Expression of a Dominant-Negative Mutant Inhibitor-κBα of Nuclear Factor-κB in Human Head and Neck Squamous Cell Carcinoma Inhibits Survival, Proinflammatory Cytokine Expression, and Tumor Growth in Vivo

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ABSTRACT

We demonstrated recently that constitutive expression of proinflammatory cytokines interleukin (IL)-1α, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor in head and neck squamous cell carcinoma is correlated with activation of transcription factor nuclear factor (NF)–κB/Rel A (p50/p65), which binds the promoter region within each of the genes encoding this repertoire of cytokines. NF-κB can be activated after signal-dependent phosphorylation and degradation of inhibitor-κBα and has been reported to promote cell survival and growth. In the present study, we expressed a phosphorylation site mutant of inhibitor-κBα (IκBαM) in head and neck squamous cell carcinoma lines UM-SCC-9, -11B, and -38 to determine the effect of inhibition of NF-κB on cytokine expression, cell survival in vitro, and growth in vivo. After transfection with IκBαM, only a few UM-SCC-9 clones were obtained that stably expressed the mutant IκB, suggesting that expression of a mutant IκBα may affect survival of the transfected UM-SCC cell lines. After cotransfection of IκBαM with a Lac-Z reporter, we found that the number of surviving β-galactosidase-positive cells in the three cell lines was reduced by 70–90% when compared with controls transfected with vector lacking the insert. In UM-SCC-9 cells that stably expressed IκBαM, inhibition of constitutive and tumor necrosis factor-α induced NF-κB activation, and production of all four cytokines was observed. Although UM-SCC-9 IκBαM-transfected cells proliferated at the same rate as vector-transfected cells in vitro, significant reduction in growth of tumor xenografts was observed in SCID mice in vivo. The decreased growth of UM-SCC-9 IκBαM-transfected tumor cells accompanied decreased immunohistochemical detection of the activated form of NF-κB in situ. These results provide evidence that NF-κB and IκB play an important role in survival, constitutive and inducible expression of proinflammatory cytokines, and growth of squamous cell carcinoma. NF-κB could serve as a potential target for therapeutic intervention against cytokine and other immediate-early gene responses that contribute to the survival, growth, and pathogenesis of these cancers.

INTRODUCTION

Autonomous production of proinflammatory cytokines has been detected in many different types of human and murine cancers, suggesting that their expression or the mechanism underlying their activation may provide a selective survival or growth advantage to cancer cells. We and others have observed previously that human head and neck SCCs autonomously produce proinflammatory cytokines IL-1α, IL-6, IL-8, and GM-CSF in vitro and in vivo (1–5), and these cytokines have also been detected in tumor specimens and cell lines from patients with SCCs arising within the lungs, cervix, and skin (6–8). We reported that transformed murine keratinocytes that express homologues of IL-1α, IL-6, IL-8, and GM-CSF at increased levels exhibit more aggressive growth and metastasis in vivo (9–11). IL-1α has been shown to promote autocrine proliferation of transformed human keratinocytes and cervical SCC cell lines (12). Elevated expression of IL-8 by human lung SCC has been shown to promote angiogenesis and tumor growth in experimental models in vivo (8). Expression of GM-CSF has been associated with homing of CD3+ granulocyte precursors that promote increased metastatic potential of human and murine SCC of the upper aerodigestive tract (13). Both IL-1 and IL-6 have been implicated in the increase in cataolism often observed in patients with these cancers (14). The molecular mechanisms that contribute to activation of this repertoire of cytokines in SCC have not been defined.

The expression of proinflammatory cytokines can be induced in response to a variety of stimuli through activation of transcription factor NF-κB/Rel A (p50/p65), which is a heterodimer formed by members of the Rel family of proteins (15). We noted that the 5′ promoter region of the IL-1α, IL-6, IL-8, and GM-CSF genes expressed by HN SSC contain binding sites for the NF-κB/Rel family of transcription factors (16–19), and we showed recently that the autonomous expression of proinflammatory cytokines in HNSSC is correlated with constitutive activation of NF-κB/Rel A p50/p65 (20). Constitutive activation of NF-κB/Rel family members has also been demonstrated recently in other laboratories in several different neoplasms, including lymphoma, melanoma, and carcinoma of the colon and breast (21–26). Members of the Rel family were originally identified as viral and cellular oncogenes (27, 28). At present, the mechanisms by which they can promote tumor growth have not been fully elucidated.

