SPANDIDOS PUBLICATIONS

Constitutive NF-κB activity regulates the expression of VEGF and IL-8 and tumor angiogenesis of human glioblastoma

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Abstract. Angiogenesis is a key pathologic feature of glioblastoma, which is the most common and most lethal primary brain tumor in adults. The degree of angiogenesis has been shown to be inversely related to patient survival. However, the molecular changes leading to angiogenesis in glioblastoma remain poorly understood. In the present study, we found a direct correlation between nuclear factor (NF)-κB activation and angiogenesis in glioblastomas. Blockade of NF-κB signaling significantly inhibited glioblastoma growth and angiogenesis in nude mice. These effects were consistent with significant inhibition of the expression of multiple angiogenic molecules, including vascular endothelial growth factor, and interleukin-8, *in vitro* and *in vivo*. Furthermore, blockade of NF-κB signaling also significantly inhibited the angiogenic potential of glioblastoma cells *in vitro* and angiogenesis of brain tumors in mouse xenograft models. Collectively, these results suggest that NF-κB activation plays a critical role in the growth and progression of glioblastoma and is a potential target for therapy for human glioblastoma.

Introduction

Glioblastoma is the most common and most lethal primary malignant brain tumor in adults. The average survival duration in patients with glioblastoma is approximately 1 year despite recent advances in both diagnostic modalities and therapeutic strategies for this tumor (1-3). A key pathologic feature that distinguishes glioblastoma from lower-grade astrocytomas is angiogenesis, which is characterized by increasing production of proangiogenic molecules by the tumor cells and organ-specific environments (4). The level of angiogenesis in glioblastomas is conversely correlated with the degree of malignancy and patient prognosis (5). Thus, the presence of angiogenesis in a glioblastoma could promote its rapid growth and clinical progression. Indeed, recent studies have indicated that of all clinical and patho-logic characteristics of glioblastoma, angiogenesis has the greatest prognostic value. When angiogenesis is extensively present in a glioblastoma, the prognosis is consistently poor (6-8). Based on the clinical implications of and potential for therapeutic interventions for glioblastoma, the mechanisms leading to angiogenesis in this tumor must be identified.

Early studies have demonstrated a complex molecular interplay underlying angiogenesis (9-14). Vascular endothelial growth factor (VEGF) (15), also known as vascular permeability factor (VPF), has been shown to induce the proliferation of endothelial cells, to increase vascular permeability, to induce the production of plasminogen activator by these cells, and to prolong their survival (11,12). Interleukin-8 (IL-8), a chemoattractant cytokine, has been shown to attract and activate neutrophils in inflammatory regions and to be angiogenic (13,14). Recent studies indicated that the expression levels of VEGF and IL-8 in human glioma cells directly correlated with the level of angiogenesis in glioma (16,17).

Numerous studies have demonstrated that hypoxia and Akt and Ras activation can lead to nuclear factor (NF)-κB activation (18-25). Hypoxia causes the activation of NF-κB through the phosphorylation of IκBα on tyrosine residues (19). Akt stimulates the activation potential of the RelA/p65 subunit of NF-κB through the use of IκB kinase and activation of the mitogen-activated protein kinase p38 (20-22). Conversely, PTEN blocks tumor necrosis factor and Her2/neu-induced NF-κB activation (23). Because the above-described molecular pathways that are involved in angiogenesis of glioblastoma can activate NF-κB, NF-κB activation is likely a common step leading to angiogenesis formation in glioblastomas. Indeed, NF-κB has been reported to be constitutively activated in high-grade gliomas (24,25), and the activation status of NF-κB has been significantly correlated with glioma grade (25).

Recent studies from our and other laboratories demonstrated that NF-κB activity regulates tumor progression and

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metastasis in a variety of tumors (26-29). However, whether NF-κB activity is relevant to angiogenesis in human glioblastoma is unknown. In the present study, we show that transfection of malignant glioma cancer cells with IκBαM, a mutated form of IκBα, decreased the expression of VEGF and IL-8 and, hence, angiogenesis and tumorigenicity.

Materials and methods

Cell lines and culture conditions. The human neuroglia cell line H4, anaplastic astrocytoma (AA) cell lines SW1088 and Hs683 and glioblastoma cell lines U-118 MG, U-87 MG and T98G were obtained from the American Type Culture Collection (Rockville, MD). The glioblastoma cell line HF U-251 MG (30) was also used. All of the cell lines were maintained as adherent monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids, L-glutamine and a vitamin solution (Flow Laboratories, Rockville, MD).

