

Fully Human Anti-Interleukin 8 Antibody Inhibits Tumor Growth in Orthotopic Bladder Cancer Xenografts via Down-Regulation of Matrix Metalloproteases and Nuclear Factor- κ B¹

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ABSTRACT

Purpose: We previously demonstrated that overexpression of interleukin 8 (IL-8) in human transitional cell carcinoma (TCC) resulted in increased tumorigenicity and metastasis. This increase in tumor growth and metastasis can be attributed to the up-regulation in the expression and activity of the metalloproteinases MMP-2 and MMP-9.

Experimental Design: To investigate whether targeting IL-8 with a fully human anti-IL-8 antibody (ABX-IL8) could be a potential therapeutic strategy for controlling TCC growth, we studied its effects on TCC growth *in vitro* and in an *in vivo* mouse model. Human TCC cell lines 253J B-V and UM UC3 (high IL-8 producers), 253J (low IL-8), and 253J transfected with the IL-8 gene (high producer) were used.

Results: ABX-IL8 had no effect on TCC cell proliferation *in vitro*. However, in the orthotopic nude mouse model, after 4 weeks of treatment (100 μ g/week, *i.p.*), a significant decrease in tumor growth of both cell lines was observed. IL-8 blockade by ABX-IL8 significantly inhibited the expression, activity, and transcription of MMP-2 and MMP-9, resulting in decreased invasion through reconstituted basement membrane *in vitro*. The down-regulation of MMP-2 and MMP-9 in these cells could be explained by the modulation of nuclear factor- κ B expression and transcriptional activity by ABX-IL8.

Conclusions: Our data point to the potential use of ABX-IL8 as a modality to treat bladder cancer and other solid tumors, either alone or in combination with conventional chemotherapy or other antitumor agents.

INTRODUCTION

TCC⁴ of the bladder is the fifth most commonly diagnosed malignancy in the United States. In 2002, an estimated 56,500 new cases will be diagnosed, resulting in an estimated 12,600 deaths (1). Radical cystectomy with urinary diversion is currently the standard treatment for patients with muscle-invasive tumors of the bladder. For patients with locally advanced or metastatic disease, systemic cytotoxic chemotherapy is the only viable option (2–5). Over the past two decades, the advances made in chemotherapy for TCC have resulted in a more favorable side effects profile, but no significant improvement in the overall survival rate has been noted (6). Despite the reported initial response rates of 20–40%, most patients with invasive or advanced bladder cancer die from progression of their disease. Thus, a continued search for more effective therapeutic agents against this aggressive disease is mandatory.

The process of tumor invasion and metastasis is highly regulated and involves multiple tumor-host interactions (7–9). Angiogenesis, which is essential for tumor growth and metastasis (9–11), is regulated through a fine balance between stimulatory (VEGF, basic fibroblast growth factor, and IL-8) and inhibitory (IFN, endostatin, angiostatin, and thrombospondin) factors produced by the tumor or its microenvironment (12). Bladder tumors produce high levels of a several of factors that promote tumor growth and metastasis, including VEGF (13, 14), basic fibroblast growth factor (15, 16), and IL-8 (17, 18).

IL-8, which was originally described as a leukocyte chemoattractant (19, 20), was subsequently found to possess mitogenic and angiogenic properties (21–23). Overexpression of IL-8 is associated with increasing tumor stage, disease progression, and recurrence in human melanoma, breast, gastric, ovarian, and prostate cancers (24). Furthermore, there is a direct correlation between high levels of IL-8 and tumor angiogenesis, progression, and metastasis in nude mouse xenograft models of human melanoma, ovarian, pancreatic, and prostate cancer cells (24, 25). In addition, Inoue *et al.* (18) who transfected the nontumorigenic human TCC cell line 253 J-P with an IL-8

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⁴ The abbreviations used are: TCC, transitional cell carcinoma; MMP, matrix metalloprotease; EGF, vascular endothelial growth factor; IL-8, interleukin-8; NF- κ B, nuclear factor- κ B; FBS, fetal bovine serum; CMEM, complete minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ISH, *in situ* hybridization; TBS, Tris-buffered saline; EMSA, electrophoretic mobility shift assay; EGFR, epidermal growth factor receptor.

expression vector found that this produced an increase in invasion, tumorigenicity, and metastases. Similarly, an antisense IL-8 transfection of a highly metastatic bladder cancer cell line resulted in a significant decrease in angiogenesis, tumor growth, and metastases (18). These effects of IL-8, confirmed in human melanoma, pancreatic, gastric, and glioma cells (24, 25), have established IL-8 as a valid target for therapy.

