Tumor Metastasis and the Reciprocal Regulation of Prometastatic and Antimetastatic Factors by Nuclear Factor κB

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Abstract

To investigate the role of the transcription factor nuclear factor κB (NFκB) in tumor metastasis, we generated a murine lung alveolar carcinoma cell line (Line 1) defective in NFκB-signaling by retroviral delivery of a dominant negative inhibitor of NFκB. The NFκB signal blockade resulted in the down-regulation of prometastatic matrix metalloproteinase 9, a urokinase-like plasminogen activator, and heparanase and reciprocal up-regulation of antimetastatic tissue inhibitors of matrix metalloproteinases 1 and 2 and plasminogen activator inhibitor 2. NFκB signal blockade did not affect tumor cell proliferation in vitro or in vivo but prevented intravasation of tumor cells in an in vivo chick chorioallantoic membrane model of metastasis as well as spontaneous metastasis in a murine model. These findings suggest that NFκB plays a central and specific role in the regulation of tumor metastasis and may be an important therapeutic target for development of antimetastatic cancer treatments.

Introduction

NFκB is a dimeric transcription factor that is involved in the regulation of genes associated with pathological processes such as inflammation and apoptosis (1, 2, 3). NFκB has been reported to selectively enhance the expression of proinflammatory cytokines such as tumor necrosis factor α, IL-1, and IL-6 as well as the expression of degradative enzymes such as MMPs. Thus NFκB has a pivotal role in sustaining chronic inflammatory reactions (4, 5). Many of the cellular events that occur during inflammation, such as vascular penetration by inflammatory cells, tissue degradation, and neoangiogenesis, also occur during tumor metastasis. Both NFκB (6, 7) and proinflammatory cytokine expression are increased in malignancies (8, 9), supporting the concept of metastasis as an analogue of inflammation. Whereas the role of NFκB in malignancies has been explored in relation to regulation of apoptosis, tumor progression, and responses to chemotherapy and ionizing radiation (2, 7, 10), its role specifically in the process of metastasis has not been examined.

MMP9 and its activator, uPA, are essential for basement membrane transgression by tumor cells and have recently been implicated as critical requirements for tumor cell intravasation and extravasation during metastasis (11). The fact that NFκB is a transcriptional activator of MMP9 and uPA (12, 13) supports a role for the NFκB signal transduction pathway in the metastatic process. Recent data also implicate another degradative enzyme, heparanase, as a critical element in tumor metastasis, allowing degradation of the heparan sulfate proteoglycan components of basement membranes (14, 15). MMP 9 and heparanase are known to colocalize in neutrophil tertiary granules, suggesting possible coordinate regulation of their production and extracellular delivery (16).

In resting cells, NFκB is sequestered in the cytoplasm by IkB proteins. After a variety of stimuli that activate the NFκB pathway, IkBα is phosphorylated and degraded, resulting in the release and nuclear translocation of NFκB. A dominant negative mutant form of IkBα (mIkBα) has been engineered that cannot be phosphorylated, and thus does not dissociate from NFκB in response to stimulation of the IkB kinase pathways (17, 18). Thus, mIkBα effectively blocks the NFκB signal transduction pathway. In the present study we examined the effect of NFκB signal blockade on tumor metastasis through its regulation of prometastatic and antimetastatic factors using mIkBα transfection in a murine lung alveolar carcinoma cell line (Line 1). These cells were infected with recombinant retroviruses containing the LacZ (control) or mIkBα gene, and stably expressing clones were obtained (17, 18). This report provides evidence for NFκB as a major controller of the metastatic phenotype through its reciprocal regulation of known prometastatic and antimetastatic genes.

Materials and Methods

Cell Culture Conditions, Cell Lines, and Transformation. Line 1 tumor cells were grown in DMEM supplemented with 10% fetal bovine serum and 0.1% penicillin/streptomycin and maintained at 37°C in an atmosphere of 5% CO2.