Animals. Female athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 6-8 weeks of age. The animals were maintained according to institutional regulations in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care and in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and National Institutes of Health.

Northern blot analysis. Cellular mRNA was extracted from glioma cells by using the FastTrack mRNA isolation kit (Invitrogen, Carlsbad, CA). mRNA was fractionated on a 1% denaturing formaldehyde agarose gel, electrotransferred onto a nylon membrane, and ultraviolet-crosslinked. Northern blot hybridization was performed by using [^{32}P]dCTP-radiolabeled TF (American Type Culture Collection) cDNA probe. Equal loading of mRNA was monitored by hybridizing the same membrane with a ß-actin cDNA probe.

Stable transfection of glioma cells with IκBαM and a control vector. U-87 MG and HF U-251 MG cells ($1x10^6$) were transfected with a pLXSN-IκBαM expression vector (30) or control pLXSN vector by using lipofectamine (Life Technologies, Inc., Rockville, MD) or a control pLXSN vector. Cells were selected with a standard medium containing 200 μg/ml G418. Fourteen days later, neo-resistant colonies were isolated by trypsinization and established as subcultures. The expression of exogenous IκBαM was verified by using Western blot analysis.

Western blot analysis. Whole-cell lysates were prepared from glioma cells. Standard Western blotting was performed with a polyclonal rabbit anti-human and anti-mouse IκBα (Santa Cruz Biotechnology) to detect endogenous and mutant IκBα. Standard Western blotting was also performed with a polyclonal rabbit antibody against human VEGF (Santa Cruz Biotechnology), and a second antibody (anti-rabbit IgG or anti-mouse IgG; Amersham Life Sciences, Arlington Heights, IL). The same membranes were stripped and blotted with an anti-ß-actin antibody (Sigma Chemical Co., St. Louis, MO) and used as loading controls. The probe proteins were detected by using the Amersham enhanced chemiluminescence system according to the manufacturer's instructions.

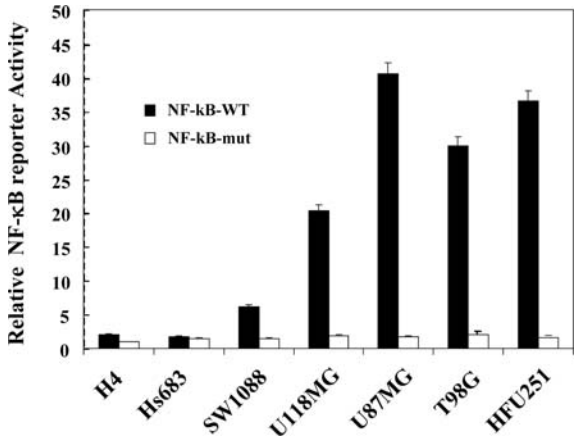

Figure 1. Constitutive NF-κB activity level in glioblastoma cells. The neuroglioma cell line H4, AA cell lines SW1088, and Hs683, together with four glioblastoma cell lines U-118 MG, U-87 MG, T98G and HF U-251 MG were transiently transfected with 2xNF-κB-Luc (containing two-copy wild-type NF-κB responsive elements) or 2xNF-κB mut-Luc (containing two-copy mutant-NF-κB responsive elements), respectively. The transfection efficiency was normalized by co-transfection with a pß-actin-RL reporter. Forty-eight hours post transfection, the relative luciferase activity was measured. Values are mean ± SD for triplicate samples from a representative experiment.

Promoter reporters and dual luciferase assays. Luciferase reporters driven by two-copy wild-type (2x NF-κB-Luc) NF-κB responsive elements were used (26-28). Glioma cells ($1x10^5$) growing in 6-well plates were transfected with the indicated reporter plasmids with the use of lipofectamine. The transfection efficiency was normalized by co-transfection with a pß-actin-RL reporter containing a Renilla luciferase gene under the control of a human ß-actin promoter (27). Both the firefly luciferase and Renilla luciferase activity was quantified by using a dual luciferase assay system (Promega, Madison, WI).

Endothelial cell tube formation assay. The tube formation assay was performed as described previously. Briefly, 250 μl of growth-factor-reduced Matrigel (Collaborative Biomedical Products, Bedford, MA) were pipetted into each well of a 24-well plate and polymerized for 30 min at 37˚C. Human umbilical vein endothelial cells (HUVECs) were harvested after trypsin treatment and suspended in a conditioned medium from $1x10^6$ glioblastoma cells. Next, $2x10^4$ HUVECs in 300 μl of a conditioned medium were added to each well and incubated at 37˚C in 5% CO_2 for 20 h. The cultures were photographed under a bright-field microscopy by using a Sony digital camera equipped with the Optimas software program (version 6.2).