IL-8 is thought to exert its effects through an autocrine/paracrine loop (24–27) by stimulating tumor and stromal cells to express angiogenesis-related factors, thus promoting endothelial cell proliferation, tumor growth, and metastasis. Indeed, we have recently demonstrated that IL-8 may exert its angiogenic activity by up-regulating of MMP-2 and MMP-9 in tumor cells (18, 25). The activation of MMP-2/9 by IL-8 can, in turn, enhance the invasion of host stroma by tumor cells, increase angiogenesis, and, hence, metastasis. In addition, IL-8 has been shown recently to act directly on vascular endothelial cells to promote survival (28). Thus, multiple mechanisms seem to be involved in IL-8 action, including direct effects on tumor and vascular endothelial cell proliferation, angiogenesis, and migration. On the basis of these observations, we hypothesize that disruption of this autocrine/paracrine loop should decrease tumor invasion and growth by TCC. In this study, we used a fully human anti-IL-8 antibody, ABX-IL8 (Abgenix, Inc., Fremont, CA) to neutralize the IL-8 secreted by human TCC cells, thus blocking its autocrine/paracrine feedback mechanism. We found that ABX-IL8 inhibited the growth of bladder cancer cells implanted into the bladder of nude mice. The decrease in tumor growth was attributable to the down-regulation in the expression and activity of MMP-2 and MMP-9. ABX-IL8 also regulated the expression and the transcriptional activity of NF- κ B. These results suggest that ABX-IL8 could be beneficial in treating bladder cancer patients, either alone or in combination with other chemotherapeutic or antiangiogenic agents.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Human TCC UMUC-3 and 253J B-V cell lines (highly tumorigenic and highly metastatic) and the 253J-P line (poorly tumorigenic and nonmetastatic) were grown as monolayer cultures in modified Eagle's MEM supplemented with 10% FBS, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin (CMEM) (29).

ABX-IL-8. ABX-IL8 is a human IgG2 monoclonal antibody directed against human IL-8 that was generated using Abgenix's proprietary Xenomouse mice. The Xenomouse technology is one in which the murine heavy and light chain loci have been inactivated and subsequently replaced with a majority of human heavy- and κ light-chain immunoglobulin loci. When immunized, these mice produce fully human antibodies. The mice used for this immunization contained only the human IgG2 heavy-chain sequences and human κ light chain. ABX-IL8 binds to human IL-8 with high affinity ($K_d = 2 \times 10^{10}$ M) and fails to cross-react with a panel of closely related chemokines. ABX-IL8 blocks the binding of IL-8 to its receptors and inhibits IL-8-dependent neutrophil activation, migration, and degranulation (30). Chemopure human IgG control antibody was purchased from Jackson ImmunoResearch (West Grove, PA) and

was used at the same concentration as ABX-IL8 in all experiments.

Cell Proliferation Assay. A total of 5×10^3 253J B-V and UMUC-3 cells was plated in 96-well plate and then treated with 1–100 μ g/ml ABX-IL8, control IgG, or CMEM for 1–7 days. A MTT assay was performed daily to determine the relative cell numbers based on the conversion of MTT to formazan in viable cells. MTT (40 μ g/ml) was added to each well and incubated for 2 h. The medium was then removed, and 100 μ l of DMSO were added to lyse the cells and solubilize the formazan. A standard microplate reader was used to determine the optical density.

Collagenase Activity. Type IV collagenase (MMP-2 and MMP-9) activity was determined using gelatin-impregnated SDS-PAGE gels, as previously described (31) with minor modifications. The metastatic cells 253J B-V (5×10^4) and UMUC-3 (5×10^4) were plated in 6-well plates and treated with 100 μ g/ml ABX-IL8, 100 μ g/ml IgG, or CMEM for 3 days. On day 3, CMEM was removed and replaced with serum-free medium, containing the antibodies, overnight. The supernatant was collected, the volume was adjusted for cell number, and separated on gelatin-impregnated (1 mg/ml; Difco, Detroit, MI) SDS/8% polyacrylamide gels under non-reducing conditions, followed by 30 min of washing twice, in 2.5% Triton X-100 (BDH, Poole, United Kingdom). The gels were then incubated overnight at 37°C in 50 mM Tris, 0.2 M NaCl, 5 mM CaCl₂, and 0.02% Brij 35 (w/v) at pH 7.6. At the end of the incubation, the gels were stained with 0.5% Coomassie G 250 (Bio-Rad, Richmond, CA) in methanol/acetic acid/H₂O (30:10:60) for 15–30 min. The gels were destained with a methanol/acetic acid/H₂O (30:10:60) solution and dried in a gel drier for storage and densitometry. The intensity of the various bands was determined on a computerized densitometer type 300A (Molecular Dynamics).

Invasion Assay. Cells that express high levels of IL-8 (253J B-V, UMUC-3, and 253J S-IL8) were plated in 6-well plates (5×10^3) and treated with ABX-IL8 (100 μ g/ml), control IgG (100 μ g/ml), or CMEM for 3–5 days. Cells were released from the plates by a brief exposure to Trypsin-EDTA (Life Technologies, Inc.), centrifuged, counted, and resuspended in serum-free medium containing appropriate antibodies to a final concentration of 5×10^4 cells/ml. Biocoat Matrigel invasion chambers with polyvinylpyrrolidone-free polycarbonate filters (8-mm pore size; Becton-Dickinson, Franklin Lakes, NJ) were primed according to the manufacturer's directions. Then 500 μ l of cell suspensions (containing 2.5×10^4 cells) were placed in the upper compartment of the Boyden chamber. Ten percent FBS-CMEM was placed in the lower compartment as a source of chemoattractant. After incubation for 12 h at 37°C, the cells on the lower surface of the filter were stained with Diff-Quick (American Scientific Products, McGraw Park, IL) and quantified with an image analyzer (Optomax V) attached to an Olympus CK2 microscope. The data were expressed as the average number of cells from five fields that had migrated to the lower surface of the filter in each of three experiments performed \pm SD (32).