Activation of NF-κB has been shown to involve signal-induced phosphorylation and degradation of cytoplasmic IκB proteins, which release NF-κB for nuclear translocation (29–32), and for binding to the promoter sites of target genes. Studies in several laboratories have shown that mutations in the serine phosphorylation sites at S32 and/or S36 of IκBα can inhibit the signal-dependent activation of NF-κB to a variety of stimuli (29–32). Such phosphorylation mutants can therefore exert a dominant-negative effect, preventing the activation of NF-κB-dependent genes. By expression of a dominant-negative IκBα mutant, NF-κB has been shown to be important in activation of genes necessary for survival and protection of cells from injury by a variety of cytokotoxic stimuli, including cytokine TNF-α, chemotherapy, and radiation therapy (33–35). In the present study, we provide evidence that the expression of a phosphorylation mutant of IκBα (IκBαM) can reduce survival, constitutive and inducible activation of NF-κB, expression of proinflammatory cytokines, and tumor growth in vivo.

3 The abbreviations used are: SCC, squamous cell carcinoma; HNSSC, head and neck SCC; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IκB, inhibitor κB; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MTT, methyl thiazol tetrazolium salt; SCID, severe combined immunodeficient; β-gal, β-galactosidase.

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EMSA assays were performed using standard methods (42), and nuclear extracts were obtained from UM-SCC-9 in log phase growth using the methods of Dignam et al. (43) and Lee et al. (44). Double-stranded DNA oligonucleotide probes for NF-κB and OCT-1 were purchased commercially (Promega, Madison, WI and Santa Cruz Biotechnology). The oligonucleotide sequences used were: NF-κB, 5′-AGTTAGGGAGACATTCCACCGG3′; and OCT-1, 5′-TGTTCAATGCGAATCATCAGA3′. EMSA reactions included 5 μg of nuclear extract protein incubated for 25 min at 20°C in buffer containing 20 mM Hepes (pH 7.9), 4.6 mM MgCl2, 63 mM KCl, 1.1% glycerol, and 1 mM DTT, 1 μg poly(dexylinosinic-dexylic acid) (Pharmacia, Piscataway, NJ), and 60,000 cpm of 32P-labeled oligonucleotide probe. For competition experiments, nuclear extracts were preincubated with a 100-fold excess of each probe before the addition of labeled probe. Binding complexes were resolved on 5%, 0.25× TBE polyacrylamide gel, and imaged with a Packard Instant Imager (Packard Technologies, Downers Grove, IL). The pglbB Luc reporter construct containing two IκB-x chain NF-κB binding sites and the luciferase gene was described previously (45), and the pCMV Lac-Z reporter containing the CMV promoter and Lac-Z gene in pcDNA3 (Invitrogen, Carlsbad, CA) used for normalization of the assay was prepared by Dr. G. Thomas in our laboratory. UM-SCC-9 cell cultures at 70% confluence were cotransfected with pglbB Luc plasmid (2 μg/ml) and pCMVluc (0.4 μg/ml) in Opti-MEM media containing 10 μg/ml LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD). After 4 h, the medium was removed, and cells were incubated in complete media for 48 h. The relative luciferase activity was determined with a Dual Light Reporter Gene Assay kit (Tropix, Bedford, MA) with a Monolight 1000 luminometer (Analytical Luminescence Laboratories, San Diego, CA).

Quantitation of Cytokine Secretion by ELISA. UM-SCC cells (3×106) were cultured in 12 ml of fresh medium in 75-cm2 flasks, and culture supernatants were collected after 5 days. Standardized ELISA assay kits were used to quantify IL-1α, IL-6, IL-8, and GM-CSF (R&D System, Minneapolis, MN). The absorbance of the reaction product was quantitated with a multichannel plate spectrophotometer at 450 nm (Biotek 311; Biotek Systems, Winonosi, VT). All samples were assayed in triplicate. Results were standardized for amount of cytokine secreted as pg/106 cells/5 days/g SDF.