Intracranial human glioma xenograft model. Glioma cells ($1x10^6$) were injected intracranially into nude mice as

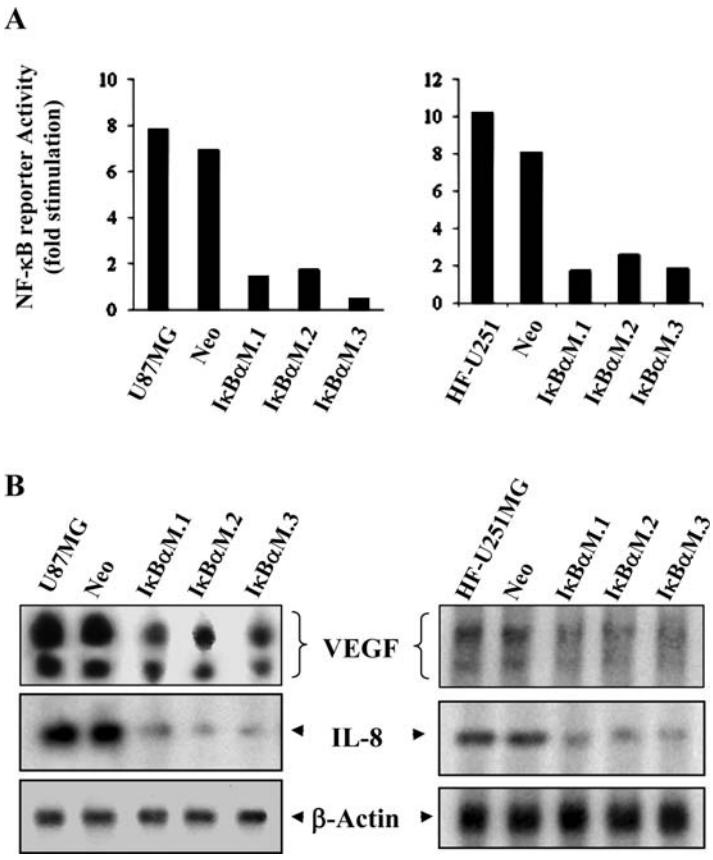

Figure 2. Downregulation of constitutive NF-κB activity and VEGF, IL-8 mRNA expression in glioma cells by transfection of IκBαM. (A) NF-κB activity in U-87MG and HFU-251 glioblastoma cells were stably transfected with IκBαM 3 independent colonies of each cell line (IκBαM-1, IκBαM-2, and IκBαM-3), together with parental and pLXSN-transfected (neo), were transiently co-transfected with 2xNF-κB-Luc and pß-actin-RL reporter. Luciferase activity was measured 48 h posttransfection. (B) Northern blot analysis of VEGF, IL-8 mRNA expression of IκBαM-1-transfected (IκBαM-1, IκBαM-1-2, and IκBαM-1-3), pLXSN-transfected (neo), and U-87 MG and HFU-251 MG cells. mRNA of each cells were extracted and Northern blot was performed according to our standard protocol. Equal loading of mRNA was monitored by hybridizing the same membrane with a ß-actin cDNA probe.

described previously (31). Two independent experiments with five mice per group were performed. Animals showing general or local symptoms were sacrificed; the remaining animals were sacrificed 45 days after glioma-cell injection. Each mouse's brain was harvested, fixed in 4% formaldehyde and embedded in paraffin. Tumor formation and the angiogenesis markers were examined with the use of histologic analysis of immunohistochemistry staining.

Immunohistochemistry. Sections (5 μm thick) of formalin-fixed, paraffin-embedded experimental glioma samples were stained with anti-NF-κB/p65 antibody (Boehringer Mannheim, Indianapolis, IN) (32). The level of expression of CD34, VEGF and IL-8 was determined by using our standard immunohistochemical staining method with anti-CD34 (Pharmingen, San Diego, CA), anti-VEGF (Santa Cruz Biotechnology) and anti-IL-8 (Biosource International, Camarillo, CA). Tissue sections immunostained with non-specific IgG were used as negative controls.

