represent a subset of brain tumors previously investigated for the presence of intravascular thrombosis (4). Among 23 GBMs, 22 (96%) contained microscopic intravascular thrombosis as noted by the complete occlusion of vascular lumens by an organizing clot, in addition to the diagnostic features of necrosis and/or microvascular hyperplasia. None of the seven AA specimens contained intravascular thrombosis (and by definition did not contain necrosis or vascular hyperplasia).

**Table 1. Immunohistochemical analysis of EGFR protein expression in human GBM and AA specimens**

<table>
<thead>
<tr>
<th>Score</th>
<th>GBM/EGFR&lt;sup&gt;amp+&lt;/sup&gt; (n = 9)</th>
<th>GBM/EGFR&lt;sup&gt;amp−&lt;/sup&gt; (n = 14)</th>
<th>AA (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (0%)</td>
<td>7 (50%)</td>
<td>6 (86%)</td>
</tr>
<tr>
<td>+</td>
<td>0 (0%)</td>
<td>3 (21%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>++</td>
<td>5 (56%)</td>
<td>3 (21%)</td>
<td>1 (14%)</td>
</tr>
<tr>
<td>+++</td>
<td>4 (44%)</td>
<td>1 (8%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*Analyzed by one-way ANOVA based on the scores of each group.

**Figure 5.** EGFR-induced TF expression through AP-1 activation is signaled via the PI3K/Akt/mTOR and MAPK/ERK1/2 pathways. A, Western blot of cell lysates from U87MG-EGFRvIII gliomas. B, Western blot of cell lysates from U87MG-wt-EGFR gliomas under basal and EGF-stimulated conditions. Inhibition of PI3K (LY294002), MAPK/ERK1/2 (U0126), and mTOR (Rapamycin) caused reduced basal TF in U87MG-EGFRvIII (A) and reduced EGF-stimulated TF in U87MG-wt-EGFR gliomas (B). All the inhibitors were used at 10 μmol/L for 24 h. β-actin, loading control. C, Western blot of cell lysates from U87MG-EGFRvIII gliomas shows that inhibition of PI3K (LY294002) and MAPK/ERK1/2 (U0126) reduced expression of p-JNK1/2. Inhibition of mTOR by rapamycin led to a moderate increase of p-JNK1/2. Basal p-JunD levels were reduced by all three inhibitors. D, Western blot of cell lysates from U87MG-wt-EGFR gliomas shows that EGF-stimulated p-JNK1/2 and p-JunD levels were attenuated by all three inhibitors. β-actin, loading control.
Among the GBMs, 39% (9 of 23) showed EGFR amplification by FISH (Fig. 6A, left top and bottom). None of the AAs (0 of 7) showed EGFR amplification. In our analysis of EGFR expression by immunohistochemistry, we found that 86% (6 of 7) of AA samples showed no appreciable expression (Fig. 6A, middle top); in GBM specimens, 22% (5 of 23) showed strong (3+) and 35% (8 of 23) showed moderate (2+) EGFR staining (Fig. 6A, middle bottom; Table 1). None of the GBMs with weak (1+) or absent (0) EGFR expression showed EGFR amplification. On ANOVA analysis, GBMs as a group had significantly greater EGFR expression than AAs (P < 0.001). Those GBMs with EGFR amplification had a higher protein expression of EGFR than tumors without amplification (P < 0.001). Immunohistochemistry for TF in AA showed weak (1+; 6 of 7) or absent (0; 1 of 7) staining (Fig. 6A, right top), whereas 30% (7 of 23) of GBMs showed strong (3+) TF expression and 39% (9 of 23) showed moderate (2+) expression (Fig. 6A, right bottom; Table 2). GBMs as a group showed much greater TF protein expression than AAs (P = 0.002). The expression of TF in EGFR-amplified GBMs was greater than that of nonamplified GBMs, but this difference did not achieve statistical significance on ANOVA analysis (P = 0.22). When all high-grade astrocytoma specimens (GBMs and AAs) were considered, there was a strong positive correlation between the expression of EGFR and TF (ρ = 0.5, Spearman’s rank test). Thus, human GBMs show greater EGFR protein expression than AA and this is associated with both a higher expression of TF and a higher frequency of intravascular thrombosis.