In Situ mRNA Hybridization Analysis. 253J B-V, UMUC-3, and 253J S-IL8 cells (5×10^3) were plated in 6-well plates and treated with ABX-IL8 (100 μ g/ml), control

IgG ($\mu\text{g/ml}$), or CMEM for 2 days. Cells were released from the plates by brief exposure to Trypsin-EDTA (Life Technologies, Inc.), plated on silane-treated Probe On slides (Fisher Scientific, Pittsburgh, PA) in triplicate, and treated for 3 days. Specific AS oligonucleotide DNA probes complementary to the mRNA transcripts were designed based on the MMP-2 and MMP-9 cDNA sequence. The specificity of the oligonucleotide sequence was initially determined by a Gene Bank European Molecular Biology Library database search with the help of the Genetics Computer Group sequence analysis program (Genetics Computer Group, Madison, WI) based on the Fast A algorithm.

In situ mRNA hybridization was performed as previously described, with minor modifications (33, 34). ISH was performed using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 35). The slides (three from each group) were placed in Microprobe slide holder, and the probe was hybridized for 45 min at 45°C. The samples were then washed three times with $2\times$ SSC for 2 min at 45°C, incubated with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed in 50 mM Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer for 1 min, and incubated with a chromogen substrate for 15 min at 45°C. To enhance a weak reaction, samples were incubated a second time with fresh chromogen substrate. A red stain indicated a positive reaction. To control for the endogenous alkaline phosphatase, the sample was treated in the absence of the biotinylated probe using chromogen alone.

Stained sections were examined under Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip, charge-coupled device color camera (model DXC-969 MD; Sony Corp.). The images were analyzed using the Optimas image analysis software (version 4.10; Bothell, WA). The slides were prescreened by one of the investigators to determine the range in the staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue mode of the color camera. All subsequent images were quantified during one sitting based on this threshold by two independent investigators in blinded manner. The samples were not counterstained; therefore, the optical density was attributable solely to the product of the ISH reaction. Five different fields in each slide were quantified to derive an average value. The intensity of staining was corrected for the integrated optical density of poly d(T) to account for mRNA integrity. The results were presented as mean optical density \pm SD.

Promoter Analysis and Dual Luciferase Assay. The luciferase reporter gene driven by either MMP-2 or MMP-9 promoter was used. The pGL3-MMP-2 (or pGL3-MMP-9) is a pGL3-basic reporter construct containing a full-length firefly luciferase gene under the control of an MMP-2 (or MMP-9) promoter. Metastatic cells, 253J B-V and UMUC-3, were treated with ABX-IL8 (100 $\mu\text{g/ml}$), control IgG (100 $\mu\text{g/ml}$), or CMEM for 3 days. The cells were then transfected with 10 ng of pB-actin-RL reporter containing a full-length *Renilla* luciferase gene (Promega Corp., Madison, WI) under the control of the human β -actin promoter and 2 μg of plasmid DNA consisting of either luciferase basic vector,

SV40-positive control, or a full-length MMP-2 (or MMP-9) promoter vector (36), using 10 μl of Lipofectin Reagent (Life Technologies, Inc., Rockville, MD). The medium was changed after 24 h, and the cells were treated for another 24 h with the appropriate antibodies. The cells were harvested in passive lysis buffer (Promega Corp.). As reported previously, the activities of firefly luciferase and *Renilla* luciferase were quantified using the dual luciferase assay system (Promega Corp.) and Ascent Lumiskan plate reader (37, 38).

Western Blot Analysis For NF- κ B. Cells were treated with ABX-IL8 (100 $\mu\text{g/ml}$), control IgG (100 $\mu\text{g/ml}$), or CMEM for 3 days. Cell nuclei were isolated as previously described (37, 38), and isolated nuclei were lysed using Triton X-100 lysis buffer [150 mM NaCl, 25 mM Tris (pH 7.5), 1% w/v Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g/ml}$ trypsin inhibitor, 20 mM leupeptin, and 0.15 units/ml aprotinin] on ice for 20 min. The soluble protein in the lysates was then separated by centrifugation at 14,000 rpm for 20 min at 4°C. The protein concentration was determined by the Bio-Rad protein assay reagent (Bio-Rad Laboratories), and the protein was stored at -70°C . Before loading, protein samples were boiled in sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% w/v glycerol, 100 mM DTT, 2.3% SDS, and 0.002% bromophenol blue] for 2–5 min and cooled on ice for 5–10 min. Samples (30 μg) were loaded and separated on 10% SDS-PAGE at 150 V for 60 min in electrophoresis buffer [25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 0.1% SDS]. Proteins in the gels were electrophoretically transferred onto immobilon-P transfer membrane (Millipore) in $1\times$ transfer buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol) at 100 V for 2 h at 4°C. The membranes were washed in blocking buffer [TBS 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 3% BSA, 1% OVA, and 0.02% NaN_3] for 2 h at room temperature with shaking and then rinsed once briefly with TTBS (99.9% TBS, 0.1% Tween 20). The membrane was incubated in 1:500 anti-NF- κ B polyclonal rabbit antibody [NF- κ B p50(H-119); Santa Cruz Biotechnology, Santa Cruz, CA] overnight at 4°C. The membranes were rinsed twice briefly with TTBS and washed three times with TBS at room temperature and then incubated with a second antibody (antirabbit immunoglobulin, horseradish peroxidase-linked F(ab)2 fragment from mouse) at dilution of 1:5000 for 1 h at room temperature with shaking. The membranes were rinsed twice briefly and washed three times with TBS at room temperature with shaking. The probed proteins were detected with Amersham enhanced chemiluminescence system according to the manufacturer's instructions.

