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 α5β1, 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); 1–10. ©2012 AACR.
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, engagement of integrin α6β1 in prostate cancer cells, androgen signaling induces cell survival through activation of NF-κB and induction of Bel-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 seems 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 Biotechnologies. 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), and rabbit anti-TNFα (Cell Signaling; dilution 1:200), mouse mAb anti–von-Willebrand 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 (#402, 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-e-jun-NH$_2$-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.

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Figure 1. Double-label immunofluorescent staining of vWF and TNFα, TNF-R1, or TNF-R2 in tumor-associated ECs of GBM. Representative frozen sections of GBM reacted with mAb anti-vWF and rabbit anti-TNFα (A), rabbit anti-TNF-R1 (B), or rabbit anti-TNF-R2 (C), followed by mouse and rabbit-specific secondary antibodies conjugated with Alexa-594 or Alexa-488 are shown. Nuclei were stained with DAPI, and sections were viewed and photographed with a Leica DMRB 4000 microscope (Mag ×40; scale bar, 20 µm). A survival probability plot of all 49 GBM samples was constructed based on positive or negative TNFα expression in the tumor-associated ECs and the number of months of survival from diagnosis (D).
transcriptase PCR (qRT-PCR) was conducted using the cFLIP was normalized to SYBER Green reagent (Bio-Rad) as described previously.

Quantitative reverse transcriptase PCR

The number of apoptotic cells was determined using the TUNEL protocol. cFLIP mRNA level at each time point.

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 adhesion 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. IA–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 intussusception 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 < 0.0001, <0.0001, and <0.001, 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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TNFα</th>
<th>TNF-R1</th>
<th>TNF-R2</th>
</tr>
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<tbody>
<tr>
<td>Normal Brain (n = 38)</td>
<td>wk+ (3 of 38)</td>
<td>wk+ (12 of 38)</td>
<td>wk+ (9 of 38)</td>
</tr>
<tr>
<td></td>
<td>Negative (35 of 38)</td>
<td>Negative (26 of 38)</td>
<td>Negative (29 of 38)</td>
</tr>
<tr>
<td>GBM Tumor (n = 49)</td>
<td>wk+ (28 of 49)</td>
<td>wk+ (30 of 49)</td>
<td>wk+ (35 of 49)</td>
</tr>
<tr>
<td></td>
<td>Negative (21 of 49)</td>
<td>1+ (16 of 49)</td>
<td>1+ (10 of 49)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2+ (2 of 49)</td>
<td>2+ (1 of 49)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative (1 of 49)</td>
<td>Negative (3 of 49)</td>
</tr>
</tbody>
</table>

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).
Treated with 25 ng/mL TNF α as a monolayer on wells coated with 10 ng/mL of type I collagen, laminin, or fibronectin in M199 media with 1% FBS for 3 hours and treated with 25 ng/mL of TNF α 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 described in the Materials and Methods, treated with TNF α overnight, and analyzed by the TUNEL assay. For TUNEL assays, conditions were carried out in replicates of 2 and 10 × 20 fields in each well were counted. The data are presented as the mean ± SEM and were analyzed by a Wilcoxon rank-sum test.

in GBM than the normal brain (P < 0.0001 and <0.0001, respectively; Supplementary Fig. S2; ref. 31).

**TNF α proapoptotic signaling is regulated by the ECM and the integrin that is engaged**

Analysis of TNF α-induced apoptosis of primary brain ECs indicated that maximal apoptosis was induced with TNF α 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 caspase-7. We found that collagen and fibronectin attachment were permissive for the proapoptotic signal of TNF α/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, attachment to laminin was largely mediated by integrins α6β1 and α6β4, with a smaller contribution by integrin α6β6, 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 (Supplementary 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 TNF α stimulation. Both of the EC isolates expressed 2 markers of ECs, that is, vWF and CD31 (Fig. 3A and data not shown). TNF α stimulation induced apoptosis in a dose-dependent manner; furthermore, attachment of both ECs to collagen or fibronectin was permissive for the proapoptotic signal of TNF α, whereas attachment to laminin was inhibitory (Fig. 3B and data not shown).

**Integrin α6β1 is inhibitory for the proapoptotic signal of TNF α**

To identify which of the laminin-binding integrins (α6β1 or α6β4) inhibited the proapoptotic signal of TNF α, 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 TNF α. Apoptosis was determined by the TUNEL assay. TNF α stimulation of brain ECs plated on immobilized mAb anti-integrin α3 or the control mAb anti-integrin α2β1 resulted in apoptosis, whereas TNF α stimulation of ECs plated on immobilized mAb anti-integrin α6 inhibited the proapoptotic signal of TNF α (Fig. 2C), suggesting that integrin α6β1 signals to protect the ECs from the proapoptotic effect of TNF α.

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

To determine the mechanism by which engagement of integrin α6β1 or attachment to laminin protects the brain ECs from the proapoptotic signal of TNF α, we examined whether this event induced expression of an antiapoptotic protein(s). We found that attachment to laminin induced 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 laminin (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 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 α2β1, α3β1, or α5β1 is permissive for TNFα/TNF-R1 proapoptotic signaling. In contrast, engagement of integrin α6β1, either by specific antibodies or by the ECM ligand laminin, is inhibitory. This observation was supported by specific activation of p38MAP kinase on TNFα stimulation of ECs attached to collagen, but not in those attached to laminin. The finding that the TNFα 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 αvβ5 and α6β1 and the heparin sulfate proteoglycan, syndecan-4 on fibroblasts to promote TNFα proapoptotic signaling (40).

Figure 5. New transcription and translation are necessary for the induction of cFLIP in primary brain ECs plated on laminin. A, primary brain ECs were plated on laminin for the indicated time periods then lysed in NP-40 and immunobotted with the indicated antibodies. B, brain ECs were plated on laminin for 2 hours, treated with actinomycin D (ActD) or with cycloheximide (CHX) for the indicated time periods, lysed in NP-40, and immunobotted with the indicated antibodies. C and D, mRNA was isolated from primary brain ECs and ECs isolated from GBM that were plated on laminin for the indicated time periods. qRT-PCR for cFLIP was carried out and normalized to actin, as described in the Materials and Methods and data analyzed by an unpaired t test.

As shown here, integrin α6β1 on the ECs of the neovascu- lature of GBM inhibits the proapoptotic signal of TNFα/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 α6β1-mediated inhibition disappeared rapidly when germinal center B cells were removed from their microenvironment (41). Expression of cFLIP has been reported in ECs in Hodgkins 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 TNFα, we found that p38MAP kinase was activated rapidly and was necessary for TNFα-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 TNFα. We also found a small early transient activation of JNK kinase in the ECs adherent to collagen and stimulated with TNFα and less activation of JNK kinase in the cells adherent to laminin. Activation of JNK kinase can induce or contribute to the ubiquination and proteosomal degradation of cFLIP (43). A number of chemotherapeutic agents are known to reduce the expression of cFLIP, likely through a ubiquination and proteosomal degradation pathway (44, 45).

As shown here, integrin α6β1 on the ECs of the neovascu- lature of GBM inhibits the proapoptotic signal of TNFα/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 α6β1-mediated inhibition
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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Endothelial Expression of TNF Receptor-1 Generates a Proapoptotic Signal Inhibited by Integrin α6β1 in Glioblastoma

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