Statistical analysis. The significance of the *in vitro* results was determined by using Student's t-test (two-tailed), whereas the significance of the *in vivo* data was determined by using the Mann-Whitney U test. P values of ≤0.05 were deemed statistically significant.

Results

Glioblastoma cells had high level of constitutive NF-κB activity. We examined the constitutive NF-κB activity in the four glioblastoma (U-118 MG, U-87 MG, T98G and HF U-251 MG), two AA (SW1088 and Hs683) and a neuroglioma (H4) cell lines by using dual luciferase assays. As shown in Fig. 1, all of the glioblastoma cell lines had NF-κB luciferase reporter activity. SW1088, Hs683 and H4 also had NF-κB luciferase activity, but the levels were significantly lower than that in the glioblastoma cell lines. These results indicated that glioblastoma cells had high level of constitutive NF-κB activity.

Downregulation of constitutive NF-κB activity in glioma cells by transfection of IκBαM. To inhibit the NF-κB activity in U-87 MG and HFU-251 MG cells, we stably transfected them with IκBαM, which encodes a mutated IκBα with mutations at S32 and S36 of the NH2 terminus and a COOH-terminal PEST sequence mutation (25-29). We analyzed the NF-κB luciferase activity in 3 independent stable colonies of each cell line as well as vector-transfected and parental cells. As shown in Fig. 2A, NF-κB reporter activity was decreased ~7-9 fold in IκBαM-transfected (IκBαM-1, IκBαM-2 and IκBαM-3) U-87 MG and HF U-251 MG cells, respectively,

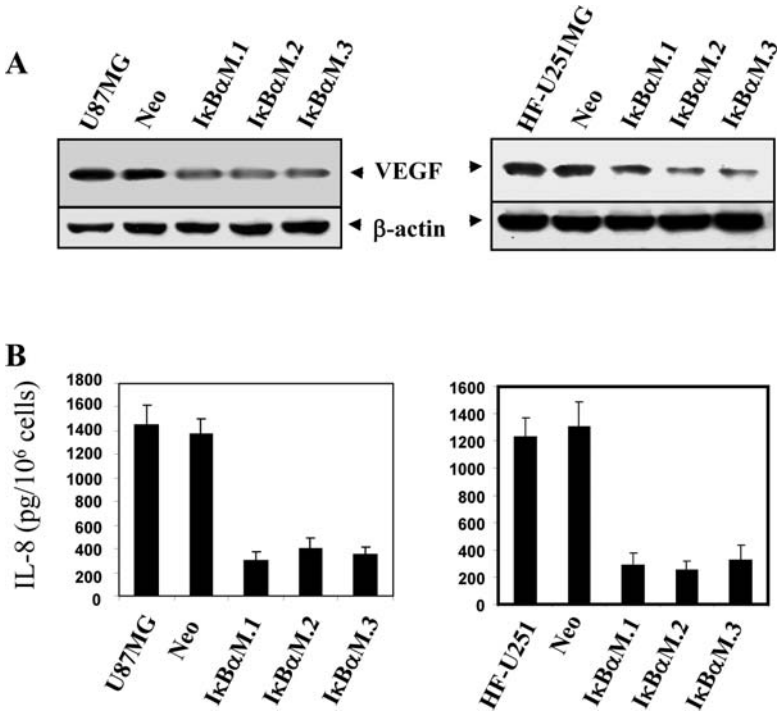

Figure 3. Inhibition of activation of NF-κB and decreased expression of VEGF and IL-8 protein level in IκBαM transfectant glioblastoma cells. (A) Downregulation of VEGF protein expression by IκBαM transfection. VEGF protein expression in IκBαM-transfected (IκBαM-1, IκBαM-2 and IκBαM-3), pLXSN-transfected (neo) U-87 MG and HF U-251 MG cells were analyzed by Western blotting. (B) Downregulation of IL-8 secretion by IκBαM transfection. IL-8 protein secretion in IκBαM-transfected (IκBαM-1, IκBαM-2 and IκBαM-3), pLXSN-transfected (neo) and U-87 MG and HF U-251 MG cells were analyzed by ELISA.

compare with parental and pLXSN-transfected (neo) groups. While there were no significant changes after NF-κB mutant reporter transfection (data not shown). Therefore, we showed that these cells had a constitutive level of NF-κB activity, which could be inhibited by transfection of the IκBαM expression vector.

