**Endothelial Expression of TNF Receptor-1 Generates a Proapoptotic Signal Inhibited by Integrin α6β1 in Glioblastoma**

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### Abstract

Activation of TNF receptor 1 (TNF-R1) can generate signals that promote either apoptosis or survival. In this study, we show that these signals can be determined by the character of the extracellular matrix in the tumor microenvironment. Specifically, through studies of glioblastoma, we showed that TNFα stimulation induced apoptosis of primary brain endothelial cells (EC) attached to collagen or fibronectin (which engage integrins α2β1/α3β1 and α6β1, respectively), but did not induce apoptosis of ECs attached to laminin (which engages integrins α6β1 and α3β1). TNF-R1 expression was significantly higher in ECs in glioblastoma (GBM) tumors compared with ECs in normal brain specimens. TNFα was also expressed in GBM tumor-associated ECs, which was associated with longer patient survival. ECs plated on anti-integrin α2 or α3 antibody were susceptible to TNFα-induced apoptosis, whereas those plated on anti-integrin α6 antibody were not. Moreover, the ECs plated on laminin, but not collagen, expressed cellular FLICE inhibitory protein (cFLIP) and TNFα stimulation of laminin-attached cells in which cFLIP had been downregulated resulted in the induction of apoptosis. In contrast, attachment to laminin did not induce cFLIP expression in GBM tumor stem cells. Together, our findings indicate that the laminin receptor integrin α6β1 promotes the survival of brain ECs by inhibiting prodeath signaling by TNF-R1, in part by inducing cFLIP expression. *Cancer Res; 72(6); 1428–37. ©2012 AACR.*

### Introduction

The highly vascularized nature of glioblastoma (GBM) tumors suggests that antiangiogenic therapy may be efficacious in this devastating disease; however, little is known about the mechanisms that regulate the antiangiogenic responses of the tumor-associated endothelial cells (EC). The tumor-associated neovasculature is malformed with frequent dilatation and tortuosity of the blood vessels. These malformations, the altered repertoire of pericytes (1) and other abnormalities are thought to reflect the perturbations in the pro- and antiangiogenic stimuli within the tumor microenvironment (reviewed in refs. 2–4). Proangiogenic molecules are secreted by both tumor cells and host cells and are released during remodeling of the extracellular environment by the tumor cells as well as during the process of angiogenesis itself. These stimuli can, however, elicit counteractive responses. For example, normal ECs in tumor-bearing organs can respond to the proangiogenic stimuli from tumor cells by upregulating their antiangiogenic pathways (3).

It is well established that TNFα, a ligand for both TNF receptor (TNF-R) 1 and 2, can initiate either a proapoptotic or a prosurvival signal in ECs. The proapoptotic signal is mediated through activation of TNF-R1, which results in the activation of caspases-8 and -3 (5). Unlike TNF-R1, TNF-R2 does not contain a death domain and so cannot promote apoptosis directly. The prosurvival signal mediated through TNF-R1 or TNF-R2 occurs via the activation of NF-κB and the resultant expression of antiapoptotic proteins, including cellular FLICE inhibitory protein (cFLIP) and XIAP (6). TNF-R2 activates the Bmx/Etk kinase promoting cell migration and proliferation, with the promotion of proliferation being due, at least in part, to Bmx transactivation of VEGF-R2 (7).

The environmental cues that determine whether TNF-R1 sends a proapoptotic signal or a prosurvival signal in ECs have
not yet been elucidated fully. Primary brain microvascular ECs in culture proliferate and resemble reactive ECs and are therefore used as a surrogate model of tumor-associated ECs. Using this model, we found previously that activation of the TNF-R1 by the extracellular matrix (ECM) proteins thrombospondin-1 and 2 (TSP-1 and TSP-2), which are endogenous inhibitors of angiogenesis, can induce apoptosis of these ECs (8), and that a small peptide (ABT-510) from the type-1 repeat of TSP-1 also induces apoptosis of primary brain ECs through a death receptor pathway (9). Specific environmental cues may also regulate the levels of expression of TNF-R1 and TNF-R2 on ECs as indicated by the reports that, in mouse models, inflammation associated with bacterial infection of the lung upregulates TNF-R1 on lung ECs, and that ischemic injury of the hind limb upregulates TNF-R2 on the hind limb ECs (7, 10).