EMSA for NF- κ B Nuclear Binding Activity. Nuclear protein extracts were prepared as described above from cells treated with ABX-IL8 and from untreated (CMEM) control cells. The sequence of the NF- κ B oligonucleotide probe was 5'-AGTTGAGGGACTTCCAGGC-3'. EMSA was performed as previously described with minor modifications (38). Five μg of nuclear extract protein and 30,000 cpm of end-labeled double-stranded DNA probe were then added to the mixture. The binding reaction was allowed to proceed for 25 min at 22°C. For supershift reactions, extracts were preincubated with anti-p65, anti-p50, or anti-cRel antibodies [(NF- κ B p50 (sc-7178); NF- κ B p65(sc-109); c-Rel (sc-272); Santa Cruz

Table 1 Tumorigenicity of UMUC-3 and 253J B-V cells implanted orthotopically in the bladder wall of nude mice

Values are the average of two representative experiments of four. *P*s were calculated using the Mann-Whitney U test.

Group	Median tumor weight (range), mg			
	UMUC-3 ^a	Incidence	253J B-V ^b	Incidence
Untreated Control	853 (227–1301)	10/10	194 (114–269)	10/10
Control IgG	534 (182–979)	10/10	179 (51–296)	10/10
ABX-IL8	380 ^c (151–762)	10/10	145 (56–195)	10/10

^a UMUC-3 cells (5×10^4) were implanted in the bladder wall of athymic nude mice (10 mice/group) and treated with ABX-IL8 or control IgG, 1000 μ g, i.p., once/week for 4 weeks.

^b 253J B-V cells (25×10^4) were implanted and treated as above with ABX-IL8 or control IgG, 100 μ g, i.p., three/week for 3 weeks.

^c $P < 0.02$ versus untreated controls.

^d $P < 0.05$.

Biotechnology] for 20 min on ice. Protein-DNA complexes were resolved on a 6% nondenaturing polyacrylamide gel. After which, the gels were dried and exposed to X-ray film at -80°C overnight.

Animals. Male 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 8 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH.

Orthotopic Implantation of Tumor Cells. Cultured 253J B-V and UMUC-3 cells (60–70% confluent) were prepared for injection as described previously (29). Mice were anesthetized with methoxyflurane. For orthotopic implantation, a lower midline incision was made, and viable tumor cells, 25×10^4 (253J B-V) or 25×10^4 (UMUC-3) in 0.05 ml of HBSS, were implanted into the bladder wall. The formation of a bulla indicated a satisfactory injection. The bladder was returned to the abdominal cavity, and the abdominal wall was closed with a single layer of metal clips. Ten mice/group were used to study the effect of ABX-IL8 on the tumorigenicity of UMUC-3 and 253J B-V cells (Table 1).

In Vivo Therapy of Orthotopic Human TCC Tumors. Four days after tumor implantation, animals were treated with control IgG or ABX-IL8 (100 μ g i.p., Q optical density for 3 weeks for 253J B-V tumors, and 1000 μ g, i.p., once/week for 4 weeks for UMUC-3 tumors). At the end of the treatment period, the mice were killed by CO_2 inhalation and cervical dislocation. The primary tumors were removed, weighed, and fixed in 10% buffered formalin for tissue analysis.

Statistical Analysis. The *in vitro* data were analyzed for significance by the student's *t* test (two-tailed) and the *in vivo* data were analyzed by the Mann-Whitney *U* test.

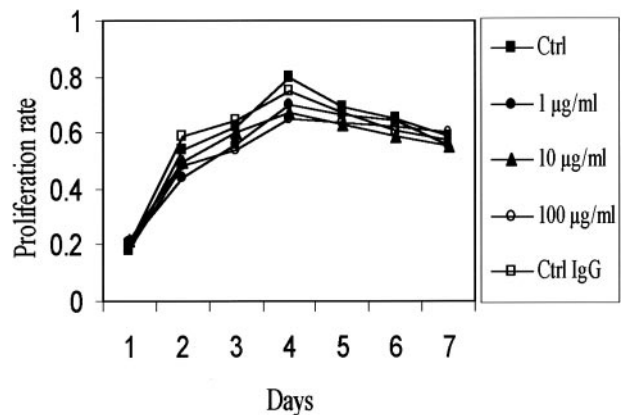


Fig. 1 Effect of ABX-IL8 on proliferation rate of TCC of bladder cells. 253J B-V cells were treated with increasing concentrations of ABX-IL8 and control IgG for 7 days. The cell proliferation rate was determined by the MTT assay. No significant difference in the proliferation of these cells was detected. This is one representative experiment of three.

RESULTS

Suppression of TCC Tumor Growth by ABX-IL8.

ABX-IL8 acts as a neutralizing antibody to IL-8. It binds to human IL-8 with high affinity and blocks the binding of IL-8 to its receptors. In the first set of experiments, we determined the effect of ABX-IL8 on tumor growth of human TCC in nude mice. To determine whether blocking IL-8 affects tumor growth, we implanted 253J B-V and UMUC-3 cells into the bladder wall of athymic nude mice. The tumors were allowed to establish and then treated with ABX-IL8 or control IgG. The animals with UMUC-3 tumors were treated with 1 mg i.p. once/week and those injected with 253J B-V were treated with 100 μ g i.p. three times/week. Tumors were observed in all mice injected (see "Incidence," Table 1). However, although some inhibitory effect was observed with the control of IgG antibody, there was a significant suppression in the growth of the orthotopically implanted tumors for both 253J B-V ($P < 0.05$) and UMUC-3 ($P < 0.02$). The inhibitory effect of ABX-IL8 was more pronounced in the UMUC-3 tumors, which were treated with 1 mg/week (Table 1).