Blockade of NF-κB activation suppresses the expression of VEGF and IL-8 in glioblastoma cells in vitro. The effect of NF-κB inhibition on the expression of VEGF and IL-8 was also studied in IκBαM-transfected U-87MG and HF-U251MG cells *in vitro*. First, the mRNA levels of VEGF and IL-8 genes in IκBαM-transfected cells were analyzed in IκBαM-transfected and control cells. Cellular mRNA was extracted from glioblastoma cells and Northern blot analysis was performed. As shown in Fig. 2B, there was a significant decrease in VEGF and IL-8 mRNA expression in IκBαM-transfected cells compared to parental and neo cells for both U87 and HF-U251MG cell lines.

The expression of VEGF and IL-8 in IκBαM-transfected cells was further determined at protein level. Consistently, the protein level of VEGF in IκBαM-transfected cells significantly decreased as determined by Western blot analysis (Fig. 3A). The protein level of IL-8 in IκBαM-transfected cells significantly decreased as determined by quantitative IL-8 ELISA (Fig. 3B).

To further investigate the mechanism of how NF-κB regulates VEGF and IL-8 expression, we performed the promoter assay by using the VEGF and IL-8 promoters in U87MG, U87MG neo, U87MG IκBαM-transfected cells. As shown in Fig. 4A, we found that in IκBαM-transfected U87MG cells, the promoter activity of VEGF and IL-8 downregulated significantly compared with parental and neo cells. Similar results were observed in HFU-251 and IκBαM-tansfected cells (Fig. 4B).

Blockade of NF-κB activation suppresses the angiogenic potential of glioblastoma cells. VEGF as well as IL-8 have also been shown to be strong regulators of human glioma angiogenesis. Thus, we determined whether decreased NF-κB/RelA activity and the subsequent decrease in VEGF and IL-8 production led to suppression of angiogenic activity in glioma cells *in vitro*. The conditioned media of U-87MG-neo, U87MG IκBαM-transfected, HFU-251MG-neo and HFU-251MG IκBαM-transfected were collected and the ability of each of the conditioned media to induce endothelial cell tube formation was determined. As shown in Fig. 5, the conditioned media from IκBαM-transfected U-87MG and HFU-251MG cells appeared to be less angiogenic than that from control cells as determined by an endothelial cell tube formation assay.

Blockade of NF-κB activation suppresses angiogenesis and tumor growth of human glioblastoma cells. To evaluate whether NF-κB activity regulates angiogenesis of glioblastoma cells, we used an orthotopic xenograft model of human glioma by intracranially injecting glioma cells into nude mice. Intracranially implanted HFU251 MG and HFU251 MG-neo cells ($1x10^6$ cells/mouse) produced brain tumors in all of the mice injected with these cells (Fig. 6A). In contrast,

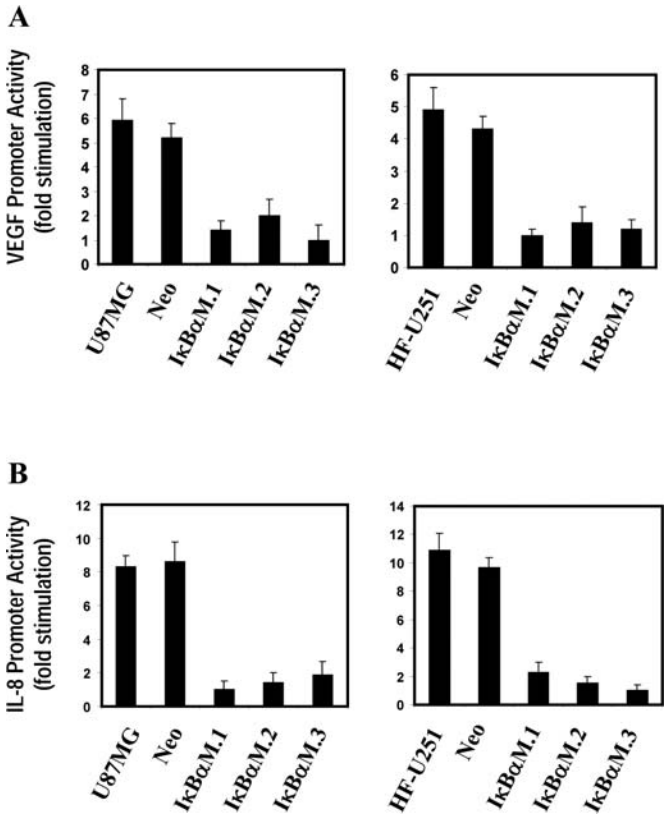

Figure 4. Blockade of NF-κB activation suppresses VEGF and IL-8 promoter activity in glioblastoma cells. IκBαM-transfected (IκBαM-1, IκBαM-2 and IκBαM-3), pLXSN-transfected (neo) U-87 MG and HF U-251 MG cells were transfected with VEGF promoter and IL-8 promoter, respectively. The transfection efficiency was normalized by co-transfection with a pß-actin-RL reporter. Forty-eight hours post transfection, the relative luciferase activity was measured. Values are mean ± SD for triplicate samples from a representative experiment.

