# Aberrant NF-κB activity is critical in focal necrosis formation of human glioblastoma by regulation of the expression of tissue factor

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**Abstract.** Focal necrosis is a key pathologic feature that distinguishes glioblastoma from lower grade glioma. The presence of necrosis in a glioblastoma could promote its rapid growth and clinical progression. Focal necrosis of glioblastoma seems to be associated with thrombosis that result from hyper-coagulability. In the present study, we found that glioblastoma cells had a high level of constitutive nuclear factor (NF)-κB activity, which was directly correlated with necrosis in glioblastomas. We also found a direct correlation between NF-κB activity and the expression of tissue factor (TF), a potent procoagulant factor in gliomas. Inhibition of TF by an inhibitory antibody prevented the procoagulant activity of glioblastoma cells, indicating a TF-dependent mechanism. Blockade of NF-κB activation significantly inhibited TF expression and the procoagulant activity of glioblastoma cells *in vitro*. Blockade of NF-κB activation also significantly inhibited *in vivo* expression of TF, which was directly correlated with decreased necrosis formation and tumor growth of glioblastoma cells in nude mice. Collectively, these results suggest that elevated NF-κB activity in glioblastomas cells plays a critical role in necrosis formation of glioblastoma and that inhibition of NF-κB activity in glioblastoma can suppress necrosis formation and progressive growth.

## 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 pseudopalisading necrosis: a dense collection of neoplastic cells that surround a central necrotic focus (4,5). Necrosis may trigger angiogenesis by inducing hypoxia within a tumor, which ultimately leads to tumor angiogenesis, invasion, and growth (6,7). Thus, the presence of necrosis in a glioblastoma could promote its rapid growth and clinical progression. Indeed, recent studies have indicated that of all clinical and pathologic characteristics of glioblastoma, necrosis has the greatest prognostic value (5,8,9). Based on the clinical implications of and potential for therapeutic interventions for glioblastoma, the mechanisms leading to focal necrosis in this tumor must be identified.

Recent studies have also suggested that necrosis of glioblastoma is largely associated with the presence of intravascular thrombosis in pseudopalisades (6,7,10). Intravascular thrombosis may result from hypercoagulability in the abnormal tumor microcirculation. Furthermore, hypercoagulation in glioblastoma may be mediated by procoagulation factors, such as tissue factor (TF). TF has been shown to play a significant role in the steps leading to coagulation under physiologic conditions. Recently, more widespread recognition of the importance of TF under these conditions - mainly, that it triggers processes leading to thrombosis - has emerged (11). The expression of TF in tumor cells in a malignant glioma correlates directly with the histologic grade of the tumor (12), supporting TF as a major procoagulant in glioblastoma. Moreover, a recent study demonstrated that PTEN loss and tumor hypoxia, the major events in the development of glioblastoma, upregulated TF expression and promote plasma clotting by glioma cells, suggesting that these mechanisms underlie intravascular thrombosis and pseudopalisading necrosis in glioblastoma (7). Additionally, that study demonstrated that both Akt and Ras pathways modulate TF expression.

Numerous studies have demonstrated that hypoxia and Akt and Ras activation can lead to nuclear factor (NF)-κB activation (13-20). Hypoxia causes the activation of NF-κB through the phosphorylation of IκBα on tyrosine residues (15). Akt stimulates the activation potential of the RelA/p65

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subunit of NF-κB through use of the IκB kinase and activation of the mitogen-activated protein kinase p38 (16). Conversely, PTEN blocks tumor necrosis factor- and Her2/neu-induced NF-κB activation (19). Also, the H-Ras oncogene initiates signal transduction cascades that ultimately lead to the activation of NF-κB (20). Because the above-described molecular pathways that are involved in necrosis of glioblastoma can activate NF-κB, NF-κB activation is likely a common step leading to necrosis formation in glioblastomas. Indeed, NF-κB has been reported to be constitutively activated in high-grade gliomas (21-23).

Our recent studies demonstrated that NF-κB activity regulates tumor progression and metastasis in a variety of tumors (24-26). However, whether NF-κB activity is relevant to necrosis in human glioblastoma is unknown. In the present study, we have investigated whether aberrant NF-κB activity in glioblastoma cells regulates expression of the major procoagulation factor TF and procoagulant activity that could promote necrosis formation in glioblastomas.

## Patients and methods

*Patient specimens*. Human glioma samples were used for this study, all of which were obtained from the Brain Tumor Center Tissue Bank of The University of Texas M.D. Anderson Cancer Center. A total of 28 AAs and 43 GBM cases were analyzed. Grading of the gliomas was performed according to the St. Anne/Mayo system (5,8). This system grades an astrocytic neoplasm as a GBM when it contains three criteria: nuclear atypia, mitosis, and endothelial proliferation. The 43 GBMs were further histopathologically graded as positive or negative based on the presence of necrosis on H&E slide. Use of the patient data and archival tissue blocks were approved for this research project by the M.D. Anderson Cancer Center Institutional Review Board.

*Immunohistochemistry*. Sections (5-$\mu$m thick) of formalin-fixed, paraffin-embedded glioblastoma samples and experimental glioma samples were stained with anti-NF-κB/p65 antibody (Boehringer-Mannheim, Indianapolis, IN) (27). The level of expression of TF was determined by using our standard immunohistochemical staining method with anti-TF (1:200 dilution; American Diagnostica, Stamford, CT). Tissue sections immunostained with non-specific IgG were used as negative controls. The sections were visualized by using a diaminobenzidine substrate kit. Staining was classified using a three-tiered system according to the percentage of positive cells and staining intensity as we described previously: negative, moderate positive, or strong positive (28). Due to the heterogeneous nature of staining, five random fields on each slide were selected and scored by two independent researchers while blinded to clinical data.

*Cell lines and culture conditions*. The human anaplastic astrocytoma (AA) cell lines SW1088 and SW1783 and glioblastoma cell lines U-118 MG, LN-229, and U-87 MG were obtained from the American Type Culture Collection (Rockville, MD). The glioblastoma cell line HF U-251 MG and transformed human AA cell line HNA-E6/E7/hTERT/Ras (29) were 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 US 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. The intensity of each band was quantitated by densitometry readings of autoradiograms using the Image Quant software program (Molecular Dynamics).

*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 $\mu$g/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.