Effect of ABX-IL8 on Cell Proliferation *in Vitro*. IL-8 has previously been shown to be an autocrine growth factor in various tumor cells. Therefore, we tested whether ABX-IL8 had a direct effect on cell growth and proliferation of both TCC cell lines *in vitro*. To that end, we treated 253J B-V cells with ABX-IL8 and control IgG. The cells were treated with increasing doses of ABX-IL8 (1–100 μ g/ml) or IgG for 7 days. An MTT assay was performed daily to determine growth rate. Treatment with ABX-IL8 did not have a direct effect on cell proliferation rate *in vitro*, despite increasing doses (Fig. 1). This experiment was performed with several concentrations of FBS (0.5, 1, 5, and 10%) with the same outcome. The same results were obtained for UMUC-3 cells (data not shown). These data suggest that the inhibition in tumor growth *in vivo* was not because of differences in cell division time.

Down-Regulation of MMP-2/MMP-9 Activity in TCC by ABX-IL8 *in Vitro*. The growth and metastatic potential of tumor cells depend on proper vascularization of the tumor and

Fig. 2 Collagenase activity of MMP-2 and MMP-9. 253J-P cells, 253J-S-IL8, 253J B-V, and UMUC-3 cells were treated with ABX-IL8 and IgG for 3 days. The conditioned media (without FBS) was collected, and gelatin zymography was performed. This is one representative experiment of three.

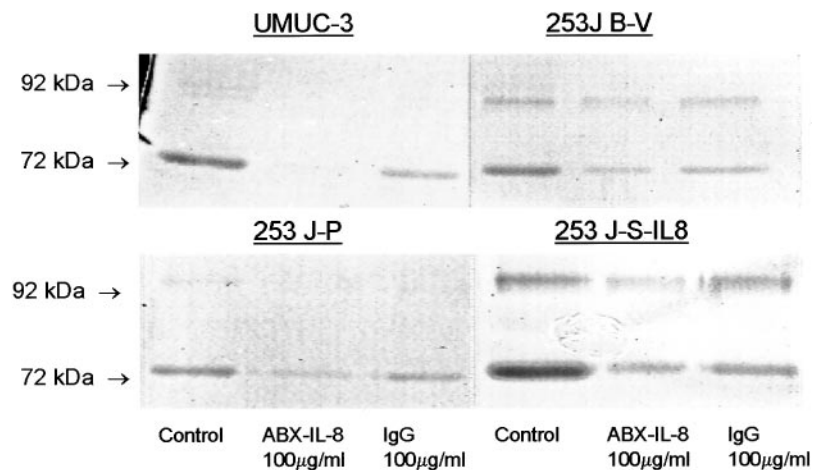


Table 2 Expression of MMP-2 and MMP-9 mRNA *in vitro* after treatment with ABX-IL8 and control IgG

	Mean optical density \pm SD ^a			
	MMP-9		MMP-2	
	Control IgG	ABX-IL8	Control IgG	ABX-IL8
253 J B-V	3.8 \pm 0.77	2.5 \pm 0.40 ^b	3.9 \pm 0.46	2.9 \pm 0.44 ^b
253 J S-IL8	4.2 \pm 0.46	3.8 \pm 0.46 ^b	4.4 \pm 0.37	3.6 \pm 0.39 ^b
UMUC-3	N/A ^c	N/A	3.7 \pm 0.42	3.1 \pm 0.38 ^b

^a Colorimetric *in situ* hybridization was performed in triplicates, and the intensity of color reaction was quantified in 5–10 areas in at least five high power fields/slide. This is one representative experiment of two.

^b All *P*s < 0.01, when compared with controls.

^c N/A, not available as MMP-9 expressed at very low levels in UMUC-3 cells.

their ability to degrade type IV collagen. We recently demonstrated that IL-8 exerts its angiogenic activity through the induction of MMP-2/MMP-9 in melanoma and bladder cancer cells (18, 25). Activation of MMP-2/MMP-9 by bladder cancer cells may provide a mechanism for the increase in their tumorigenicity. We, therefore, tested whether neutralizing IL-8 by ABX-IL8 had any effect on MMP-2/MMP-9 activity by TCC. There was an increase in MMP-2 and MMP-9 activity in 253J-P cells transfected with the IL-8 gene (253J-S-IL8; Fig. 2). We found, however, that treatment with ABX-IL8 resulted in a 5–8-fold decrease in MMP-2 activity in UMUC-3 cells and 2–3-fold activity of MMP-2 and MMP-9 in 253JB-V and 253J-S-IL8 cells, respectively (Fig. 2).

Decreased Expression of MMP-2/MMP-9 in TCC Treated with ABX-IL8. To determine whether ABX-IL8 suppressed the expression of MMP-2/MMP-9 by bladder cancer cells, a colorimetric *in situ* mRNA hybridization assay was performed. Results summarized in Table 2 demonstrate a moderate but significant fold reduction in the mRNA expression of MMP-2 in all cells treated *in vitro* with ABX-IL8 as compared with control-IgG treated cells and in MMP-9 mRNA transcript in 253JB-V and 253J-S-IL8 cells.