Members of the integrin family of cell adhesion receptors play a role in the interactions of many types of cells with the ECM and act to regulate a number of biologic processes, including cell survival, proliferation, and motility in a context-dependent manner (reviewed in refs. 4, 11). The ligation or engagement of integrins can initiate prosurvival signals, such as activation of the mitogen—activated protein kinase (MAPK) pathway downstream of Src, focal adhesion kinase, and Ras (12), or promote cell survival by inhibiting caspase-8 activity (4). Recently, expression of the α6β1 integrin, which mediates adhesion to laminin, has been shown to contribute to the tumorigenicity of several types of cancer cells, including malignant glioma, melanoma, breast, and prostate cancer cells (13–16). Specifically, on engagement of integrin α6β1 in prostate cancer cells, androgen signaling induces cell survival through activation of NF-κB and induction of Bcl-xL expression (17). Integrin α6β1 is enriched on glioma stem cells and necessary for maintenance of their capacity for self-renewal (18) as well as affecting the function of normal neural stem cells (19). A direct effect of cell attachment to laminin on the sensitivity of glioma cells to proapoptotic agents has been reported, and phosphoinositide 3-kinase seemed to be necessary for this effect (20). Collectively, the current literature suggests that the expression of integrin α6β1 is necessary for the promotion of cell proliferation and the inhibition of apoptosis in tumor cells propagated in vitro and in vivo in mouse models, as well as in promoting tumor cell migration and metastasis. In terms of EC function, integrin α6β1 seems to be necessary for delta-like 4/Notch signaling that controls tip versus stalk cell selection in angiogenesis (21). The ability of other integrins to promote the survival of ECs is suggested by the report that αvβ3, which binds to multiple ECM proteins, is associated with the activation of NF-κB and the induction of osteoprotegerin (22).

We therefore investigated the expression of TNFα, TNF-R1, and TNF-R2 on tumor-associated ECs in GBM as compared with normal brain and the integrin regulation of TNFα-induced proapoptotic signal on brain and GBM-derived ECs.

Materials and Methods

Reagents

Both laminin from human placenta, which is a mixture of laminins (Sigma Aldrich; catalog no. L6274) and laminin-1 (R&D system; catalog no. 3400-010-01) for plating of ECs with highly similar results (data not shown). Fibronectin was from EMD Biosciences, Inc., and collagen from MP Biomedicals, Inc. The neutralizing monoclonal antibodies (mAb) anti-integrin α1, α2β1, α3β1, α4, α5β1, α6, β4, αvβ5, and αvβ3 were from Invitrogen and Santa Cruz Biotechs. Antibodies used for immunohistochemistry were the following: rabbit anti-TNF-R1 (Abcam Inc.; dilution 1:2,000), rabbit anti-TNF-R2 (Abcam; dilution 1:300), rabbit anti-TNFα (Abcam; dilution 1:200), rabbit anti-TNFα (Cell Signaling; dilution 1:200), mouse mAb anti-von-Willibrand factor (vWF; Abcam; dilution 1:1,000), rabbit anti-vWF (Chemicon; dilution 1:500), rabbit anti-vWF (DAKO; dilution 1:1,000), mouse mAb anti-CD31 (Sigma Aldrich; dilution 1:500); AlexaFluor-488 goat anti-rabbit (Invitrogen; dilution 1:800), AlexaFluor-488 goat anti-mouse (Invitrogen; dilution 1:800), AlexaFluor-594 goat anti-mouse (Invitrogen; dilution 1:1,000), biotinylated goat anti-rabbit (Vector Laboratories Inc.; dilution 1:250), and biotinylated goat anti-mouse (Vector Laboratories Inc.; dilution 1:250).