*Knockdown of NF-κB p65 by small interfering RNA (siRNA)*. U-87 MG cells ($1x10^6$) were transfected with NF-κB p65 (Rel A) SMARTpool SiRNA (100 nM) (Upstate Cell Signaling Solutions) by using Lipofectamine (Life Technologies). A non-specific siRNA with limited homology with any known sequences in the human, mouse, and rat genomes was used as a negative control (Ambion). Transfection reagent and undelivered siRNA were removed 24 h post-transfection by washing the cells twice with Dulbecco's modified Eagle's medium.

*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 (C-21; Santa Cruz Biotechnology) to detect endogenous and mutant IκBα. Standard Western blotting was also performed with a polyclonal rabbit antibody against human TF (American Diagnostica), 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. The intensity of each band was quantitated by densitometry readings of autoradiograms using the Image Quant software program (Molecular Dynamics).

*Electrophoretic mobility shift assay*. Nuclear protein extracts were prepared as described previously (24-26). The sequence of the NF-κB oligonucleotide probe was 5'-AGTTGAGGGA CTTTCCCAGGC-3'. Electrophoretic mobility shift assay (EMSA) was performed as described previously (24) with minor modifications: 5 $\mu$g of a nuclear extract protein and a 30,000-cpm end-labeled double-stranded DNA probe were added to the mixture. The binding reaction was allowed to proceed for 25 min at 22˚C.

*Promoter reporters and dual luciferase assays*. Luciferase reporters driven by two-copy wild-type (2x NF-κB-Luc) NF-κB-responsive elements were used (24-26). 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 pB-actin-RL reporter containing a Renilla luciferase gene under the control of a human ß-actin promoter (31). Both the firefly luciferase and Renilla luciferase activity was quantified by using a dual luciferase assay system (Promega, Madison, WI).

*Tilt tube plasma-clotting assay*. Plasma-clotting times induced by tumor cells or conditioned media were measured in triplicate by using a tilt tube assay with all reagents maintained at 37˚C (7). Tumor cells ($3x10^6$) were cultured in a serum-free medium for 24 h; the medium was then collected and briefly centrifuged to remove any cell debris before its use as a conditioned medium in clotting assays. Tumor cells were rinsed three times in phosphate-buffered saline, scraped from the dish, and resuspended in 1 ml of phosphate-buffered saline. For the clotting assay, 200 $\mu$l of a cell suspension or conditioned medium was added to 200 $\mu$l of citrated human plasma (Precision Biologic, Dartmouth, Nova Scotia, Canada), and 200 $\mu$l of 25 mmol $CaCl_2$ was added to the tube to initiate the clotting process. The clotting time was assayed visually by noting when the liquid formed a semisolid gel that did not flow during tube tilting. In some experiments, cell suspension was pretreated with anti-TF monoclonal antibody (100 $\mu$g/ml; American Diagnostica), anti-VEGF antibody Avastin (10 $\mu$g/ml, Bevacizumab; Genentech), or anti-IL-8 monoclonal antibody (0.4 $\mu$g/ml; R&D System), for 1 h at 37˚C prior to assessing clotting time.

*Intracranial human glioma xenograft model*. Glioma cells ($1x10^6$) were injected intracranially into nude mice as described previously (32). 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 necrosis phenotype were examined with the use of histologic analysis of hematoxylin and eosin stained sections.

*Statistical analyses*. The significance of the patient specimen data was determined by using the Person's $\chi^2$ test. 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, which correlated with TF expression*. We examined the constitutive NF-κB activity in the four glioblastoma (U-118 MG, LN-229, U-87 MG, and HF U-251 MG) and three AA (HNA-E6/E7/hTERT/Ras, SW1088, and SW1783) cell lines by using EMSA. As shown in Fig. 1A1, all of the glioblastoma cell lines had NF-κB-binding activity. SW1088 and SW1783 also had NF-κB-binding activity, but the levels were 3-5-fold lower than that in the glioblastoma cell lines. HNA-E6/E7/hTERT/Ras cells had a negligible level of NF-κB activity (Fig. 1A1). Furthermore, the NF-κB DNA-binding complex of HF U-251 MG cells was competed out by an unlabeled NF-κB consensus probe but not by a mutant NF-κB probe. Moreover, the DNA-protein complex was super-shifted by an anti-NF-κB (p65) antibody, indicating that the shifted band represented an NF-κB DNA-protein complex (Fig. 1A2). These results indicated that glioblastoma cells had high level of constitutive NF-κB activity.

Next, we prepared whole-cell lysates from those glioma cell lines and analyzed TF expression using Western blotting. Significantly higher expression of TF protein was evident in U-118 MG, LN-229, U-87 MG, and HF U-251 MG glioblastoma cells than in SW1088, SW1783, and HNA-E6/E7/hTERT/Ras (Fig. 1B). TF expression in glioma cells was directly correlated with NF-κB activity (Fig. 1C).

*Inhibition of TF attenuated the procoagulant activity of glioblastoma cells*. We determined the effects of TF, VEGF and IL-8 expression on the coagulative ability of glioblastoma cells by using a tilt tube assay. We observed that plasma clotting occurred at 52±6 sec with a U-87 MG-cell suspension (Fig. 1D1). However, the plasma-clotting time of U-87 MG cell preincubated with a 100-$\mu$g/ml anti-TF antibody was prolonged to 416±28 sec. The TF antibody also prolonged the plasma-clotting time of HF U-251 MG (from 42±6 to 446±32 sec; $P<0.01$) (Fig. 1D2). In contrast, the use of non-specific IgG, anti-VEGF antibody Avastin or an anti-IL-8 neutralization antibody has no effect (Fig. 1D1 and D2). These results showed that TF, but not VEGF and IL-8, was mainly responsible for promoting plasma clotting.