Retrovirus was produced using 293 kidney fibroblasts stably expressing the Moloney gag and pol under control of the cytomegalovirus promoter-enhancer as the packaging cell line. Twenty μg of pLXSN retroviral vector containing: (a) either mIkBα or the bacterial LacZ gene upstream of the neomycin resistance gene; (b) 5 μg of plasmid pSVSVG, containing the vesicular stomatitis virus envelope downstream of the cytomegalovirus promoter-enhancer; and (c) 1 μg of pRSV β-gal containing the bacterial LacZ gene downstream of the Rous sarcoma virus promoter-enhancer to aid in assessing transfection efficiency. Twenty μg of pRSV β-gal containing the bacterial LacZ gene downstream of the Rous sarcoma virus promoter-enhancer to aid in assessing transfection efficiency was used in the case where the retroviral vector contained mIkBα. Two weeks with 0.5 mg of G418 (Life Technologies, Inc., Gaithersburg, MD) as we harvested and selected for 2 g of pRSV β-gal containing the bacterial LacZ gene downstream of the Rous sarcoma virus promoter-enhancer to aid in assessing transfection efficiency. In the case where the retroviral vector contained mIkBα were transfected into the packaging cell line by CaPO4 precipitation.

Line 1 tumor cells were transformed by retroviral infection with recombinant virus-bearing mutant IkBα or the bacterial LacZ gene and selected for 2 weeks with 0.5 mg of G418 (Life Technologies, Inc., Gaithersburg, MD) as we have described previously (18). G418-resistant clones were pooled and constituted a heterogeneous population of mIkBα-expressing cells. This result excludes the confounding possibility of a random mutagenic effect of transgene insertion.

NFκB DNA-binding and Functional Activity. EMSA was used to assess NFκB DNA-binding by a method previously described. Briefly, 5 μg of nuclear extracts from cells were mixed with 2 μg of poly(dI-dC) and RNA-binding buffer (50 mM NaCl, 5 mM HEPES (pH 7.5), 5 mM EDTA, 0.5 mM EGTA, 30% Glycerol, and 1.25 μg BSA) in a total volume of 10 μl and incubated on ice for 20 min. NFκB and Oct1 oligonucleotides (Santa Cruz Biotech) were end-labeled by use of T4 polynucleotide kinase and [32P] cytosine triphosphate (DuPont NEN) and 20,000 cpm of [32P]-labeled oligonucleotide added to the binding reaction and incubated for 30 min at room temperature.
temperature. The complexes were then separated on 6% polyacrylamide gel under nondenaturing conditions at 125 V for 3 h. Gels were dried on 3-m Whatman papers and the DNA-protein complexes visualized by autoradiography. To identify NFκB subunits of the heterodimeric complex, EMSA supershifts were done by preincubating nuclear extracts with antibodies specific for each subunit (p50, p52, p65, c-rel antibody; Santa Cruz Biotech) for 10 min on ice.

Functional NFκB activity was assessed by cotransfecting 1.5 μg pNFκB-Luc (firefly luciferase driven by a TATA box with 5 NFκB sites in the enhancer element) and 2 ng of pRL-SV40 (renilla luciferase driven by SV-40) reporter constructs (Promega) complexed to 3 μg of Fugene transfection reagent (Boehringer Mannheim) in 100 μl of serum-free culture medium. Three hundred thousand cells seeded in six-well plates ( Falcon) in 3 ml of culture medium were transfected by adding 100 μl of the Fugene- gelatin complex and assaying for luciferase activity 24 h after using the Dual Luciferase Reporter Assay System (Promega) following the manufacturer’s instructions. The EMSA and heterologous promoter/reporter assays were carried out on cells with and without a 30-min stimulation with 20 nm PMA (Sigma) and 20 μg/ml ionomycin A23187 (Calbiochem), a commonly used regimen for the induction of NFκB in cultured cells through activation of the protein kinase C/mitogen-activated protein kinase pathway (3).

Assessment of Cell Proliferation. Thymidine incorporation assays were used to assess cell proliferation by a method described previously (19). Briefly, cell cultures of 20,000 cells in 24-well plates (Falcon) were labeled with 8μCi/ml [3 H]thymidine (New England Nuclear, Boston, MA) in the presence of 5% of unlabeled thymidine in culture medium. After 4 h of incubation, the DNA was precipitated and centrifuged and the pellet resuspended in NaOH. Radioactivity was determined by liquid scintillation spectrometry. All samples were assayed in quadruplicate.