Human tissues

Primary human brain microvessel ECs were purchased from Cell Systems and propagated as recommended and described previously (23). Fresh snap-frozen, formalin-fixed, and paraffin-embedded normal adult brain (tissue adjacent to a seizure focus resected to gain access to the seizure focus) and GBM samples were obtained from the Cleveland Clinic Brain Tumor Bank in accordance with the guidelines and policies of the Cleveland Clinic Institutional Review Board (#2559). For isolation of ECs from human GBM, fresh aliquots of GBM were obtained from the University Hospital Brain Tumor and Neuro-Oncology Program in accordance with the guidelines and policies of the University Hospitals Institutional Review Board (#Y02, 10Z07, and 1307). The diagnosis of GBM tumor was according to the classification of the World Health Organization (24).

Isolation of ECs from human GBM

The fresh aliquots of GBM were placed in cold sterile saline, minced, digested with papain and DNase I ( Worthington Biochemical Corporation; 37°C, 15–60 minutes), filtered (40 μm), and the material trapped on the filter resuspended and digested further in 2 mg/mL collagenase type II [Invitrogen (GibcoBRL); 10 to 30 minutes; refs. 25, 26]. The suspension was filtered (70 μm), the cells in the filtrate pelleted (200 g, 5 minutes), resuspended in EC media, and seeded onto laminin-coated wells. Confluent cells were detached with Accutase (Innovative Cell Technologies) and passaged into a 0.5% gelatin-coated flask in EC media. Pure EC populations were then obtained by magnetic bead separation using CD31-coupled MicroBeads (Miltenyi Biotech; ref. 26).

Immunofluorescence and immunohistochemical analysis

Double-label immunofluorescence analysis of frozen sections (27) and immunohistochemistry (23) were conducted as
described previously. Staining in more than 5% of ECs was the threshold for positivity.

**Downregulation of gene expression using siRNA**

SMARTpool siRNA consisting of a pool of 4 SMARTselection-designed siRNA duplexes directed toward p38 MAPK and extracellular-regulated kinase (ERK) 1 were purchased from Dharmacon, as were the controls. Predesigned siRNA duplexes directed toward cFLIP and controls were purchased from Ambion. Transient transfections were carried out as described previously (23). Cells were exposed to siRNA for 72 hours, followed by preparation of whole-cell extracts and immunoblotting.

**Apoptosis assays**

TUNEL assays were conducted and analyzed as described previously (23). Staurosporine (Sigma) was used as a positive control.

**Immunoblot analysis**

Cells were lysed in NP-40 lysis buffer with protease inhibitors and immunoblotted as described previously (23). Anti–phospho-p38 MAPK, total p38 MAPK, anti–phospho-c-jun-NH₂-kinase (JNK), and total JNK were obtained from Cell Signaling; anti-FLIP from Alexus; anti-cleaved (active) caspase 7 from EMD Biosciences; and anti-Bcl2, anti-Bcl-xL, and anti-glyceraldehyde-3-phosphate dehydrogenase from SantaCruz Biotech.
Cell attachment assays

Ninety-six-well plates were coated overnight with 10 μg/mL of ovalbumin, fibronectin, collagen, or laminin in PBS then washed, blocked with 5% heat-denatured bovine serum albumin (BSA; 30 minutes). The cells (20,000 cells per well) plus-minus blocking anti-integrin antibodies were plated onto coated wells and allowed to attach (20 minutes, 37°C), as described previously (28). Adherent cells were fixed, stained, and quantitated by spectrophotometric absorbance. Attachment to ovalbumin was subtracted. Conditions were assayed in replicates of 5, and the data are presented as the mean ± SEM.

Integrin immobilization assays

Eight well chamber slides were coated with goat anti-mouse immunoglobulin G (IgG: 20 μg/mL, 45 minutes, 25°C), blocked with 1% heat-denatured BSA (1 hour, 37°C), incubated with 10 μg/mL anti-integrin mAb (45 minutes, 37°C), washed, 20,000 cells in cell adherence buffer added to each well and allowed to attach (37°C, 3 hours) as described previously (29). This was followed by TNFα treatment (overnight incubation, 37°C, 5% CO2), after which the number of apoptotic cells was determined using the TUNEL assay.