*Direct correlation of NF-κB activity with TF expression and necrosis in glioma*. In order to study the relationship between necrosis and the NF-κB activity, the level of activated NF-κB/p65 in anaplastic astrocytomas (AAs), which by definition are gliomas that are not associated with necrosis, was determined and compared with the level in GBMs with necrosis, which

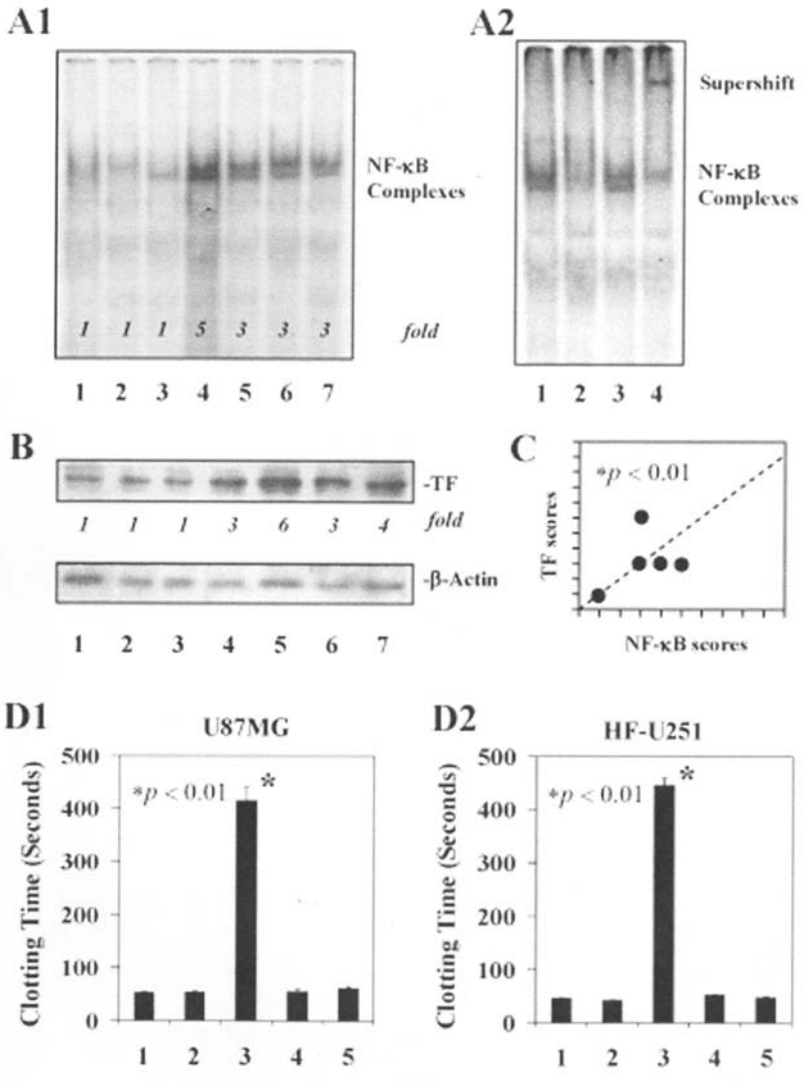


Figure 1. Constitutive NF-κB activity and TF expression in and plasma clotting activity of glioma cell lines. A1, Constitutive NF-κB binding activity. Nuclear protein was extracted from HNA-E6/E7/hTERT/Ras, SW1088, SW1783, U-118 MG, LN-229, U-87 MG, and HF U-251 MG cells (lanes 1-7, respectively), and EMSA was performed with a NF-κB consensus probe. A2, NF-κB binding activity in HF U-251 MG cells. Nuclear protein of HF U-251 MG cells was subjected to EMSA with the NF-κB consensus probe. In some reactions, unlabeled NF-κB probe (lane 2) or its mutant (lane 3) or an anti-NF-κB antibody (anti-p65, lane 4) were added. The positions of the NF-κB and supershifted complexes were marked. B, The expression of TF. Protein was extracted from HNA-E6/E7/hTERT/Ras, SW1088, SW1783, U-118 MG, LN-229, U-87 MG, and HF U-251 MG cells (lanes 1-7, respectively), and Western blot analysis was performed with an anti-TF antibody. C, Relationship between NF-κB activity and TF expression. The intensities of NF-κB complex (A1) and TF protein bands (B) were determined by image scanning. Intensity of lane 1 was given a value of 1 and intensity levels of the rest samples were expressed as fold changes. Relative levels of NF-κB activity directly correlated with the levels of TF protein expression ($P<0.01$, Person's $\chi^2$ test). D, Plasma clotting activity of glioblastoma cells. Two hundred microliters of a cell suspension of U-87 MG (D1) or HF-U251 cells (D2) was added to a plasma-clotting assay (lane 1). In some experiments, cell suspension was pretreated with a control IgG (lane 2), anti-TF monoclonal antibody (100 $\mu$g/ml, lane 3), anti-VEGF antibody Avastin (10 $\mu$g/ml, lane 4)), or anti-IL-8 monoclonal antibody (0.4 $\mu$g/ml, lane 5), for 1 h at 37˚C prior to assessing clotting time. Treatment with the anti-TF monoclonal antibody prolonged the plasma-clotting time induced by the cell suspension ($P<0.01$, Student's t-test).

are gliomas characterized by extensive amount of necrosis. The level of activated NF-κB/p65 in GBMs without necrosis was also determined and compared with the level in GBMs with necrosis. We performed nuclear staining of the activated form of NF-κB/p65 with an antibody that specifically recognizes its nuclear localization sequence in tumor specimens (28 AAs, 32 GBM with necrosis and 11 GBM without necrosis). In general, staining was heterogeneous with cells in near regions of microscopic necrosis exhibiting higher NF-κB expression than those without nearby necrotic regions. Fig. 2A shows representative sections of strong staining, moderate staining, and negative staining. We observed that 17.8% of the AAs were strongly positive, 42.9% were moderately positive, and 39.3% were negative for NF-κB/p65 expression. Similarly, 27.2% strong positive, 27.3% moderate positive and 45.5% negative NF-κB staining was observed in the GBMs without necrosis. In contrast, 75% of the GBMs with necrosis were strongly positive, 21.9% were moderately positive, and only 3.1% were negative for NF-κB/p65 expression (Fig. 2B1). We found significantly higher levels of NF-κB activity in the GBMs with necrosis than in the AAs and the GBMs without necrosis ($\chi^2$ test; $P<0.05$). We also analyzed the TF expression on the above-described specimens and found significantly higher levels of TF expression in the GBMs with necrosis