MTT Assay. The cell proliferation rate of UM-SCC-9 and transfected cells mixed in vitro were compared by using the MTT reaction assay (Boehringer Mannheim, Mannheim, Germany). Replicate cultures of each of the cell lines were plated in 96-well, flat-bottomed microplates at an initial concentration of 5×103 cells/100-μl medium/well. On days 1–5, 10 μl of MTT labeling agent was added, and cells were incubated for an additional 4 h. One hundred μl of solubilization solution was added to each cell line at each dose. Tumors were measured weekly. The data are presented as mean ± SE. Animals were sacrificed if the tumor exceeded 10% of the animal’s body weight or exceeded 2.5 cm3 in volume.

Electromobility Shift Assay. EMSA were performed using standard methods (42), and nuclear extracts were obtained from UM-SCC-9 in log phase growth using the methods of Dignam et al. (43) and Lee et al. (44). Double-stranded DNA oligonucleotide probes for NF-κB and OCT-1 were purchased commercially (Promega, Madison, WI and Santa Cruz Biotechnology). The oligonucleotide sequences used were: NF-κB, 5′-AGTTAGGGAGACATTCCACCGG3′; and OCT-1, 5′-TGTTCAATGCGAATCATCAGA3′. EMSA reactions included 5 μg of nuclear extract protein incubated for 25 min at 20°C in buffer containing 20 mM Hepes (pH 7.9), 4.6 mM MgCl2, 63 mM KCl, 1.1% glycerol, and 1 mM DTT, 1 μg poly(dexylinosinic-dexylic acid) (Pharmacia, Piscataway, NJ), and 60,000 cpm of 32P-labeled oligonucleotide probe. For competition experiments, nuclear extracts were preincubated with a 100-fold excess of each probe before the addition of labeled probe. Binding complexes were resolved on 5%, 0.25× TBE polyacrylamide gel, and imaged with a Packard Instant Imager (Packard Technologies, Downers Grove, IL).
(mouse IgG1; Dako, Carpenteria, CA) diluted in 1% BSA in PBS were used as controls. The samples were incubated with horse anti-mouse antibody (1:200 in PBS containing 1.5% horse serum; Vector Lab), followed by a 30-min incubation with biotin/avidin horseradish peroxidase conjugates (Vectastain Elite ABC kit; Vector Lab), and an 8-min incubation with the chromogen diaminobenzidine tetrahydrochloride (Vector Lab). Stained sections were dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, New Jersey).

RESULTS

Expression of a Phosphorylation Mutant of IkB in UM-SCC Inhibits Survival of Transfected Cells. We transfected a phosphorylation site-deficient mutant of murine IkBα (IkBαM, Ref. 35) or a control vector lacking an insert into head and neck squamous cell carcinoma lines UM-SCC-9, -11B, and -38 to determine whether we could obtain a UM-SCC line that stably expressed a dominant-negative mutant of IkBα. After selection in G418 and cloning, we obtained UM-SCC-9 cells expressing IkBαM, but we did not detect expression of IkBαM in over 30 UM-SCC-11B or 38 clones plated from two independent transfections, despite similar transfection efficiencies and evidence of transient expression of mutant IkBα in transfected cultures by Western blot (data not shown). Because NF-κB/Rel transcription factors have been reported to regulate genes involved in cellular survival (21–25, 33–35), we examined the possibility that expression of an IkBα mutant might reduce survival of transfected UM-SCC cells. UM-SCC-9, -11B, and -38 cell lines were cotransfected with a Lac-Z reporter in the presence of a 4-fold excess of vector containing IκBαM transfectants. The number of IκBαM transfectants was significantly reduced in the three cell lines by 70–90%.