Zymography. Zymography was used for the analysis of MMP and uPA activity secreted into the culture medium of cell lines as described previously. Briefly, a confluent 10-cm dish was maintained in 2 ml of serum-free medium (Centricon 10; Amicon, Stonehouse, Gloucester, United Kingdom) before analysis. Aliquots of conditioned medium were then separated on a 6% SDS-polyacrylamide gel copolymerized with 1 mg/ml type-1 gelatin from Zymogenetics prior to gel electrophoresis with formaldehyde and transferred to nylon membranes (Hybond N; Amersham); blots were hybridized overnight with 32 P-labeled RNA probes using the Quikhyb hybridization solution (Stratagene) at a temperature of 68°C, washed twice for 15 min at room temperature with 2 × SSC buffer and 0.1% (w/v) SDS wash solution and once for 30 min at 60°C with 0.1 × SSC buffer and 0.1% (w/v) SDS wash solution, and then overnight exposure to Biomax scientific imaging films (Kodak) Oligonucleotide primer pairs (5’-ACACGAGATGGCACAATGCT-3’ and 5’-AGAAC-CTTGGGATGACAGTT-3’) for plasminogen activator inhibitor 2 and 5’-ATGGAATGATGATCGGCGC-3’ and 5’-GCTTGAAGGGTGGACATT-3’) for β-actin were used for the reverse transcription of 100 μg of total cellular RNA and PCR amplification under the following conditions: (a) initial denaturation at 90°C for 30 s; (b) annealing at 60°C (PAI 2) and 55°C (β-actin) for 30 s; and (c) extension at 72°C for 1.30 min for 26 cycles.

Chick Embryo Metastasis Model. The CAM of 9-day-old chick embryos were exposed by creating a window on their eggshells. Tumor cells (1 × 106) were then seeded on the upper CAM and the eggs maintained at 37°C in an egg incubator for 48–50 h. The upper pole of the egg was then discarded and genomic DNA extracted from the chorioallantoic membrane of the lower CAM to detect metastatic tumor cells. Oligonucleotide primers pairs (5’-GGGCGAG- GTGGCACAACGCTTTATCCC-3’- and 5’-AGGCCACACAGAACAC- CCGTGTCC-3’) for mouse ALU were used to detect tumor cell DNA in the genomic DNA extract from the lower CAM. One μg of genomic DNA was PCR-amplified under the following conditions: (a) 95°C for 10 min; (b) 95°C for 30 s; (c) 58°C for 45 s; (d) 72°C for 45 s; and (d) 72°C for 10 min. Samples were then separated on a 2% agarose gel and the bands visualized by ethidium bromide staining.

In Vivo Pulmonary Metastasis Model. Thirty-six syngeneic BALB/cByJ mice (Jackson Laboratories, Bar Harbor, ME) were used to create 3 study groups of 12 each. All mice received a single i.m. injection of 0.05 ml of suspension containing 2 × 105 Line 1 cells (Parental, mIκBα, and LacZ control). At 25 days, the mice were euthanized by CO2 asphyxiation. All mice then had their tracheas canulated with a 21-gauge i.v. catheter under loupe magnification and their lungs insufflated with 2.5 ml of India ink. The lungs were dissected from the mice and fixed in Fekete’s solution (61% ethanol, 32% formaldehyde, and 4.3% acetic acid) and the primary tumors excised and fixed in 10% formalin. Lung metastasis, evident as white nodules on a black background, were counted and measured using a dissecting microscope and graduated measuring reticule.

Immunohistochemistry. Fixed and paraffin-embedded tissue specimens were deparaffinized, rehydrated, denatured for 5 min in a microwave oven and treated to block nonspecific staining. For this, sections were incubated for 30 min at 25°C with 1% H2O2 in methanol and then by blocking for 30 min with 5% normal goat serum in PBS. Polyclonal antibodies to mIκBα and p65 were added (1:500 dilution) overnight at 25°C, and then by incubation for 30 min at room temperature with biotinylated secondary goat-antirabbit IgG antibodies and 30 min with avidin-biotin peroxidase conjugate (1:50 dilution). Color was developed using Sigma Fast 3,3-diaminobenzidine tablet sets (Sigma) for 10 min and then counterstained with Mayer’s hematoxylin.