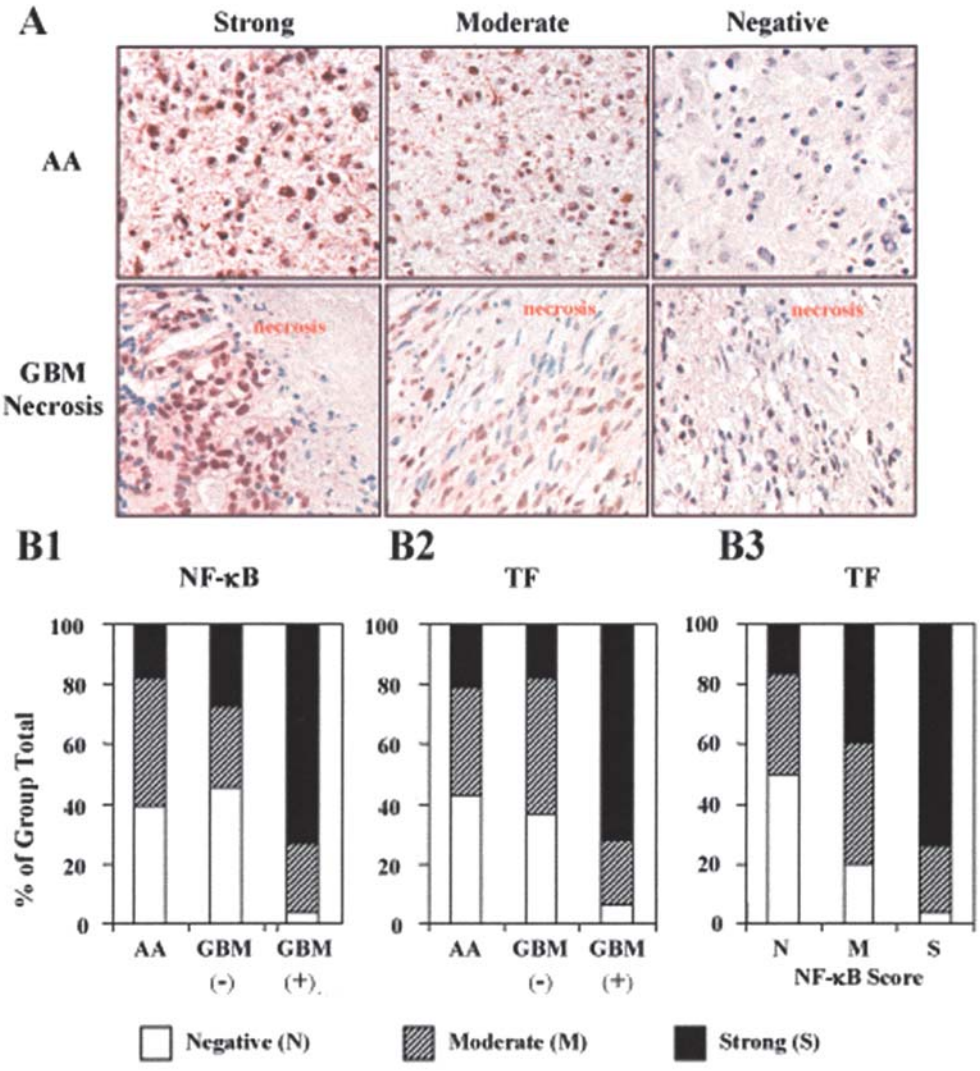


Figure 2. Constitutive NF-κB activity and TF expression in human glioma tissues. A, Immunohistochemical analysis of NF-κB/p65 activity and TF expression in human AA and GBM tissues. GBM samples was divided into two groups: one with and another without necrosis. A, Representative sections of negative staining, moderate staining, and strong staining of NF-κB/p65. B, Relationships among NF-κB activity, TF expression and necrosis formation in glioma tissue specimens [GBM(-), GBMs without necrosis; GBM(+), GBMs with necrosis]. Statistical significance of the correlations was determined by Person's $\chi^2$ when comparisons were made for NF-κB activation in glioma with or without necrosis (B1), TF expression in glioma with or without necrosis (B2); and NF-κB activation vs TF expression in all GBMs (B3).

than in the AAs the GBMs without necrosis (Fig. 2B2; $\chi^2$ test, $P<0.05$). Moreover, we observed a significant positive correlation between the levels of expression of activated NF-κB and TF ($P<0.05$; Fig. 2B3). Thus, we found that increased levels of activated NF-κB significantly correlated with expression of TF and the existence of necrosis in glioma specimens.

*Downregulation of constitutive NF-κB activity in glioma cells by transfection of IκBαM.* To inhibit the NF-κB activity in U-87 MG and HF U-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 (30). We analyzed the expression of both endogenous and mutant IκBα by using Western blot analysis. As shown in Fig. 3A, we detected endogenous IκBα in parental control, pLXSN-transfected (Neo), and IκBαM-transfected (IκBαM-1, IκBαM-2, and IκBαM-3) U-87 MG and HF U-251 MG cells, whereas we detected exogenous mutant IκBα only in IκBαM-transfected U-87 MG and HF U-251 MG cells. Consistently, constitutive NF-κB-binding activity was present in U-87 MG and HF U-251 MG cells, whereas IκBαM expression significantly inhibited the NF-κB activity in IκBαM-transfected cells but not in the control Neo cells (Fig. 3B).

Next, we confirmed the suppressive effect of IκBαM transfection on the constitutive level of NF-κB activity by using an NF-κB-dependent luciferase reporter activity assay. We transiently transfected a 2x NF-κB-Luc (wild-type) or 2x NF-κB-mut-luc (mutant) reporter into U-87 MG and HF U-251 MG cells. As shown in Fig. 3C, constitutive NF-κB reporter activity was decreased about 7-9-fold in IκBαM-transfected U-87 MG and HF U-251 MG cells respectively, which was consistent with the EMSA results (Fig. 3B). 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.