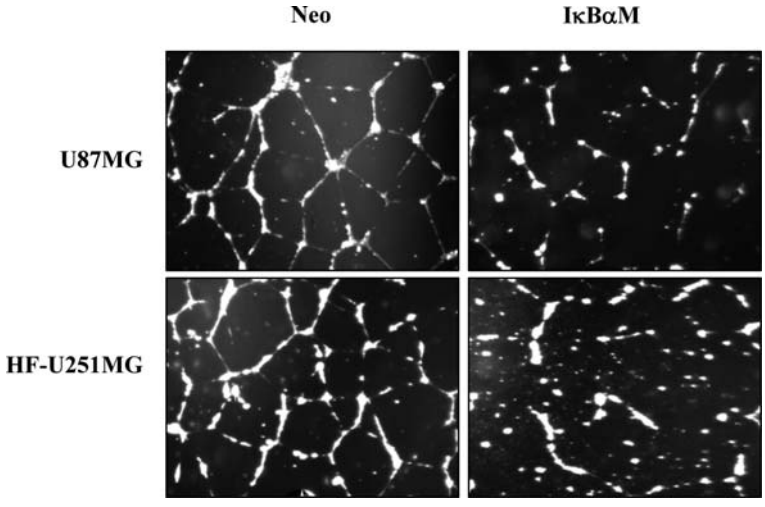

Figure 5. Effects of altered activation of NF-κB by IκBαM transfection on angiogenic potential of glioma cells. Conditioned media were prepared from $1x10^6$ pLXSN (neo)-transfected and pLXSN-IκBαM-transfected U-87 MG or HFU-251 MG cells. HUVECs were harvested after trypsin treatment and suspended in a conditioned medium from $1x10^6$ glioblastoma cells. HUVECs ($2x10^4$) in 300 μl of a conditioned medium were then plated on growth factor-reduced Matrigel to form a capillary tube. Capillary tube formation in each group was photographed. This is a representative experiment of two. Capillary tube formation in each group was photographed a representative of two experiments is shown.

HFU251MG-IκBαM-1 and HFU251MG-IκBαM-2 cells produced smaller tumors, and obtained longer survival time compared with previous groups (Fig. 6A).

Next, tumor-associated neovascularization (as indicated by MVD) was determined by IHC using anti-CD34 antibodies. As shown in Fig. 6B, tumors formed by HFU251MG neo-transfected cells were highly vascularized, whereas the tumors formed by IκBαM-transfected IκBαM cells had a significantly decreased vascular density. These studies indicated that tumor-associated neovascularization correlated