Table 3 Invasion through Matrigel-coated membrane by bladder cancer cells after treatment with ABX-IL8

Treatment	Mean number of invading cells \pm SD, per membrane ^a		
	253J B-V	UMUC-3	253J S-IL8
Control	125 \pm 44	135 \pm 49	113 \pm 43
IgG (100 μ g/ml)	90 \pm 50	115 \pm 40	97 \pm 37
ABX-IL8 (100 μ g/ml)	26 \pm 9 ^b	37 \pm 10 ^b	50 \pm 20 ^b

^a A total of 5×10^3 cells was plated in the invasion chambers after treatment with ABX-IL8 or IgG for 3 days, suspended in serum-free media. Cells that had invaded and migrated were counted on the lower surface of the membrane. Experiment performed twice in triplicates.

^b All *P*s < 0.01.

Suppression of TCC Invasion by ABX-IL8. We next analyzed whether the decreased activity and expression of MMP-2 and MMP-9 in ABX-IL8-treated cells correlated with their ability to invade through the basement membrane, an important component in the process of invasion. To that end, UMUC-3 and 253J-B-V cells that had treated with 100 μ g/ml ABX-IL8 or control IgG for 3 days were subsequently placed in the upper compartment of an invasion chamber in the presence of 100 μ g/ml ABX-IL8 or control IgG. After 22 h of incubation, the cells on the lower surface of the filter were counted. As shown in Table 3, cells treated with ABX-IL8 exhibited a significant decrease of 2.3 to 4.8 (*P* < 0.01) in invasion through Matrigel-coated filter, when compared with IgG-treated or untreated cells.

Suppression of MMP-2/MMP-9 Transcription by ABX-IL8. To examine the effect of ABX-IL8 on MMP-2 and MMP-9 transcription, the MMP-2 and MMP-9 promoters were linked upstream of a luciferase reporter gene and transfected into 253J-B-V and UM-UC3 cells which were then treated with ABX-IL8. Cells were lysed and dual luciferase assay was performed to determine the activity of MMP-2 and MMP-9 promoters. Consistent with a decreased MMP-2 and MMP-9 collagenase activity, there was a significant decrease in the MMP-2 promoter activity in both cell lines after treatment with ABX-

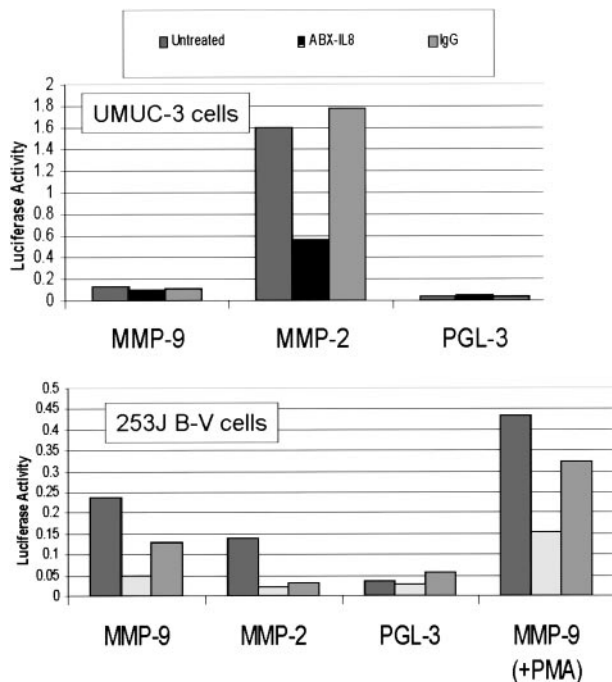


Fig. 3 Promoter activity. 253J B-V and UM-UC3 cells were treated with ABX-IL8 (100 $\mu\text{g}/\text{ml}$) for 4 days and then transiently transfected with PGL3-basic reporter, containing firefly luciferase gene, under the control of MMP-9 or MMP-2 promoters. Dual luciferase assay was used to measure the promoter activity. There was a 3–6-fold decrease in the MMP-2 and MMP-9 promoter activity. The down-regulation of MMP-9 promoter was evident for both the constitutive, as well as the inducible (with phorbol 12-myristate 13-acetate) MMP-9 activity. This is one representative experiment of three.

IL8. After treatment with ABX-IL8, 253J-B-V cells exhibited a decrease in the constitutive, as well as inducible (with phorbol 12-myristate 13-acetate) MMP-9 promoter activity (Fig. 3). These results suggest that IL-8 may directly regulate MMP-2 and MMP-9 expression at the transcriptional level and that blocking IL-8 by ABX-IL8 suppressed MMP-2/MMP-9 expression in bladder cancer cells.

Suppression of NF- κ B Expression by ABX-IL8. IL-8 regulates the expression of MMPs by modulating the expression and transcriptional activity of NF- κ B. To determine whether ABX-IL8 affected the level of NF- κ B, nuclear extracts were isolated from 253J-B-V cells after treatment with ABX-IL8 or control IgG. Western blot analysis was performed for NF- κ B (p50), and fold reduction was calculated after normalization to β -actin expression. There was a 2–3-fold decrease in the level of NF- κ B after treatment with ABX-IL8 when compared with control IgG (Fig. 4). The same results were obtained with UMUC-3 cells (data not shown).