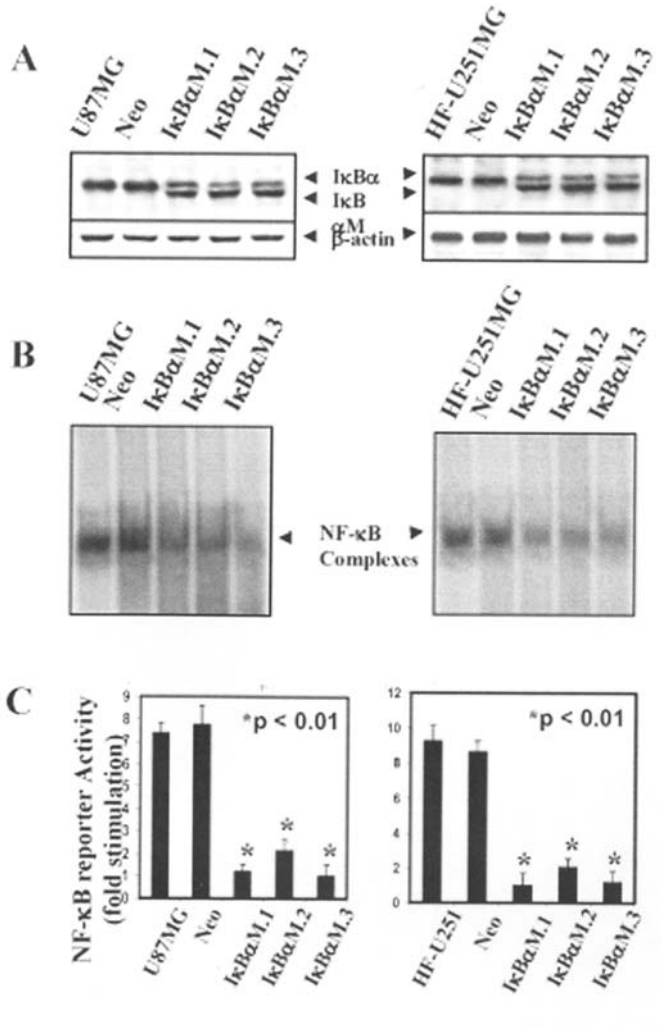


Figure 3. IκBαM transfection and NF-κB activity. A, Exogenous IκBαM expression. Cytosolic protein was extracted from parental, pLXSN (Neo)-transfected, and pLXSN-IκBαM-transfected U-87 MG and HF U-251 MG cells. Western blot analysis performed with a specific anti-IκBα antibody detected endogenous IκBα (higher molecular weight) and exogenous IκBαM (lower molecular weight) (arrows). Equal loading was monitored by hybridizing the filter with an anti-ß-actin antibody. B, NF-κB binding activity. Nuclear protein was extracted from the cells described above and subjected to EMSA with an NF-κB probe. C, 2x NF-κB-Luc reporter activity. Luciferase reporters driven by NF-κB response elements were co-transfected with pB-actin-RL into U-87 MG and HF U-251 MG cells. Firefly and Renilla luciferase activity were quantified by using the dual luciferase reporter assay system. This is one representative experiment of two.

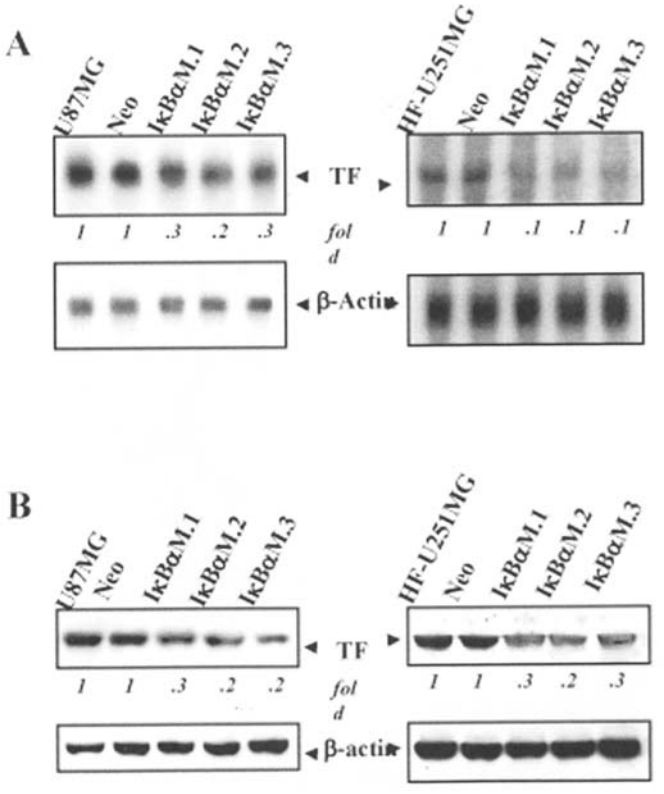


Figure 4. Suppression of the expression of TF mRNA and protein by blockade of NF-κB activation. A, Cellular mRNA was extracted from parental, pLXSN (Neo)-transfected, and pLXSN-IκBαM-transfected U-87 MG or HF U-251 MG cells. Northern blot analysis was performed with TF cDNA probe, and equal loading of mRNA was monitored by hybridizing the same membrane filter with a ß-actin cDNA probe. B, The level of TF protein also was analyzed in those cells by using Western blot analysis. The intensity of each band was quantitated by densitometry readings of autoradiograms. Intensity of the first sample was given a value of 1 and intensity levels of the rest samples were expressed as fold changes.

*Blockade of NF-κB activation suppresses the expression of TF in glioblastoma cells in vitro*. To provide evidence of the contribution of NF-κB activation to the regulation of TF expression and, hence, procoagulant activity, we studied the effect of altered NF-κB activity on TF expression. First, we analyzed the mRNA levels of TF in IκBαM-transfected and control cells. As shown in Fig. 4A, there was a significant decrease in TF mRNA expression in IκBαM-transfected U-87 MG and HF U-251 MG cells when compared with that in parental and Neo cells. We further analyzed the expression of TF in these cells at the protein level. Consistently, the level of TF protein expression in IκBαM-transfected cells significantly decreased as determined by Western blot analysis (Fig. 4B).

*Blockade of NF-κB activation suppresses the procoagulant activity of glioblastoma cells and conditioned media*. To determine the effect of NF-κB activation on the coagulative ability of glioblastoma cells, we tested IκBαM-transfected glioma cells or their conditioned media to determine whether they could inhibit plasma coagulation by using a tilt tube assay. First, we observed that plasma clotting occurred at 45±5 sec with a U-87 MG-cell suspension (Fig. 5A). The plasma-clotting time increased with the suspension of IκBαM-transfected U-87 MG cells (84±5 and 85±6 for cells transfected with IκBαM-1 and IκBαM-2, respectively; $P<0.01$). The plasma-clotting time also increased in IκBαM-transfected HF U-251 MG cells as compared with that in parental and Neo-transfected cells ($P<0.01$). We further examined the ability of conditioned media from these cells to induce plasma clotting. We found that conditioned media from U-87 MG cells caused plasma clotting at 40±7 sec. IκBαM transfection of U-87 MG cells prolonged clotting times induced by conditioned media (93±4 and 84±5 for cells transfected with IκBαM-1 and IκBαM-2, respectively; $P<0.01$); we obtained similar results with HF U-251 MG cells (Fig. 5B).