Quantitative reverse transcriptase PCR

Total RNA was isolated with QIAGEN’s RNeasy kit and quantified with RiboGreen (Invitrogen). Quantitative reverse transcriptase PCR (qRT-PCR) was conducted using the SYBER Green reagent (Bio-Rad) as described previously (8). cFLIP was normalized to β-actin to derive the relative cFLIP mRNA level at each time point.

Results

Increased TNFα, TNF-R1, and TNF-R2 expression on tumor-associated ECs in GBM

We first assessed the expression of TNFα, TNF-R1, and TNF-R2 in tumor-associated ECs in 49 GBM and 38 normal brain biopsies. vWF was used as a marker of ECs (1). We found significantly higher expression of TNFα, TNF-R1, and TNF-R2 in the tumor-associated ECs in the GBM as compared with the ECs in normal brain (P < 0.0001, <0.0001, and <0.001, respectively; Mann–Whitney test; Figs. 1A–C; Supplementary Fig. S1, and Table 1). The types of tumor-associated vessels expressing TNFα, TNF-R1, and TNF-R2 were capillaries; mother vessels, which are dilated capillaries that lack pericytes; and glomeruloid microvascular proliferations, which are groups of abnormal capillaries formed from the intussuception and bridging in a mother vessel, as classified by Nagy and colleagues (30). TNF-R1 was detected in tumor-associated ECs in 47 of the 49 GBM biopsies, and TNF-R2 was detected in tumor-associated ECs in 46 of the 49 GBM biopsies (Table 1). The levels of TNF-R1 expression in the tumor-associated ECs exhibited an inverse relationship with the levels of expression of TNF-R1 in the tumor cells as determined with a linear correlation statistic, in which TNF-R1 expression in the ECs increases as TNF-R1 expression in the tumor cells decreases (P = 0.0001, Mantel–Haenszel). TNFα expression was detected in tumor-associated ECs in only 28 of the 49 GBM biopsies. TNFα expression in the tumor-associated ECs correlated with a high TNF-R1 expression in these cells, but did not correlate with a high TNF-R2 expression in the tumor-associated ECs (P = 0.0084 and 0.7998, respectively, Mann–Whitney test). We did not find a correlation between the vessel surface area density and TNFα expression in the tumor-associated ECs in the GBM (data not shown).

When we separated out the subset of GBM tumors that were recurrent, we found significantly higher expression of TNFα, TNF-R1, and TNF-R2 in the tumor-associated ECs of 31 untreated GBM tumors than the 38 normal brains (P < 0.0001, <0.0001, and <0.001, respectively; Mann–Whitney test), and a significantly higher expression of TNFα, TNF-R1, and TNF-R2 in the tumor-associated ECs of the 18 recurrent GBM tumors than the 38 normal brains (P < 0.001, <0.0001, and <0.0001, respectively; Mann–Whitney test; Supplementary Tables S1 and S2). Notably, the 28 patients with tumors that had TNFα expression in the tumor-associated ECs (14 untreated GBM and 14 recurrent GBM) had a significantly longer survival (P = 0.0159; log-rank test; Fig. 1D). In support of these findings, data mining of the Oncomine Database revealed a significantly greater expression of TNF-R1 and TNF-R2 mRNAs

| Table 1. Increased expression of TNFα, TNF-R1, and TNF-R2 in the ECs in GBM |
|------------------|------------------|------------------|
| Tissue           | TNFα             | TNF-R1           | TNF-R2           |
| Normal Brain (n = 38)  | wk+ (3 of 38)    | wk+ (12 of 38)   | wk+ (9 of 38)    |
|                   | Negative (35 of 38) | Negative (26 of 38) | Negative (29 of 38) |
| GBM Tumor (n = 49)  | wk+ (28 of 49)   | wk+ (30 of 49)   | wk+ (35 of 49)   |
|                   | Negative (21 of 49) | 1+ (16 of 49)    | 1+ (10 of 49)    |
|                   |                   | 2+ (2 of 49)     | 2+ (1 of 49)     |
|                   |                   | Negative (1 of 49) | Negative (3 of 9) |