Expression of IkBαM in UM-SCC-9 Cells Inhibits Constitutive and TNF-α-inducible Activation of NF-κB. Fig. 2 shows a Western blot analysis demonstrating expression of IkBαM in UM-SCC-9, vector control transfecteds UM-SCC-9 C11 and C13, IkBαM transfec-

![Fig. 2. Western blot of murine IκBα phosphorylation mutant (IkBαM) and endogenous human IκBα expression in UM-SCC-9. UM-SCC-9 cells were transfected with a murine IκBαM or vector control and selected in G418 as described in “Materials and Methods.” Anti-mouse and human IκBα antibodies were used to detect IκBα species in UM-SCC-9, vector-transfected C11, C13, and IkBαM-transfected I11 and I13 cell line extracts, as described in “Materials and Methods.” Murine Pam LU-1 SCC cytoplasmic protein was used as a control for the faster migrating murine IκBα (Lane 1). UM-SCC-9 and the transfected cells were stimulated with TNF-α (+) (10,000 units/ml for 1 h) prior to extraction to demonstrate degradation of endogenous IκBα and resistance of the IkBα phosphorylation mutant protein to degradation.

Fig. 3. Inhibition of NF-κB binding activity in UM-SCC-9 IkBαM transfec-
tants I11 and I13 by EMSA. A, the NF-κB binding activity in UM-SCC-9 (Lanes 1 and 2), vector control transfec-
ts I11 and I12 (Lanes 3–6), and IkBαM transfec-
ts I11 and I13 (Lanes 7–10) was compared by EMSA as described in “Materials and Methods.” Cells were incubated in the absence or presence of 10,000 units/ml of TNF-α for 1 h before isolation of nuclear extracts. Binding specificity was confirmed by competition with unlabeled probe (Lanes 11–14). Comp., competitor; NS, nonspecific. B, probe containing Oct-1 motif was used in EMSA as a control for quality and quantity of cell extracts. The specificity of Oct-1 binding activity was demonstrated by competition with unlabeled Oct-1 probe (Lane 11) but not AP-1 probe (Lane 12).

To determine the effect of expression of IkBαM upon constitutive and TNF-α-inducible activation of NF-κB in UM-SCC-9 cells, we examined the level of NF-κB DNA binding activity in nuclear extracts from UM-SCC-9, vector control, and IkBαM transfec-
tants UM-SCC-9 I11 and I13, and a murine SCC line Pam LU-1, which serves as a control. The immunoblot in Fig. 1 confirms that murine IkBαM in UM-SCC-9 transfec-
ts I11 and I13 and murine IkBα in Pam LU-1 can be distinguished from endogenous human IkBα due to a faster rate of migration on SDS-PAGE (35). IkBαM was detected in UM-SCC-9 IkBαM transfec-
ts I11 and I13 but not in the parental or vector control lines. Fig. 2 also confirms that, 1 h after stimulation with TNF-α, the endogenous human IkBα has un-
dergone TNF-induced degradation, whereas IkBαM is resistant to TNF-induced degradation and prevents NF-κB-dependent resynthesis of human IkBα (22).
observed in the UM-SCC-9 cell line and vector control transfectants C11 and C13 and is further induced by addition of TNF-α (Fig. 3A, Lanes 1–6). The level of constitutive and TNF-α-induced NF-κB activation is significantly reduced in UM-SCC-9 IκBαM transfectants I11 and I13 (Fig. 3A, Lanes 7–10). The NF-κB DNA binding activity observed in UM-SCC-9, C11, and C13 cells could be specifically inhibited by competition with unlabeled NF-κB oligonucleotide (Fig. 3A, Lanes 11–14). Fig. 3B shows that expression of IκBαM did not inhibit DNA binding activity of the unrelated transcription factor p53.

To determine whether the reduction in NF-κB DNA binding activity observed corresponded to a reduction in the functional activation of NF-κB, we examined NF-κB reporter activity in UM-SCC-9 vector and IκBαM transected clones. Fig. 4 confirms that functional activation of NF-κB as determined by luciferase reporter assay is reduced ~10-fold in the IκBαM transfectant I11 and I13 cells relative to UM-SCC-9 vector control transfectants C11 and C13. The reporter activity was specific for NF-κB, because UM-SCC-9 cells transfected with a reporter containing a wild-type NF-κB sequence, but not a mutant sequence, exhibited luciferase signal (data not shown).