*Blockade of NF-κB activation by p65siRNA suppresses the TF expression in and the procoagulant activity of glioblastoma*

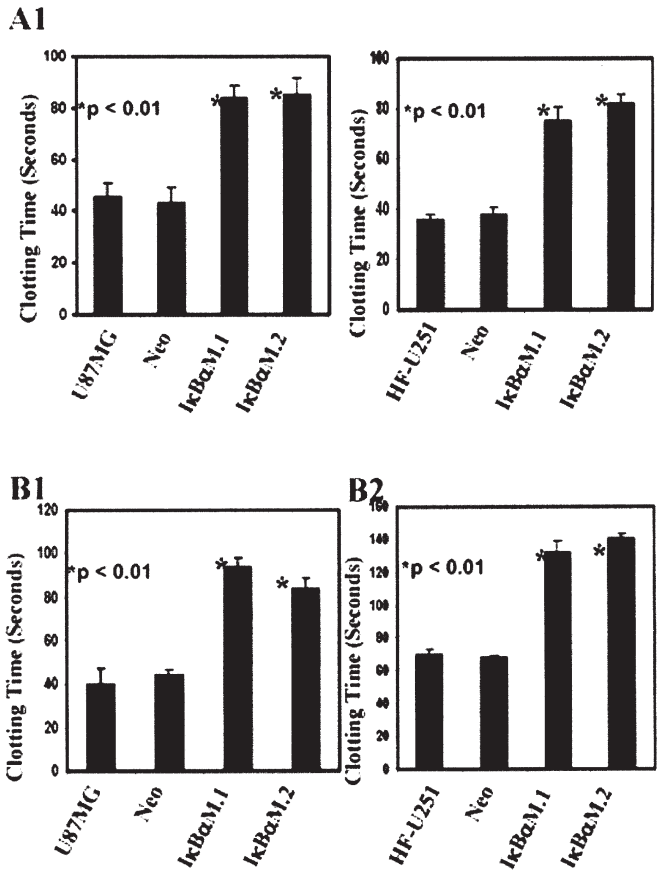


Figure 5. Suppression of plasma clotting by glioblastoma cells and conditioned media by blockade of NF-κB activation. A, Plasma-clotting time induced in a cell suspension. Two hundred microliters of a cell suspension from parental, pLXSN (Neo)-transfected, and pLXSN-IκBαM-transfected U-87 MG or HF U-251 MG cells was added to a plasma-clotting assay. Inhibition of NF-κB activation in both cell lines prolonged the plasma-clotting time induced by the cell suspension (P<0.01). B, Plasma-clotting time induced by conditioned media. Two hundred microliters of conditioned media from parental, pLXSN (Neo)-transfected, and pLXSN-IκBαM-transfected U-87 MG or HF U-251 MG cells was added to a plasma-clotting assay. Inhibition of NF-κB activation in both cell lines prolonged the plasma-clotting time induced by the conditioned media (P<0.01).

*cells*. To rule out the possibility that the effects of IκBα mutant transfection on the expression of TF was due to its other biologic effects than on NF-κB, we used p65 siRNA to knock-down NF-κB activity. First, we analyzed the protein level of NF-κB p65 in p65 siRNA-transfected U-87 MG and control cells using Western blot analysis. As shown in Fig. 5A1, there was a significant decrease (70%) in p65 expression in p65 siRNA-transfected cells when compared with that in mock and control siRNA-transfected cells. Consistently, constitutive NF-κB-binding activity in U-87 MG p65 siRNA-transfected cells was significantly decreased as compared to that in the mock and control siRNA-transfected cells (Fig. 5A2). We further analyzed the expression of TF in these cells at the protein level. We found that the level of TF protein expression in p65 siRNA-transfected U-87 MG cells significantly decreased (80%) as determined by Western blot analysis (Fig. 5A1). These results suggested that p65 siRNA transfection leads to specific suppression of p65 protein expression and NF-κB activity, and subsequent inhibition of TF expression.

Moreover, we analyzed the procoagulant activity of these cells using the plasma clotting assay. We found that the plasma-clotting time increased with the suspension of p65 siRNA-transfected U-87 MG cells as compared with the suspension of mock and control siRNA-transfected cells (Fig. 5B1) (P<0.05). The plasma-clotting time also increased with the conditioned media of p65 siRNA-transfected U-87 MG cells as compared with that of mock and control siRNA-transfected cells (Fig. 5B2) (P<0.05).

*Blockade of NF-κB activation suppresses necrosis formation and tumor growth of human glioblastoma cells*. To evaluate whether NF-κB activity regulates necrosis formation by glioblastoma cells, we used an orthotopic xenograft model of human glioma by intracranially injecting glioma cells into nude mice. Intracranially implanted U-87 MG and U-87 MG-Neo cells ($1x10^6$ cells/mouse) produced brain tumors in all of the mice injected with these cells (Table I). In contrast, U-87 MG-IκBαM-1 and U-87 MG-IκBαM-2 cells produced smaller tumors. Moreover, the incidence of necrosis produced by the IκBαM-transfected U-87 MG cells was significantly reduced as compared with that produced by the U-87 MG and Neo cells. We obtained similar results with the use of IκBαM-transfected HF U-251 MG cells. To rule out the possibility that difference in necrosis formation was mainly due to difference in tumor size, we produced small control

Table I. Suppression of growth of U-87 MG and HF U-251 MG cells in the brain of nude mice by IκBαM transfection.

| Cell line | Tumor incidence | Tumor volume ($mm^3$) | Focal necrosis incidence | P-value |
|---|---|---|---|---|
| U-87 MG-Neo | 5/5 | 60.00±29.96 | 3/5 | - |
| U-87 MG-IκBαM-1 | 4/5 | 3.80±2.45 | 0/5 | <0.01 |
| U-87 MG-IκBαM-2 | 5/5 | 4.36±2.21 | 0/5 | <0.01 |
| HF U-251 MG-Neo | 5/5 | 46.65±22.40 | 4/5 | - |
| HF U-251 MG-IκBαM-1 | 5/5 | 4.96±3.64 | 0/5 | <0.01 |
| HF U-251 MG-IκBαM-2 | 4/5 | 4.23±1.98 | 0/5 | <0.01 |

Tumor cells ($1x10^6$) were injected into the brain of nude mice. Samples were collected 45 days after injection or when the mice were moribund. Tumor volume = a x $b^2$ x 0.4, in which a is largest diameter and b is the smallest diameter. Results are shown for one representative experiment of two.