NOTE: Immunohistochemistry was conducted as described in the Materials and Methods. The staining was compared with a negative control of rabbit IgG and a positive control of anti-vWF antibody. The staining intensity was graded as negative; wk+ (weak intensity staining); 1+ (medium intensity staining); and 2+ (very strong intensity staining).
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used a concentration of 25 ng/mL for examination of the roles
25 or 50 ng/mL (Supplementary Fig. S3), so we subsequently
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respectively; Supplementary Fig. S2; ref. 31).

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P
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TNFα proapoptotic signaling is regulated by the ECM and
the integrin that is engaged

Analysis of TNFα-induced apoptosis of primary brain ECs
indicated that maximal apoptosis was induced with TNFα at
25 or 50 ng/mL (Supplementary Fig. S3), so we subsequently
used a concentration of 25 ng/mL for examination of the roles
of 3 ECM proteins known to be expressed in the EC basement
membrane (reviewed in refs. 32, 33) on apoptosis, as detected
by the terminal deoxynucleotidyl transferase–mediated dUTP
nick end labeling (TUNEL) assay or blotting for cleaved cas-
pase-7. We found that collagen and fibronectin attachment
were permissive for the proapoptotic signal of TNFα/TNF-R1
on brain ECs, whereas laminin attachment was inhibitory
(Fig. 2A and B).

To identify the integrin(s) that mediates the attachment
of the brain ECs to the 3 ECM proteins, cell attachment assays
were conducted in the presence of blocking anti-integrin
mAbs, as described previously (28). Attachment of the ECs to
collagen was mediated by integrins α2β1 and α3β1, attach-
ment to laminin was largely mediated by integrins α6β1 and
α3β1, with a smaller contribution by integrin α6β4, and
attachment to fibronectin was mediated by integrin α5β1
(Supplementary Fig. S4). To determine the relative importance
of the two α6 integrins in laminin attachment, double-label
fluorescence-activated cell sorting was conducted. We found
that integrin α6β1 was expressed on 84% of ECs, whereas
integrin α6β4 was expressed on only 14% of ECs (Supplemen-
tary Fig. S5). All of these integrins are expressed on the ECs
of the tumor-associated vasculature in GBM (34, 35).

The signaling pathways in ECs in malignant tumors may
differ from the cultured primary ECs due, for example, to the
effects of growth factors elaborated by the tumor cells and
the ongoing differentiation of cancer stem cells into ECs (36,
37). Therefore, we isolated ECs from 2 GBM and assessed their
response to TNFα stimulation. Both of the EC isolates
expressed 2 markers of ECs, that is, vWF and CD31 (Fig. 3A
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signal of TNFα, whereas attachment to laminin was inhibitory
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Integrin α6β1 is inhibitory for the proapoptotic signal
of TNFα

To identify which of the laminin-binding integrins (α6β1 or
α3β1) inhibited the proapoptotic signal of TNFα, we plated the
brain ECs on immobilized anti-integrin mAbs directed toward
integrins α6, α3, or α2β1, as we have described before (29),
and treated with TNFα. Apoptosis was determined by the TUNEL
assay. TNFα stimulation of brain ECs plated on immobilized
mAb anti-integrin α3 or the control mAb anti-integrin α2β1
resulted in apoptosis, whereas TNFα stimulation of ECs plated
on immobilized mAb anti-integrin α6 inhibited the proapo-
ptotic signal of TNFα (Fig. 2C), suggesting that integrin α6β1
signals to protect the ECs from the proapoptotic effect of
TNFα.