To further support the above findings, proteins extracted from another 11 frozen human GBM specimens were analyzed for EGFR and TF protein expression by Western blot. The five GBMs with the strongest EGFR expression also showed the strongest TF expression (Fig. 6B). The six GBMs with the least EGFR expression showed only weak to moderate TF protein levels. EGFR/actin and TF/actin expression ratios were obtained for each specimen following densitometry of protein bands (Fig. 6B). Comparison of these ratios among specimens showed a strong positive correlation between EGFR and TF expression (ρ = 0.8, Spearman’s rank test).

Discussion

Once astrocytomas have progressed to GBM, they show accelerated growth and rapid progression to death (3, 28). Mechanisms responsible for the abrupt onset of explosive growth are still being defined but are likely related to the development of necrosis and angiogenesis (microvascular hyperplasia), which are two defining features of GBM histology and powerful predictors of poor prognosis (2, 3, 5). Necrosis and the ensuing hypoxia-induced angiogenesis could be initiated or propagated by intravascular thrombosis (2, 6). Nearly all GBM specimens (grade 4) show microscopic intravascular thrombosis within tissue specimens, but lower-grade astrocytomas (grades 2 and 3) that lack necrosis only rarely do. Importantly, those grade 3 tumors that lack necrosis but show intravascular thrombosis have aggressive clinical courses, similar to GBM, suggesting that thrombosis may initiate an aggressive growth phase (4).

A strong relationship between human malignancy and abnormal blood clotting is well established but underlying mechanisms are not (3, 5). One of the most highly up-regulated prothrombotic factors in human malignancy is TF, the primary cellular initiator of plasma coagulation (29, 30). In astrocytomas, TF levels correlate with tumor grade and are highest in GBM. Indeed, publicly available data sets of gene expression7 show that TF is strongly up-regulated in GBM specimens compared with lower-grade astrocytomas (31–33). Previous reports have indicated that oncogenic events could trigger TF overexpression and coagulopathy (34). In light of this, we investigated two genetic alterations specific to the GBM histology that are rare in lower-grade astrocytomas—EGFR amplification and rearrangement and PTEN loss—to define their roles in regulating TF expression (11). We found that EGFR activation was associated with marked increases in TF expression by human GBM cells, both in constitutively active EGFRVIII-expressing cells and in EGF-stimulated wt-EGFR-expressing cells. In all cases, increased TF was associated with accelerated plasma coagulation dependent on the interaction of TF with factor VII.

These findings are supported by our investigations on human high-grade astrocytoma resection specimens. GBMs, which almost uniformly contain microscopic evidence of intravascular thrombosis, showed a higher frequency of EGFR amplification and a greater level of EGFR protein expression than AAs, and this EGFR up-regulation was strongly associated with the increased expression of TF. Thus, the expression of EGFR and TF proteins and the presence of intravascular thrombosis are tightly linked to the GBM.

Table 2. Immunohistochemical analysis of TF protein expression in human GBM and AA specimens