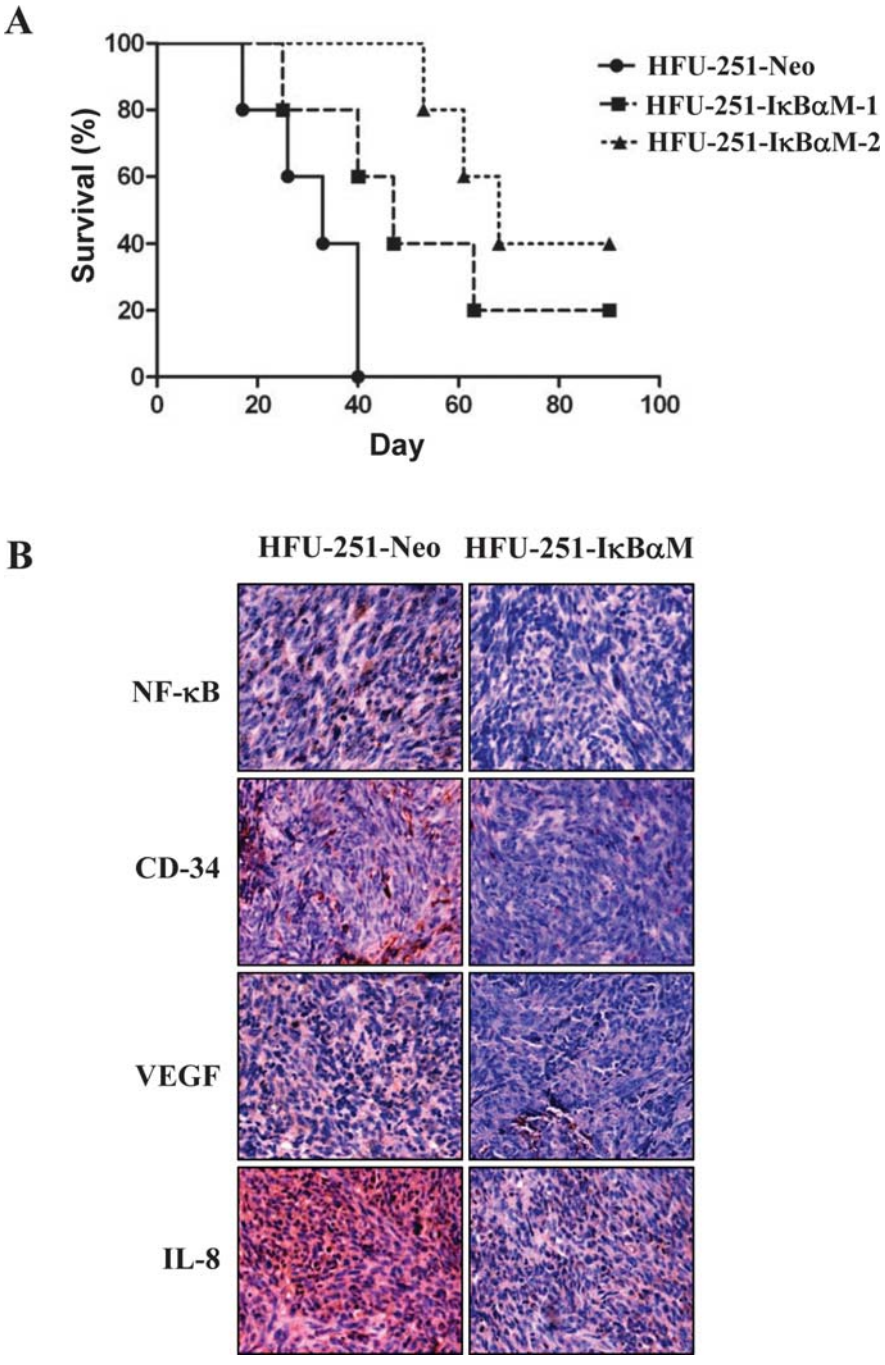

Figure 6. Effect of IκBαM transfection on glioma growth in the brain of nude mice. (A) Glioma cells ($1x10^6$) were implanted intracranially into nude mice. Mice were euthanized when animals showed general or local symptoms and the survival days were statistically analyzed. (B) The brain of each mouse was harvested, fixed in 4% formaldehyde and embedded in paraffin. Tumor formation and the necrosis phenotype were examined with the use of histologic analysis and immunohistochemistry staining with the indicated antibodies.

directly with NF-κB activity, and tumorigenicity of human glioma cells. To rule out the possibility that difference in neovascular formation was mainly due to difference in tumor size, we produced small control tumors similar in size to IκBαM-transfected tumors. We found frequent neovascularization in those small tumors, but not in IκBαM-transfected tumors of similar size, suggesting that tumor size might not be the major cause of differential neovascularization. Therefore, inhibition of NF-κB activity by IκBαM transfection suppressed both neovascularization and tumorigenicity in glioblastomas.

Inhibition of activation of NF-κB and decreased expression of VEGF and IL-8 in IκBαM transfectant tumors. To provide direct evidence of the contribution of NF-κB activation to the regulation of angiogenesis molecules, we studied the effect of altered NF-κB activity on the orthotopic xenograft model of human glioma *in vivo*. First, we sought to determine whether IκBαM transfection suppresses NF-κB activity *in vivo*. We performed immunohistochemical analysis of HFU-251MG-neo and HFU-251MG-IκBαM brain tumor specimens with the use of an antibody that recognizes the nuclear localization sequence of the activated form of NF-κB p65.

is an inhibition of activated NF-κB in the HFU-IκBαM tumors (Fig. 6B). Similar results were obtained with the use of U-87MG-neo and U-87MG-IκBαM tumors (data not shown).