Attachment to laminin or engagement of integrin α6β1
induces the expression of cFLIP and thereby inhibits
TNFα-induced apoptosis

To determine the mechanism by which engagement of
integrin α6β1 or attachment to laminin protects the brain
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robust expression of the 55-kDa form of cFLIP, whereas

Figure 2. TNFα-induced apoptosis of brain ECs is regulated by the
ECM or the integrin that is engaged. A and B, brain ECs were plated
as a monolayer on wells coated with 10 μg/mL of type I collagen,
laminin, or fibronectin in M199 media with 1% FBS for 3 hours and
treated with 25 ng/mL TNFα for 17 hours. Staurosporine (0.1 μmol/L)
was used as a positive control. A, cell death was analyzed by a
TUNEL assay and an unpaired t test. B, whole-cell extracts were
prepared and apoptosis analyzed by immunoblotting for cleaved
caspase-7 using the indicated antibodies. The normalized
densitometric ratio of cleaved caspase-7 (CC7) to tubulin is shown.
C, cells were plated on immobilized anti-integrin antibodies as
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robust expression of the 55-kDa form of cFLIP, whereas
attachment to collagen did not result in the induction of detectable levels of cFLIP (Fig. 4A and B). To determine whether cFLIP was necessary for the protection from the proapoptotic signal of TNFα in ECs plated on laminin, we downregulated cFLIP with siRNA (Fig. 4C), plated the cells on laminin and treated with TNFα. In the absence of cFLIP, TNFα induced apoptosis of the brain ECs plated on laminin (Fig. 4D) suggesting that attachment to laminin or engagement of integrin α6β1 protects brain ECs from the proapoptotic signal of TNFα by inducing the expression of cFLIP.

As cFLIP is regulated at the level of transcription and translation (6), we determined the time course of cFLIP expression in the brain ECs plated on laminin and the necessity of new transcription or translation. We found that cFLIP protein and mRNA were detectable by 1 hour (Fig. 5A, C, and D, and data not shown), and that treatment with actinomycin D or cycloheximide completely blocked cFLIP expression (Fig. 5B), suggesting the induction of cFLIP in the ECs plated on laminin requires de novo transcription and translation.

To determine whether the induction of cFLIP in the brain ECs adherent to laminin was a generalized signaling mechanism found upon engagement of integrin α6β1, we plated U-87MG human GBM cells and 3 primary human glioma tumor stem cells on laminin, fibronectin, or polylysine for 4 hours and blotted for cFLIP. cFLIP was not detected in these cells on attachment to collagen (Supplementary Fig. S6), suggesting that integrin α6β1 induction of cFLIP in the brain ECs may be cell-type specific. Low levels of cFLIP were detected in the U-87MG cells on attachment to fibronectin or polylysine, and in suspension.

On double-labeling for cFLIP and vWF in our GBM and normal brain samples, we found double-labeling for cFLIP and vWF in ECs of vessels in 32 of 33 GBM and 20 of 20 normal brain samples (Supplementary Fig. S7). Furthermore, the mean percentage of blood vessels double-labeling for cFLIP and vWF was not significantly different between the GBM and the normal brain (24% ± 3.8% and 19% ± 2.0%, respectively, mean ± SEM), \( P = 0.99 \) (nonparametric Wilcoxon rank-sum test).

**TNFα proapoptotic signaling in brain ECs requires p38 MAP kinase**

Other investigators have shown that JNK and/or p38MAP kinase can promote the proapoptotic signal of TNFα. We therefore examined the phosphorylation of p38MAP and JNK kinases on TNFα treatment of brain ECs plated on collagen or laminin. We found a time-dependent increase in p38MAP kinase phosphorylation on TNFα stimulation in the ECs adherent to collagen, whereas no phosphorylation was detected in cells adherent to laminin (Fig. 6A and B, respectively).
Downregulation of p38MAP kinase with siRNA blocked, in part, the TNFα proapoptotic signal (Fig. 6C and D), suggesting that p38MAP kinase is necessary for TNFα-induced apoptosis of the brain ECs plated on collagen. An early transient increase in JNK phosphorylation was also detected with TNFα treatment in brain ECs plated on collagen, but JNK phosphorylation was not detected with TNFα treatment in cells plated on laminin (Fig. 6E and F). These data support the concept that different integrins can act to promote or inhibit the TNFα proapoptotic signal in brain ECs.