Results

Validation of NFκB Signal Blockade in Tumor Cell Lines. To confirm the efficacy of the mIκBα in blocking NFκB signaling, we analyzed the expression of IκBα and NFκB activity in these cells with and without stimulation by PMA and ionomycin (Fig. 1). Immunoblot analysis of cellular protein extracts showed that the mIκBα clones contain elevated levels of IκBα protein, which are insensitive to induced proteolysis that degrades the IκBα in the parental cells (Fig. 1A). Extracts of nuclear proteins from the transfectant and wild-type cells were analyzed by EMSA for NFκB binding activity with and without stimulation. As shown in Fig. 1B, NFκB-binding activity under basal conditions was decreased in the mutant (Lane 3) in comparison with the wild type (Lane 1), and the stimulation of
NFκB-binding activity by PMA/ionomycin was absent (Lane 4). Oct-1 DNA binding, which was used as a constitutively expressed control, was equivalent in all of these samples (Lanes 5–8). Supershift assays revealed that the proteins in the shifted complex are the p50 and p65 isoforms of NFκB in the wild-type cells (Lanes 9 and 11). The shift in p65 (Lane 11) appears as decreased NFκB-DNA binding complexes because of conformational changes in the p65 subunit induced by antibody binding, that preclude DNA binding. Additionally, we confirmed the differential transactivational activity of NFκB in the wild-type and mutant cell lines by heterologous promoter-reporter assays. The basal NFκB activity was 7-fold lower in the mIκB-expressing transfectants, and there was no inducible luciferase activity in these cells after PMA/ionomycin stimulation (Fig. 1C). These data confirm the specific suppression of NFκB in the mIκB-expressing Line 1 cells.

The Effect of NFκB Signal Blockade on Cell Proliferation and the Expression of Prometastatic and Antimetastatic Genes. Neither the transfection nor the NFκB suppression affected cell proliferation as determined by measurement of thymidine incorporation into DNA (Average ± SE of four experiments: Wild-type, 19,700 cpm ± 270; LacZ, 19,000 cpm ± 630; and mIκB, 19,373 cpm ± 385). Northern blots for MMP9 and heparanase demonstrated markedly lower levels of these transcripts in the mIκB transfectants as compared with the wild-type cells and the control LacZ transfectants. Inhibition of uPA protein expression by mIκB was also demonstrated by immunoblot analysis of conditioned medium from the cell lines using an antibody that recognizes the β-chain of uPA, common to the inactive and active uPA. (Fig. 2A). In contrast, Northern blot analysis of TIMPs 1 and 2, known antimetastatic regulators of MMP activity (20), demonstrated significantly higher levels of these transcripts in the mIκB-transfected cells compared with the wild-type. In the case of TIMP 2, only the 3.5 kb of mRNA was regulated in this manner, whereas the expression of the 1.0-kb transcript was not regulated. Similarly PAI 2, a known antimetastatic regulator of uPA and the MMP activation cascade (21) was elevated in the mIκB Line 1 cells as determined by RT-PCR (Fig. 2B).

Functional degradative activity, assessed by zymography of conditioned medium from the transfected and wild type Line 1 cells demonstrated significant suppression of uPA activity in samples from the mIκB transfectants (Fig. 3A). NFκB inhibition thus reciprocally regulates matrix-degrading enzymes and their inhibitors resulting in a significant suppression of functional degradative enzymatic activity known to be essential for tumor metastasis.

**In Vivo Evaluation of Tumor Cell Metastasis.** On the basis of these findings, we evaluated the effect of mIκB expression on tumor cell intravasation in vivo using a chick CAM model of metastasis (11). Wild type-, control LacZ-, or mIκB-transfected Line 1 cells were seeded on the CAM of day 9 old chick embryos, and metastasis to the opposite pole of the CAM was determined by PCR amplification of murine specific Alu sequences as described in “Materials and Methods.” The method demonstrated high sensitivity to detect small numbers of metastatic cells in control experiments (Fig. 3A). The wild-type and LacZ cell lines all exhibited metastasis; in contrast there was no detectable metastasis in any of the mIκB-expressing transfectants (Fig. 3B).