Expression of Proinflammatory Cytokines IL-1α, IL-6, IL-8, and GM-CSF Is Inhibited in UM-SCC-9 Cells Transfected with IκBαM. We determined if expression of the IκBαM in UM-SCC-9 resulted in reduced expression of IL-1α, IL-6, IL-8, and GM-CSF cytokine genes that contain NF-κB sites in their 5′ promoter regions (16–19). The quantity of each of these proinflammatory cytokines secreted by vector control and IκBαM transfectants as determined by ELISA is shown in Fig. 5. Secretion of all four of the proinflammatory cytokines was reduced in IκBαM transfectants I11 and I13 relative to two vector control transfectants C11 and C13. Thus, expression by IκBαM in UM-SCC-9 inhibited activation of NF-κB and production of proinflammatory cytokines IL-1α, IL-6, IL-8 and GM-CSF, indicating that NF-κB promotes constitutive expression of these proinflammatory cytokines by UM-SCC-9.

Effect of Expression of IκBαM on Growth of UM-SCC-9 Cells in Vitro and in Vivo. We compared the growth of UM-SCC-9 IκBαM transfectants I11 and I13 and control vector transfectants C11 and C13 in vitro and as xenografts in SCID mice in vivo. The growth of the cell lines in vitro was compared in a 5-day MTT assay as described in “Materials and Methods,” and the results are shown in Fig. 6A. There was no significant difference in growth between IκBαM transfectants I11 and I13 and control vector transfectants C11 and C13 in vitro. However, when SCID mice were inoculated s.c. with 1 × 10⁷ cells of IκBαM transfectants I11 and I13 or control vector transfectants C11 and C13, the growth of the IκBαM transfectants was significantly reduced when compared with vector-transfected cell lines in two independent experiments (Fig. 6B). As shown in Fig. 6B, Experiment 1, we observed slower tumor growth of the IκBαM-transfected UM-SCC-9 I11 cell line when compared with vector transfectant C11. We observed complete tumor regression of IκBαM transfectant I13 at a tumor cell dose of 1 × 10⁷ cells, whereas the control line C13 grew progressively (Fig. 6B, Experiment 2). Similar but smaller differences were obtained in both experiments in animals inoculated with a lower dose of 5 × 10⁶ cells (data not shown). Thus, expression of IκBαM in UM-SCC-9 cells inhibited tumor growth in vivo but did not inhibit tumor cell growth in vitro.

Inhibition of Activation of NF-κB in UM-SCC-9 IκBαM Transfectant Tumor Xenografts. To establish whether there is a relative difference in constitutive activation of NF-κB in UM-SCC-9 and IκBαM transfectant tumor xenografts, we compared NF-κB activation in UM-SCC-9, C11, and I11 tumor specimens in situ by immunohistochemistry, using a monoclonal antibody specific for the nuclear localization sequence of the activated form of human NF-κB p65 (46). As shown in Fig. 7, staining for the activated form of NF-κB protein was observed in UM-SCC-9 (Fig. 7A) and vector transfectant C11 cells (Fig. 7B) but was significantly decreased in IκBαM-transfected I11 cells (Fig. 7C). All three tumor specimens stained positively with monoclonal antibody against pan cytokeratin (Fig. 7, D–F) but not with isotype-matched control antibody (Fig. 7, G–I). Thus, expression of IκBαM inhibited constitutive activation of NF-κB in vivo as well as in vitro.