Discussion

In the first part of this study, we show that the expression of TNFα and TNF-R1 is upregulated significantly on the tumor-associated ECs in GBM, and that patients with tumors in which TNFα is expressed in the tumor-associated ECs have a longer survival. In approximately 50% of the GBM biopsies tested, the ECs exhibited expression of both TNFα and TNF-R1 suggesting the possibility of an autocrine or paracrine signaling loop, which may function to inhibit angiogenesis and could potentially explain the longer survival of patients with tumors with this expression pattern. We also found that the expression of TNF-R2 was significantly increased on the tumor-associated ECs in GBM, which may be due to the hypoxic changes found in GBM as other investigators have reported upregulation of TNF-R2 on ECs in a mouse model of ischemic injury (38). As TNF-R2 cannot signal apoptosis directly, we did not pursue this finding in this study, although signaling through this molecule may contribute to angiogenesis in GBM.

Other investigators have divided GBM tumors into 4 molecular subtypes based on cDNA array data generated from total tumor tissue RNA and immunostaining of tumor tissue (39). One of these subtypes, termed the mesenchymal subtype, shows increased expression of TNF-R1 in tumor cells, as well as focal hemizygous loss of the Nf1 gene, increased expression of microglial markers, an increase in the number of inflammatory cells in the tumor, and other alterations (39). Our current results suggest that the subgroup of GBM samples with TNFα expression in the tumor-associated ECs is unlikely to fit into the mesenchymal molecular subtype of GBM as we did not find a correlation between TNFα expression in tumor-associated ECs and TNF-R1 expression in tumor cells, and we found that TNF-R1 expression in ECs increases as TNF-R1 expression in tumor cells decreases.

In the second part of this study, we examined the regulation of TNFα/TNF-R1 proapoptotic signaling in primary brain ECs and GBM-derived ECs propagated in vitro. Tumor-associated ECs in GBM have been reported to express multiple β1 integrins, including α6β1, α2β1, α3β1, and α5β1, as well as integrin α6β4 (34, 35). Expression of the two α6 integrins, α6β1 and α6β4, is decreased on tumor-associated ECs in GBM as compared with ECs in normal brain (34, 35). We found that TNFα/TNF-R1 proapoptotic signaling is regulated in these ECs by the integrin that is engaged and,
thus, by the composition of the ECM. The engagement of integrin $\alpha_2\beta_1$, $\alpha_3\beta_1$, or $\alpha_5\beta_1$ is permissive for TNF-$\alpha$/TNF-R1 proapoptotic signaling. In contrast, engagement of integrin $\alpha_6\beta_1$, either by specific antibodies or by the ECM ligand laminin, is inhibitory. This observation was supported by specific activation of p38MAP kinase on TNF$\alpha$ stimulation of ECs attached to collagen, but not in those attached to laminin. The finding that the TNF$\alpha$ activation of TNF-R1 signaling of apoptosis or survival is determined, at least in part, by integrin signaling is consistent with the report by other investigators that the matricellular protein known as CCN1/CYR61 can cooperate with integrins $\alpha_v\beta_5$ and $\alpha_6\beta_1$ and the heparin sulfate proteoglycan, syndecan-4 on fibroblasts to promote TNF$\alpha$ proapoptotic signaling (40).

The induction of cFLIP inhibition contributes to the regulation of TNF$\alpha$/TNF-R1 proapoptotic signaling on EC attachment to laminin. Attachment of the cells to laminin induced rapid expression of cFLIP (within 1 hour), in marked contrast to attachment to collagen. This cFLIP expression in brain ECs adherent to laminin required new transcription and translation, consistent with the relatively short half-life described for cFLIP in other cell types and its regulation by ubiquitination and proteosomal degradation (6).