Finally, the effect of NFκB signal blockade was evaluated in an in vivo murine model more closely simulating natural cancer metastasis. In this model, the low class I HLA-DR expression in the Line 1 cells allows transplantation in syngeneic, immunologically normal BALB/c ByJ mice. After inoculation of tumor cells, all mice developed large
primary tumors at the injection site. Immunohistochemical evaluation of the primary tumors using antibodies that recognize the nuclear localization sequence of the p65 subunit of NFκB demonstrated suppression of activated (nuclear) p65 NFκB (Fig. 4A and 4B) and MMP9 expression (Fig. 4C and 4D) in the mIkB Line 1 tumors, confirming maintenance of the mIkB effect in vivo. The primary tumor weights were not statistically different among the wild type-, LacZ-, and mIkB-transfected Line 1 cells (Fig. 4E). Thus, as expected from the in vitro thymidine assays, there was no effect of the transfection or mIkB expression on cell proliferation in vivo. However, we observed a marked suppression of pulmonary metastatic nodules in the mice inoculated with the mIkB cells (Fig. 4E and 4F). Metastatic nodules, when present in the mIkB group, were smaller, and thus the total lung tumor burden was also dramatically decreased in this group (data not shown). The in vivo metastasis experiments have been reproduced with similar findings for several different pooled populations of different mIkB clones, minimizing the possibility of clonal variation accounting for the diminished metastatic capability of the mIkB cells.

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**Fig. 3.** NFκB signaling is required for tumor cell degradative activity and tumor cell intravasation in vivo. Standard gelatin and plasminogen activator zymography carried out on conditioned medium from the Line 1 cells (A) degradative enzymatic activity associated with tumor cell intravasation. Tumor cell lines [wild type (WT), LacZ, and mIkB] were assessed for the ability to intravasate using the chick embryo metastasis model. The sensitivity of this model was determined by limiting dilution of DNA from Line 1 cells in 1 μg of chick DNA (B). Tumor cell transmigration 50 h after implantation onto the CAM of 9-day-old chick embryos by Alu PCR (C) was quantitated for each cell line in triplicate (each Lane represents an independent experiment).

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**Fig. 4.** mIkB suppresses NFκB signaling and MMP9 expression in vivo and prevents tumor metastasis in a mouse model. The in vivo Line 1 lung metastasis assay was used to evaluate the effect of the mIkB on primary tumor growth and metastasis in mice. Wild-type (WT), LacZ, or mIkB Line 1 cells (2 × 10^5) were injected into the thighs of a Balb/cByJ mice. Twenty-five days later, the animals were sacrificed and their primary tumors (A–E) and lungs (F and G) were harvested. Immunohistochemistry of the primary tumors reveal the presence of activated NFκB/p65 and MMP9 (dark brown staining) in the parental tumors (A and C), and their absence in the mIkB Line 1 cells (B and D). No statistically significant difference was observed in the sizes and weights (in grams) of the primary tumors (E), however, representative lungs harvested and stained with India ink reveal multiple large tumor nodules (arrows) as a result of Line 1 cell metastasis. In contrast, the lungs of mice injected with the mIkB cells showed marked reduction in the number of tumor nodules per lung (F). The data (G) represents the average ± SE of 12 animals.
Discussion

The defining feature of malignant neoplasms which distinguishes them from benign tumors is the capacity for metastasis. Derangement of cellular proliferation is clearly an important feature of malignant tumors but occurs in benign tumors as well; thus metastasis is more specific to the clinical problem of cancer. Metastasis is an extremely complex process, involving cell motility, basement membrane transgression through enzymatic degradative activity, intravasation with subsequent endothelial attachment and extravasation at a remote organ or tissue site, angiogenesis and additional tumor growth at the remote site, and local invasion with additional extracellular matrix degradation. Transgression of basement membranes is facilitated by expression of MMPs, and the increased expression of a number of different MMPs has been associated with malignancy and metastasis (22). In particular, MMP9 and one of its indirect activators, uPA, have recently been demonstrated to be critical elements without which tumor cell intravasation cannot occur (11).