EMSA Gel for NF- κ B Activity. To determine whether the down-regulation in NF- κ B expression in ABX-IL8-treated cells is reflected in its ability to bind to its target DNA, nuclear extracts of 253J-B-V cells treated with ABX-IL8 or control IgG were extracted and analyzed by EMSA. There was a 3-fold reduction in NF- κ B binding activity after treatment with ABX-IL8 (Fig. 5, Lane 7) as compared with control IgG (Fig. 5, Lane

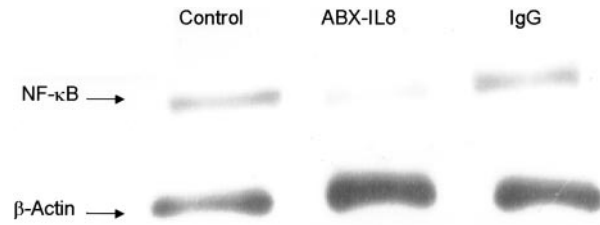


Fig. 4 Western blot analysis of NF- κ B (p50). 253J B-V cells were treated with ABX-IL8, 100 $\mu\text{g}/\text{ml}$ or control IgG, for 3 days. Nuclear protein extracts were obtained from control and treated cells, and Western blot analysis was performed using β -actin as protein loading control. There was a significant reduction in NF- κ B expression after ABX-IL8 treatment when compared with untreated or IgG control group.

2). The upper band of protein-DNA complex was supershifted with anti-p50, but not with anti-p65, for both ABX-IL8-treated (Fig. 5, Lane 8), and control IgG-treated cells (Fig. 5, Lane 5). Collectively, these results demonstrated that treatment of bladder cancer cells with ABX-IL8 resulted in down-regulation of NF- κ B expression and activity.

DISCUSSION

TCC of the bladder is partially sensitive to conventional chemotherapeutic agents; however, the responses are often short-lived because chemoresistance can develop rapidly. Despite the reported initial response rates of 20–50%, most patients with advanced or metastatic TCC of the bladder die from progression of their disease, with a median survival of <2 years. Therefore, the development of new therapeutic agents to replace or complement the current cytotoxic agents is highly desirable to improve the outlook of patients with invasive bladder cancer (39–41).

Several tumor cells express constitutively high levels of IL-8. There is a direct association between constitutive levels of IL-8 and the aggressiveness and metastatic phenotype in colon cancer (42), pancreatic cancer (43), and melanoma (25). The highly tumorigenic and metastatic bladder cancer cell lines 253J B-V and UMUC-3 express constitutively high levels of IL-8. It has also been shown that IL-8 expression is regulated by NF- κ B in several cell lines (44, 45). Furthermore, blockade of NF- κ B, after transfection with inhibitor of nuclear factor- κ B, down-regulated the expression of IL-8 and VEGF and decreased angiogenesis and tumorigenicity. IL-8 exerts its action through an autocrine and paracrine loop by inducing adjacent tumor cells and stromal cells to express increased levels of MMP-2 and MMP-9 (18), which facilitate tumor growth, invasion, and metastases. Angiogenesis, a crucial component of tumorigenicity and metastases is also regulated by IL-8. This finding, which was originally demonstrated in human bronchogenic carcinoma, has subsequently been confirmed in glioblastoma, head and neck squamous cell carcinoma, colon cancer, breast cancer, ovarian cancer, melanoma, pancreatic cancer, and prostate cancer (24). The involvement of IL-8 in angiogenesis is additionally supported by the finding that human microvascular endothelial cells express the IL-8 receptors that allow direct interaction of IL-8 with endothelial cells (28). Previously, we demonstrated

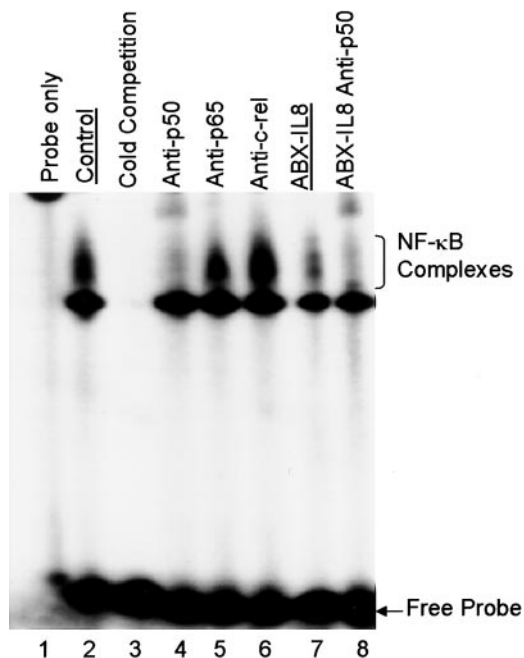


Fig. 5 NF- κ B binding activity. Nuclear protein was extracted from 253J B-V cells before and after treatment with ABX-IL8 or control IgG. EMSA was performed using probe only (Lane 1), competition with cold d.s. DNA probe (Lane 3), in the presence of specific antibodies against p50, p65, and c-rel components (Lanes 4–6). There was a 3-fold decrease in the binding activity of NF- κ B in the ABX-IL8-treated cells (Lane 7) when compared with the control (Lane 2). The NF- κ B bands were supershifted in the presence of anti-p50 antibody for both untreated and treated cells (Lanes 5 and 8, respectively). This is one representative experiment of three.

that expression of IL-8 directly correlated with angiogenesis, invasiveness, and tumorigenicity in an orthotopic bladder cancer model (18). The transfection of an IL-8 expression vector into the nonmetastatic 253 J-P human TCC cell line resulted in increased tumor angiogenesis, invasiveness, and tumorigenicity, whereas the antisense transfection of an IL-8 expression vector resulted in decreased angiogenesis, invasion, and tumorigenicity by the highly metastatic 253 J-BV cell line implanted orthotopically in the bladders of nude mice. Similar antitumor effects were noted after antisense IL-8 transfection of highly metastatic prostate cancer cell line PC3M-LN4 (46). In humans, IL-8 expression is increased in muscle-invasive bladder cancer and in carcinoma *in situ* of the bladder when compared with superficial bladder tumors (47), thus additionally establishing a link between increasing levels of IL-8 and progressive tumor grade and stage.