<table>
<thead>
<tr>
<th>Score</th>
<th>GBM/EGFR&lt;sup&gt;Amp&lt;/sup&gt; (&lt;i&gt;n&lt;/i&gt; = 9)</th>
<th>GBM/EGFR&lt;sup&gt;Amp&lt;/sup&gt; (&lt;i&gt;n&lt;/i&gt; = 14)</th>
<th>AA (&lt;i&gt;n&lt;/i&gt; = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (0%)</td>
<td>1 (8%)</td>
<td>1 (14%)</td>
</tr>
<tr>
<td>+</td>
<td>1 (11%)</td>
<td>5 (36%)</td>
<td>6 (86%)</td>
</tr>
<tr>
<td>++</td>
<td>5 (56%)</td>
<td>4 (28%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>+++</td>
<td>3 (33%)</td>
<td>4 (28%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>$p^*$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBM vs AA</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBM/EGFR&lt;sup&gt;Amp&lt;/sup&gt;+ vs GBM/EGFR&lt;sup&gt;Amp&lt;/sup&gt;−</td>
<td>N.S. (P = 0.22)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: 0, no staining; +, weak staining; ++, moderate staining; ++++, strong staining; EGFR<sup>Amp</sup>, EGFR gene amplified; EGFR<sup>Amp</sup>−, EGFR gene nonamplified.

Abbreviation: N.S., not significant.

*Analyzed by one-way ANOVA based on the scores of each group.

EGFR and PTEN Modulate Tissue Factor in GBM

7 http://www.oncomine.org
Figure 6. Analysis of EGFR and TF expression in human AA and GBM samples. A, FISH analysis of EGFR gene status and immunohistochemical (IHC) analysis of EGFR and TF protein in human GBM and AA specimens. Left, representative fluorescence images showing the EGFR (orange) and chromosome 7 centromeric (green) signals in interphase GBM cells. Left top, GBM specimen that is nonamplified for EGFR shows roughly equal numbers of EGFR and centromeric signals per nucleus (typically two EGFR signals per nucleus). Left bottom, GBM with EGFR gene amplification shows greatly increased signals for EGFR compared with centromeric signal (>10 EGFR signals per nucleus). Middle, immunohistochemical analysis of EGFR protein expression in human AA and GBM specimens using Fast Red for detection. Insets, negative controls (without primary antibodies). Middle top, representative image of AA, showing a lack of EGFR staining (scored as 0 in a 0–3+ scale). Middle bottom, representative image of EGFR staining in GBM, showing strong cytoplasmic and cell surface immunoreactivity on neoplastic cells (3+, red). Right, immunohistochemical analysis of TF protein expression in AA and GBM specimens using DAB (brown) for detection. Right top, representative image of AA, showing weak cytoplasmic TF expression (1+, brown) in scattered tumor cells. Right bottom, GBM specimen showing strong cytoplasmic TF staining (3+, brown) in nearly all tumor cells. B, Western blot analysis of EGFR and TF protein expression in 11 human GBM samples. Lanes 1, 2, 4, 8, and 9, five GBM samples with moderate to strong EGFR protein expression also showed the highest TF expression. The six other samples with lower EGFR protein expression showed modest to mild TF expression. There was a strong positive correlation between EGFR and TF protein expression in these specimens based on a comparison of the EGFR/β-actin and TF/β-actin ratios, determined following densitometry (ρ = 0.8, Spearman’s rank test). β-actin, loading control.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 5/1/2008; revised 11/17/2008; accepted 12/16/2008; published OnlineFirst 3/10/09.
Grant support: USPHS NIH awards CA-109382 and NS053727 (D.J. Brat), CA-94233 (D.L. Durden), and CA86335 and CA116804 (E.G. Van Meir).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Gabriela Denning for providing the full-length COOH terminally HA-tagged human PTEN cDNA vector, Drs. Frank Furnari and Webster Cavenee for the U87-wt-EGFR and U87-EGFRvIII cell lines, and Daniel Drake for the statistical analysis for the immunohistochemical results of human GBM specimens.


References


Epidermal Growth Factor Receptor and PTEN Modulate Tissue Factor Expression in Glioblastoma through JunD/Activator Protein-1 Transcriptional Activity

Yuan Rong, Vladimir E. Belozerov, Carol Tucker-Burden, et al.

Cancer Res  Published OnlineFirst March 10, 2009.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-08-1547

Supplementary Material  Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2009/03/05/0008-5472.CAN-08-1547.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.