**DISCUSSION**

In this study, we encountered difficulty in obtaining human HNSCC lines that stably express a dominant-negative IκBα phosphorylation site mutant, suggesting that expression of the mutant IκBα could affect survival of transfected UM-SCC cells. When the three UM-SCC cell lines were cotransfected with a Lac-Z reporter in the presence of a 4-fold excess of vector containing a human IκBα phosphorylation mutant or control vector, it was found that transfection of mutant IκBα markedly reduced the survival of
β-gal-positive cells by 70–90% in cultures within 72 h (Fig. 1). These results are consistent with recent studies that show that inhibition of activation or deletion of NF-κB/RelA inhibits survival of a variety of normal and neoplastic cells of different tissue origin (21–24, 33–35). The promotion of cell survival by NF-κB has recently been attributed to several mediators. NF-κB has been reported to induce TRAF1, TRAF2, c-IAP1, and c-IAP2, resulting in suppression of caspase-8 activation, thereby inhibiting apoptosis (47). A novel apoptosis inhibitor IEX-1 L has also been reported to be involved in NF-κB-mediated cell survival (48). Recently, the pro-survival Bcl-2 homologue Bcl-2/RelA has been shown to be a transcriptional target of NF-κB, which can block TNF-α-induced apoptosis (49). Although the pathways by which NF-κB promotes survival in HNSCCs remain to be defined, our results and those of others suggest that NF-κB may be an important target for therapeutic intervention against immediate-early responses that promote survival and resistance of cancer.

When we examined UM-SCC-9 cells that survive expression of IκBαM, we found that IκBαM suppressed constitutive and TNF-α-induced activation of NF-κB, production of proinflammatory cytokines, as well as growth in vitro. We confirmed that expression of IκBαM inhibited constitutive activation of NF-κB in UM-SCC-9 tumors in situ. Whereas expression of IκBαM inhibited growth of UM-SCC-9 in vivo, there was no significant difference in growth between the IκBαM and vector control transfectants in vitro. These results provide evidence that cytokines or other genes activated by NF-κB in UM-SCC-9 promote interactions with the host that contribute to malignant growth in vivo. These data are consistent with observations made in our laboratory in an independent murine model of SCC, in which expression of the murine homologues of these cytokines was found to be associated with more aggressive growth in vivo but not in vitro (9–11). We found that low cytokine-producing clones of the murine SCC line Pam 212 grew slowly or regressed, whereas those that expressed the cytokine homologues IL-1α, IL-6, KC, and GM-CSF grew more aggressively in vivo. However, no significant differences in proliferative rate were observed in vitro.

Among the NF-κB-induced cytokines expressed by human and murine SCCs, there is evidence that the C-X-C family of chemokines to which human IL-8 and murine KC belong can induce host angiogenesis responses and thereby provide a differential growth advantage in vivo. Smith et al. (8) have shown that expression of IL-8 by human lung SCC can promote an increase in angiogenesis and growth of tumor xenografts in SCID mice. In murine SCC, we have obtained preliminary evidence that overexpression of murine cytokine KC in the low KC expressing Pam 212 SCC line promotes an increase in
growth in vivo, whereas it has no effect on the rate of growth in vitro. The relative contribution of additional autocrine or paracrine mechanisms by which proinflammatory cytokines IL-1α, IL-6, GM-CSF, and others expressed by SCC may be found to promote growth remains to be determined. NF-κB and IκBa could serve as potential targets for therapy to inhibit expression of cytokine and other NF-κB-dependent genes that are ultimately found to contribute to the growth of SCCs in vivo.

Although NF-κB/Rel A and IκBa appear to be important in the regulation of expression of cytokines and other genes that promote growth in UM-SCC cell lines, it is possible that activation of other pathways contribute to expression of these cytokines and other NF-κB-dependent genes. The inhibition of activation of cytokine expression and growth by IκBa in UM-SCC-9 that we observed was less than complete. The promoter region of the cytokine genes expressed by SCC contains AP-1 and NF-IL6 as well as NF-κB-dependent genes. The inhibition of activation of cytokine genes expressed by SCC and in other neoplasms remains to be determined. It will be interesting to determine whether changes in expression or activation of other signal transduction molecules upstream that lead to activation of protective pathways, or inactivation of molecules that induce apoptosis, determine the requirement for activation of NF-κB.

In addition to cytokine-induced activation, NF-κB and alternative pathways may be activated by alterations in epidermal growth factor or receptor expression, Ras activation, or through cell damage and changes in oxidation-reduction state (26, 28, 53–55). Baldwin and colleagues have shown previously that Bcr-Abl and Ras promote activation of NF-κB in leukemia, and an IκBa super-repressor can inhibit Bcr-Abl-mediated tumors in nude mice (56). Thus, F-κB could serve as a common pathway for oncogenesis involving altered expression or function of a variety of components of these pathways.

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