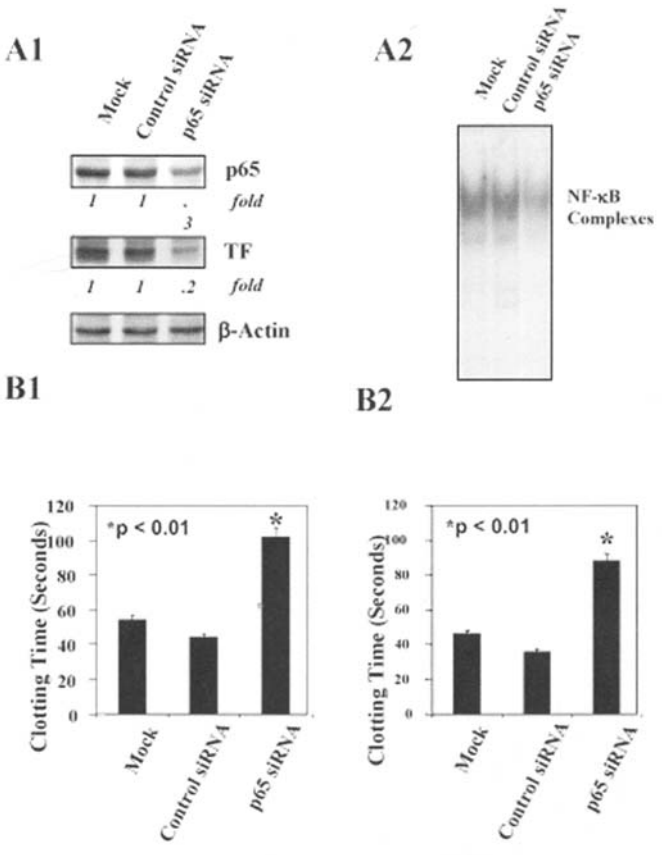


Figure 6. Suppression of the expression of TF protein and plasma clotting by p65 siRNA transfection. A1, U-87 MG cells were transfected with NF-κB p65 siRNA (100 nM) or control siRNA (100 nM) using Lipofectamine. Treatment of Lipofectamine alone was used as mock-transfection control. The levels of p65 and TF proteins were analyzed using Western blot analysis. Intensity of the first sample was given a value of 1 and intensity levels of the rest samples were expressed as fold changes. A2, Nuclear protein was extracted from mock, control siRNA or NF-κB p65 siRNA transfected U-87 MG cells, and EMSA was performed with an NF-κB probe. B1, Plasma-clotting time induced in a cell suspension. Two hundred microliters of a cell suspension from mock, control siRNA or NF-κB p65 siRNA transfected U-87 MG cells was added to a plasma-clotting assay. Inhibition of NF-κB activation in the cell line prolonged the plasma-clotting time induced by the cell suspension ($P<0.01$). B2, Plasma-clotting time induced by conditioned media. Two hundred microliters of conditioned media from mock, control siRNA or NF-κB p65 siRNA transfected U-87 MG cells was added to a plasma-clotting assay. Inhibition of NF-κB activation in the cell lines prolonged the plasma-clotting time induced by the conditioned media ($P<0.01$).

tumors similar in size to IκBαM-transfected tumors. We found frequent necrosis formation 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 necrosis formation. Therefore, inhibition of NF-κB activity by IκBαM transfection suppressed both necrosis and tumorigenicity in glioblastomas.

*Inhibition of activation of NF-κB and decreased expression of TF in IκBαM transfectant tumors*. To provide direct evidence of the contribution of NF-κB activation to the regulation of procoagulation molecules and, hence, necrosis, we studied the effect of altered NF-κB activity on the expression of TF *in vivo*. First, we sought to determine whether IκBαM transfection suppresses NF-κB activity *in vivo*. We performed immunohistochemical analysis of U-87 MG-Neo and U-87 MG-IIκBαM brain tumor specimens with use of an antibody that recognizes the nuclear localization sequence of the activated form of NF-κB p65. There was an inhibition of activated NF-κB in the U-87 MG-IκBαM tumors (Fig. 7). Similar results were obtained with the use of HF U-251 MG-Neo and HF U-251 MG-IκBαM tumors (Fig. 7).

We also evaluated the expression of TF protein *in vivo* by using immunohistochemistry. As shown in Fig. 7, we observed staining for TF in U-87 MG, HF U-251 MG, and 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 TF gene *in vivo*.

## Discussion

In the present study, we found that NF-κB activity is correlated with necrosis formation in glioblastomas. In our animal model, blockade of NF-κB activation by IκBαM transfection suppressed necrosis and tumor growth by human glioblastoma cells. Furthermore, altered NF-κB activity significantly affected expression of the major procoagulation molecule TF *in vitro* and *in vivo* and the plasma-clotting activity of glioblastoma cells. Therefore, for the first time to our knowledge, we provide clinical, experimental, and mechanistic evidence that constitutively activated NF-κB plays an important role in necrosis formation in human glioblastomas.

Necrosis is a poorly understood process. By definition, it is sudden, accidental cell death brought on by a large stimulus sufficient to kill the cell, and it is characterized by membrane damage and energy depletion (33). Necrosis is generally regarded as a process that is not under genetic control, whereas apoptosis is a process that often requires new gene transcription and the triggering of complex regulatory pathways (34). The focal necrosis of glioblastoma seems to be largely associated with the presence of multiple thrombotic foci that result from hypercoagulability in the abnormal tumor microcirculation (35-37). Furthermore, hypercoagulability in the glioblastoma tumor bed could be caused by aberrant production of procoagulation factors. The present study provided critical evidence of the molecular mechanism of aberrant expression of procoagulation molecule in glioblastoma. Specifically, we found that NF-κB activity is at least in part responsible for the aberrant expression of the major procoagulation factor TF in glioblastoma cells.