The levels of cFLIP contribute to the ability of cFLIP to promote either survival or apoptosis in various other cell types (6). High levels of cFLIP are thought to inhibit the death-inducing signaling complex activation of caspase-8. The possibility that the levels of cFLIP are regulated by the microenvironment is consistent with the report that cFLIP expression disappeared rapidly when germinal center B cells were removed from their microenvironment (41). Expression of cFLIP has been reported in ECs in Hodgkin lymphoma tissues (41), although the percentage of vessels with EC expression of cFLIP was not indicated, and in the ECs of normal coronary artery (42).

In determining the contribution of MAPK signaling to the apoptosis induced by TNF$\alpha$, we found that p38MAP kinase was activated rapidly and was necessary for TNF$\alpha$-induced apoptosis in the brain ECs adherent to collagen; however, activation of p38 MAP kinase was not seen in ECs adherent to laminin and treated with TNF$\alpha$. We also found a small early transient activation of JNK kinase in the ECs adherent to collagen and stimulated with TNF$\alpha$ and less activation of JNK kinase in the cells adherent to laminin. Activation of JNK kinase can induce or contribute to the ubiquitination and proteosomal degradation of cFLIP (43). A number of chemotherapeutic agents are known to reduce the expression of cFLIP, likely through a ubiquitination and proteosomal degradation pathway (44, 45).

As shown here, integrin $\alpha_6\beta_1$ on the ECs of the neovascularure of GBM inhibits the proapoptotic signal of TNF$\alpha$/TNF-R1 by inducing expression of cFLIP. This underscores the importance of the remodeling of the ECM that occurs in the neovessels of GBM, as well as the altered expression of integrins that occurs on these ECs, the activity state of the EC integrins, the availability of growth factors, and likely other factors in determining whether prosurvival or antisurvival signals are generated. The integrin $\alpha_6\beta_1$-mediated inhibition

![Figure 5](image-url)
of the proapoptotic signal of TNFα/TNF-R1 on the ECs would be expected to promote the growth of the GBM tumors. This indicates another mechanism by which integrin α6β1 can promote tumor growth in addition to the effects of its expression on the tumor cells, in which it promotes proliferation and motility.

In this study, we focused on the regulation of the proapoptotic signal generated by TNF-R1 on tumor-associated ECs as this approach had the possibility of suggesting chemotherapeutic targets. The mechanisms that regulate expression of TNFα and TNFR1 on GBM-associated ECs, which is obviously of interest in terms of the classification of these tumors and determinants of their behavior, are unknown and likely complex. TNFα expression is known to be regulated in other cell types at many different levels, including transcription, premRNA processing, mRNA stability, translation, and retention at the plasma membrane (reviewed in refs. 46, 47). Upregulation of TNF-R1 expression in lung ECs has been reported in bacterial infection of the lung (10).

These studies suggest various potential chemotherapeutic strategies for GBM that may be particularly effective when used in combination. They support the emerging concept that integrin α6β1 and/or p38MAPK may be promising chemotherapeutic targets in GBM as well as suggesting other candidate downstream targets, including cFLIP. They also suggest that activating TNF-R1 specifically on tumor-associated ECs in GBM may be a useful antiangiogenic therapeutic approach.

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

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Figure 6. p38MAP kinase is necessary for the proapoptotic effect of TNFα in brain ECs adherent to collagen. A, B, E, and F, whole-cell extracts were prepared from primary brain ECs plated on collagen or laminin and treated with TNFα or staurosporine as described in Fig. 2. Proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies. C, primary brain ECs were treated with siRNA for p38 MAPK (150 nmol/L) or siRNA to ERK1 (100 nmol/L) or scrambled siRNA control for 72 hours followed by lysis in NP-40 and immunoblotting with the indicated antibodies. D, primary brain ECs treated with siRNA to p38MAPK or ERK1 for 48 hours were harvested, replated onto chamber slides coated with collagen, and treated with specific siRNA for another 24 hours, followed by treatment with TNFα for 17 hours. A TUNEL assay was conducted and the data analyzed by an unpaired t test.


Endothelial Expression of TNF Receptor-1 Generates a Proapoptotic Signal Inhibited by Integrin α6β1 in Glioblastoma

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