In this study, our goals was to test the hypothesis that disruption of the IL-8 autocrine loop would result in decreased tumorigenicity and to determine the cellular pathways that may be involved in the suppressive effect of ABX-IL8. We observed that the treatment with ABX-IL8 had no direct cytotoxic effect on bladder cancer cell lines *in vitro*. However, the athymic nude mice bearing human TCC cells in the bladder wall had significantly smaller tumors after treatment with ABX-IL8. Treatment with 1 mg once weekly was more efficient to inhibit tumor

growth and MMP-2/MMP-9 expression *in situ* as compared with a treatment of 100 μ g/ml three times/week. *In vitro*, however, treatment with 100 μ g/ml ABX-IL8 was sufficient and enough to inhibit NF- κ B expression and DNA binding activity as well as MMP-2/MMP-9 activity. The control IgG antibody used in our studies had some inhibitory effect on tumor growth in nude mice. The nature of this inhibition is not clear. However, this is commercially purchased antibody and its inhibitory effect might be because of its purity. There was also a moderate but significant decrease in the expression of MMP-2 and MMP-9 mRNA as measured by ISH as well as MMP-2 and MMP-9 activity. Nevertheless, these changes resulted in decreased invasion through Matrigel-coated filters. Our promoter analyses provide direct evidence that down-regulation of MMP-2 and MMP-9 by ABX-IL8 is regulated at the transcription level. The data also suggest that the transcriptional regulation of MMPs is mediated, in part, through NF- κ B. After treatment with ABX-IL8, there was a significant decrease in the nuclear protein level of NF- κ B, as well as its nuclear binding activity. Although NF- κ B was reported to regulate the expression of IL-8, here, we demonstrate that the expression and transcriptional activity of NF- κ B could be modulated by IL-8, thus providing a feedback mechanism of NF- κ B's action.

Our findings are consistent with previous studies of the effects of IL-8 on tumorigenicity and metastases. Inoue *et al.* (48) treated highly metastatic human TCC cell lines implanted into the subcutis of athymic nude mice with intralesional adenoviral vector containing antisense IL-8. The tumor growth was significantly inhibited when compared with the control groups. There was a significant decrease in the expression of IL-8, MMP 9, and microvessel density. Other studies involving antisense IL-8 transfection have shown similar results mediated through a decrease in MMP expression.

In addition to tumor cells, IL-8 receptors are also expressed on vascular endothelial cells, and IL-8 has been demonstrated to act as survival factor for vascular endothelial cells. In a recent study, we have demonstrated that ABX-IL8 directly inhibited the formation of capillary-like network by human umbilical vascular endothelial cells (49). It should be noted that treatment with ABX-IL8 did not affect the existing vessel-like tube formation *in vitro* and disrupted only the formation of the newly formed blood vessels *in vivo*. Although the effect of ABX-IL8 on bladder cancer cells is very well documented in our study, its effect on angiogenesis needs to be additionally established. Nevertheless, ABX-IL8 may act on both tumor and the micro-environment surrounding the tumor, including the stroma and vascular endothelial cells to affect growth of bladder cancer cells.

The potential of antibody therapy represented by ABX-IL8 fits recent discoveries. Antibody immunotherapy provides a novel approach for the treatment of a broad spectrum of diseases, including cancer. Cetuximab (IMC-C225), a mouse-human chimeric anti-EGFR monoclonal antibody and fully human anti-EGFR (ABX-EGF), have been shown to inhibit the proliferation of a variety of cultured human tumor cell lines that overexpress EGFR and to inhibit tumor growth in several xenograft models. Currently, both of these antibodies are being evaluated in clinical trials. The therapeutic modalities to control tumor growth and metastasis of human TCC are very limited.

The idea of using fully humanized antibodies to neutralize IL-8 is especially appealing because multiple dose regimens of the antibody could be administered to the patients with little risk of mounting an immune reaction. A Phase II, single-dose clinical trial and Phase I/II multiple-dose clinical trial with ABX-IL8 have been conducted in patients with moderate to severe plaque psoriasis. Both trials were designed as dose-escalating trials to examine the safety of administering a range of dose levels of ABX-IL8 by i.v. infusion; ABX-IL8 was shown to be safe and well tolerated in both the single-dose and the multiple-dose trials. No serious or unexpected adverse events have been reported, no immunogenicity has been detected, and there has been no evidence of cytokine release syndrome in either of the trials. Our studies should promote a serious consideration for initiating a Phase I/II clinical trial with ABX-IL8 in patients with bladder cancer. Because ABX-IL8 did not completely inhibit tumor growth of TCC, it should most likely be used in combination with chemotherapy or other anticancer agents to increase its efficacy.

In addition, chemotherapeutic drugs have been recently demonstrated to induce the expression and secretion of IL-8 by tumor cells. This might be one of the mechanisms used by tumor cells to escape the cytotoxic effects of the drugs. Thus, ABX-IL8 should be used to potentiate the efficacy of the therapeutic drugs by neutralizing the secreted IL-8 induced by chemotherapy. Furthermore, ABX-IL8 should be considered as a treatment modality for other solid tumors in which IL-8 plays an angiogenic role, including melanoma, prostate, ovarian, and lung cancers.

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