NF-κB is an inducible dimeric transcription factor that belongs to the Rel/NF-κB family of transcription factors, whose prototype in most nonlymphoid cells is a heterodimer consisting of the RelA (p65) and NF-κB1 (p50) subunits (38). NF-κB complexes are typically retained in the cytoplasm by inhibitory IκBα 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

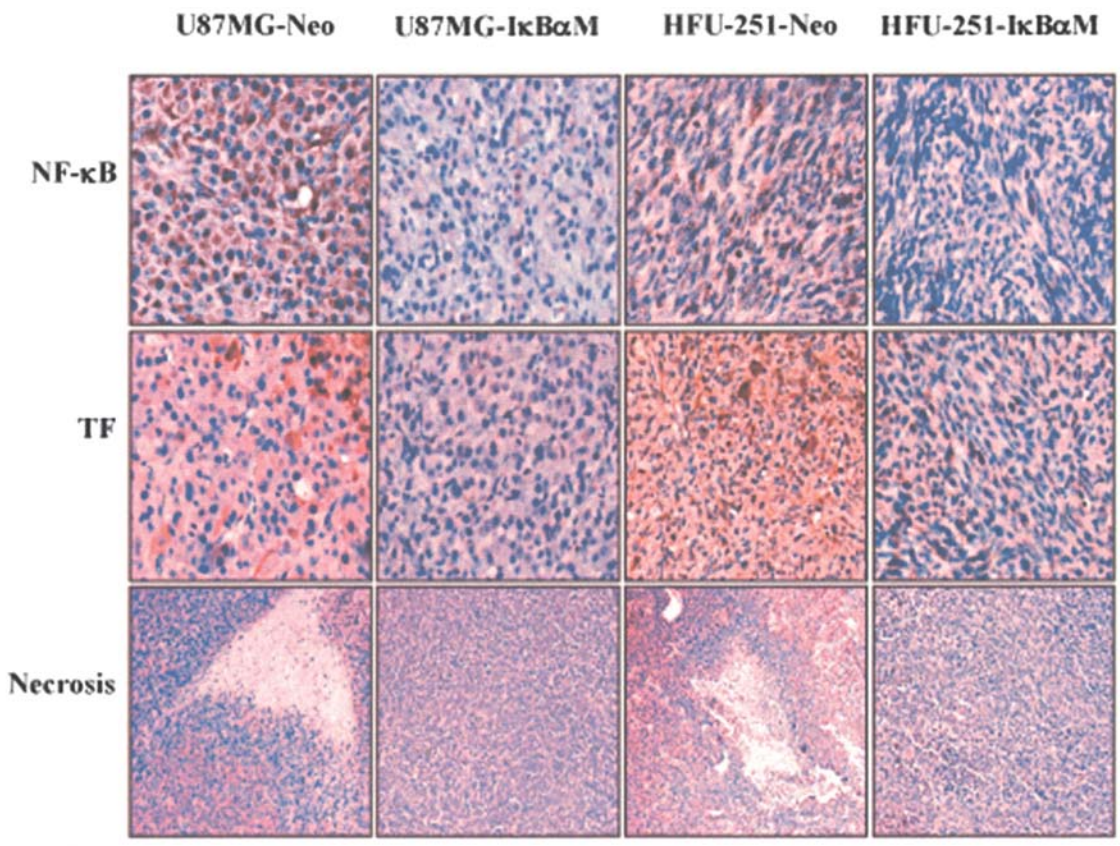


Figure 7. Immunohistochemical analysis. Brain tumors produced by pLXSN (Neo)-transfected and pLXSN-IκBαM-transfected U-87 MG or HF U-251 MG cells were harvested and processed for immunohistochemical analysis. The NF-κB activity in the tumors was evaluated by using antibodies that recognize the nuclear localization sequence of the activated form of NF-κB p65. Sections of the tumors also were immunostained with anti-human TF antibodies to detect TF protein. The staining patterns shown are representative of those observed in at least five random fields.

in glioblastoma cells suppressed the coagulability of glioblastoma cells *in vitro* and necrosis formation in brain tumors in nude mice. Blockade of NF-κB activity also inhibited the expression of procoagulation molecule TF in glioblastoma cells, suggesting that increased NF-κB activity may contribute to the aberrant expression of TF and coagulability of glioblastomas.

Previous studies suggested that cell-type-specific pathways regulate TF gene expression (39). AP-1 sites and an NF-κB site mediate the induction of the human TF promoter in monocytic and endothelial cells (40,41). Similarly, AP-1 sites regulate the induction of the murine TF promoter in fibroblast-like cells. In contrast, Egr-1 and Sp1 sites mediate the induction of human and rat TF promoters in epithelial-like cells and vascular smooth muscle cells, respectively (42,43). In the present study, our data showed that NF-κB activity critically regulated the transcription of TF in human glioma cells. However, this study mainly focused on TF, which directly involves in the process of coagulation. It also has been shown by Golaman *et al* (6), that VEGF released by glioma cells promotes hypercoagulability manifesting as focal necrosis in glioblastoma (6). VEGF may do so by increasing TF expression (44) and thromboplastin activity in human endothelial cells and by inducing vWF release from endothelial cells (6,45). Interestingly, VEGF promoter contains NF-κB binding site and NF-κB activation may regulate the expression of VEGF (25,46-48), which then play an indirect, but important role in hypercoagulation induced by NF-κB activation.

Finally, some have proposed that the presence of necrosis in a glioblastoma could promote rapid tumor growth (6,7,10,49). Necrosis may promote tumor growth by triggering massive angiogenesis in glioblastomas, and VEGF and other angiogenic factors seem to be important mediators. The most likely explanation is that hypoxia in necrotic areas induces VEGF expression, which in turn induces angiogenesis to counter hypoxia. In our present study, blockade of NF-κB activation inhibited tumor growth *in vivo* and this effect was related to the inhibition of necrosis formation. Both suppression of tumor growth and necrosis formation did not appear to be due to reduced cell proliferation, because the *in vitro* growth of the IκBαM-transfected cells was similar to that of the control cells (data not shown). Moreover, to find out whether tumor size dictated the necrosis formation, we have compared necrosis formation in tumor samples of similar sizes from both control xenografts and IκBαM xenografts and found that more frequent necrosis formation was observed in control xenografts than in IκBαM xenografts, suggesting that inhibition of necrosis by NF-κB blockade is not mainly due to decreased size of tumors. Thus, the inhibition of tumorigenicity by NF-κB blockade may in part occur by inhibition of necrosis.

In conclusion, we found that human glioblastomas with necrosis have higher levels of constitutive NF-κB activity than gliomas without necrosis. Suppression of NF-κB activity

through expression of a mutant IκBα inhibited necrosis formation, and retarded tumor growth, in part through down-regulation of the expression of TF. These data indicate that NF-κB activation plays an important role in hypercoagulability manifesting as focal tumor necrosis. Therefore, our findings strongly suggest that NF-κB is a potential therapeutic target for glioblastoma.

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