We also evaluated the expression of VEGF and IL-8 protein *in vivo* by using immunohistochemistry. As shown in Fig. 6B, we observed staining for VEGF and IL-8 in HFU-251MG neo tumors; we observed significantly decreased staining in IκBαM transfectant tumors. Thus, expression of IκBαM in glioblastoma cells inhibited constitutive activation of NF-κB and subsequently suppressed expression of the angiogenesis gene *in vivo*.

Discussion

In the present study, we found that glioblastoma cells have high NF-κB activity playing an important role in angiogenesis of glioblastomas. Our present data demonstrate that U-87MG parental, U-87MG neo, HFU-251 parental and HFU-251 neo cells (high expression level of VEGF/VPF and IL-8) expressed significantly higher levels of NF-κB/RelA activity than the IκBαM-transfected cells (low expression level of VEGF/VPF and IL-8). In our animal model, blockade of NF-κB activation by IκBαM transfection suppressed angiogenesis and tumor growth by human glioblastoma cells. Furthermore, altered NF-κB activity significantly affected expression of the major angiogenesis molecules VEGF and IL-8 *in vitro* and *in vivo* in glioblastoma cells. Therefore, we provide mechanistic evidence that constitutively activated NF-κB plays an important role in angiogenesis in human glioblastomas.

The regulation of both VEGF and IL-8 expression during tumor progression may involve diverse mechanisms. In this study, we sought to determine whether NF-κB regulates VEGF and IL-8 expressions in glioblastoma cells. IκBαM transfection, which blocks NF-κB activation (25-29), suppressed the production of both IL-8 and VEGF under *in vitro* and *in vivo* conditions. The significant decrease in VEGF and IL-8 promoter activities found in the IκBαM-transfected cells suggested that the regulation of VEGF by NF-κB occurred at the transcriptional level.

NF-κB is an inducible dimeric transcription factor that belongs to the Rel/NF-κB family of transcription factors, whose prototype in most non-lymphoid cells is a heterodimer consisting of the RelA (p65) and NF-κB1 (p50) subunits (33,34). NF-κB complexes are typically retained in the cytoplasm by inhibitory IκB proteins, including IκBα. Upon stimulation, IκBα is rapidly phosphorylated and degraded via the ubiquitin-proteasome pathway, permitting activation and nuclear import of NF-κB. Dominant-negative mutant forms of IκBα that cannot be phosphorylated and degraded and thus prevent the activation of NF-κB have been engineered. Indeed, we found that NF-κB activation was suppressed when we transfected a dominant-negative mutant form of IκBα-IκBαM into the glioblastoma cells. Additionally, blockade of NF-κB activity in glioblastoma cells suppressed the angogenesis of glioblastoma cells *in vitro* and the formation of brain tumors in nude mice. Blockade of NF-κB activity also inhibited the expression of the angiogenic molecule VEGF, IL-8 in glioblastoma cells, suggesting that increased NF-κB activity may contribute to the over-activity of angiogenesis in human glioblastomas.

NF-κB activation can protect tumor cells from apoptosis; thus, suppression of tumor growth by blocking NF-κB activity could have been due to increased apoptosis (34,35). Previous reports showing that stable inhibition of NF-κB in cancer cells by stable transfection of IκBαM does not inhibit cell growth *in vitro* (36,37). Thus, the inhibition of tumorigenicity by suppression of NF-κB activity had to occur by other mechanisms, such as inhibition of cell adhesion (37), inhibition of proinflammatory cytokine production (38), or inhibition of plasminogen activator and matrix metalloproteinase (39), which contribute to neoplastic angiogenesis, growth and metastasis. NF-κB has also been shown to play a role in retinal neovascularization (40) and in oxidative stress-induced tubular morphogenesis of endothelial cells (41). Our data showing that NF-κB activity in glioblastoma cells regulates the expressions of VEGF and IL-8 support the role that NF-κB may play in angiogenesis, as an essential feature of glioma growth.

In summary, we show that human glioblastoma cells with high-malignant potential express high levels of constitutive NF-κB activity. Suppression of NF-κB activity through expression of a phosphorylation mutant IκBαM decreased angiogenesis, retarded tumor growth, in part through down-regulation of the angiogenic molecules VEGF and IL-8. These data provide the first direct evidence for the essential role of NF-κB/RelA in angiogenesis, tumor growth, and formation of malignant glioblastoma. Targeting NF-κB may therefore be a potential approach in controlling angiogenesis and growth of human glioblastoma.

Acknowledgements

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