Another enzyme critical for metastasis, the mammalian heparanase, has been recently cloned and may represent the dominant endoglycosidase activity in mammalian tissues (14, 15). Enhanced expression is found in metastatic malignant tumors, and transfection of heparanase into nonmetastatic cell lines enhances metastatic capability. Furthermore, pharmacological inhibition of heparanase has been shown to have a dramatic effect on reducing metastasis in a rodent model (23). In the present study, we found that heparanase was expressed in the Line 1 cells, and its expression was significantly inhibited by blockade of the NFκB signal transduction pathway. This is the first demonstration of heparanase regulation by NFκB, which may contribute to the efficacy of the mIκB transfection in suppressing metastasis. The reciprocal up-regulation of antimetastatic TIMPs 1 and 2 and PAI 2 by NFκB, may further enhance the antimetastatic effects of NFκB signal blockade. In a previous report, NFκB up-regulated MMPs and proinflammatory cytokines in inflammatory cells but did not up-regulate anti-inflammatory cytokines or MMP inhibitors (4). However, the present study represents the first identification of a reciprocal regulation of MMPs and TIMPs by NFκB in tumor cells. The NFκB p50/p65 heterodimer identified as the DNA binding complex in the Line 1 tumor cells is a transcriptional activator, although the effect exerted on antimetastatic factors appears to be inhibitory. Therefore, the reciprocal suppression of antimetastatic genes may be an indirect effect mediated by an as-yet unidentified other factor(s). This is further supported by the absence of classic NFκB-DNA binding sites in the promoters of TIMPs 1, 2 and PAI 2.

NFκB proteins have been implicated as playing a role in cellular transformation by either providing continued positive growth stimuli such as that mediated by cytokines, or through inhibition of apoptotic pathways (24). The role of NFκB in regulating tumor growth remains unclear because of inconsistent findings among different cell types, and may be cell-specific. In the present study, inhibition of NFκB did not influence tumor cell growth in vitro or in vivo with the Line 1 cell model, and the observed effects on phenotype were more specific to genes involved in metastasis. NFκB is known to stimulate the expression of a number of proinflammatory cytokines such as tumor necrosis factor, IL-6, and IL-1, as well as the degradative enzymes MMP 9 and uPA that may enhance tumor metastasis. Because proinflammatory cytokines can, in turn, stimulate NFκB activation, a positive feedback loop could occur to sustain enhanced levels of NFκB activity, cytokine production, and tumor cell degradative activity, thus sustaining the metastatic phenotype (25). Through local cytokine production, tumor cells may also recruit host stromal cells to participate in the metastatic cascade. The fact that NFκB inhibition markedly suppressed, but did not eliminate, metastasis in the in vivo murine model may reflect production of some of the essential proteins for metastasis by host stromal cells stimulated by unknown mediators from the tumor cells or local inflammatory processes. Alternately, it is possible that the mIκB expression in some of the tumor cells could be eliminated by “in vivo promoter shut-off” of the long terminal repeat. Consequently, these cells could revert to their metastatic phenotype. Nonetheless, the lack of an effect of NFκB suppression on cell proliferation in vitro, and of tumor growth in vivo, indicates a relatively specific role of NFκB in the metastatic aspects of malignancy rather than at the level of growth regulation. Data from the chick CAM model of metastasis suggest that tumor cells defective in NFκB signaling are incapable of intravasation, consistent with the observed suppression of metastatic nodule development in the lungs of the mice inoculated with the mIκB-expressing cells.

In conclusion, the finding that a single factor (NFκB) can reciprocally regulate the expression of several different prometastatic and antimetastatic genes implicates NFκB as an important regulator of the metastatic phenotype and implies that dysregulation of NFκB in tumor cells can have a dramatic impact on metastatic potential. In other experiments, we have found similar effects of NFκB signal blockade on prometastatic and antimetastatic gene expression in multiple clones of human prostatic carcinoma (PC3) and breast carcinoma cells (MDA-MB 231; data not shown), suggesting that our findings are not specific to either particular clones or cell types, and that this regulatory pathway may play a more widespread role in the control of metastasis. Our findings suggest that NFκB may be an important therapeutic target for the development of future antimetastatic cancer treatments.

References

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