Interleukin 8 Expression Regulates Tumorigenicity and Metastasis in Human Bladder Cancer

Keiji Inoue, Joel W. Slaton, Sun Jin Kim, Paul Perrotte, Beryl Y. Eve, Menashe Bar-Eli, Robert Radinsky, and Colin P. N. Dinney


ABSTRACT

Interleukin 8 (IL-8) is mitogenic and chemotactic for endothelial cells. Within a neoplasm, IL-8 is secreted by inflammatory and neoplastic cells. The highly tumorigenic and highly metastatic human transitional cell carcinoma (TCC) cell line 253J B-V over-expresses IL-8 relative to the nontumorigenic and nonmetastatic 253J-P cell line. To determine whether IL-8 expression regulates tumorigenesis and metastasis in human TCC, 253J B-V cells were transfected with the full-sequence antisense (AS) cDNA for IL-8, whereas 253J-P cells were transfected with the full-length IL-8 cDNA, and control cells for each were transfected with the neomycin resistance (Neo) gene. In vitro, sense-transfected 253J-P cells over-expressed IL-8-specific mRNA and protein, whereas both of these were markedly reduced in AS-IL-8-transfected 253J B-V cells relative to controls. Moreover, sense-transfected cells showed up-regulation in matrix metalloproteinase type 9 mRNA, collagenase activity, and increased invasiveness through Matrigel-coated filters, whereas these measures were lower in AS-transfected cells relative to controls. After implantation into the bladders of athymic nude mice, the sense-transfected 253J-P cells acquired increased tumorigenicity and metastasis, whereas the AS-transfected cells significantly inhibited tumorigenicity and metastases in the 253J B-V cell lines. This effect was accompanied by reduced IL-8 expression and microvessel density. These studies demonstrate that IL-8 expression enhances angiogenic activity through the induction of matrix metalloproteinase type 9 and subsequently regulates the tumorigenesis and production of spontaneous metastases of human TCC.

INTRODUCTION

TCC of the bladder is the fifth most common malignancy diagnosed in the United States (1). Although modest improvements in therapy have occurred (2, 3), most deaths from bladder cancer are caused by metastases that resist conventional therapy. Rather, a continued empiricism in the treatment of advanced TCC is unlikely to cause marked improvement over current therapy. Therefore, in the present study, we used an orthotopic model of human TCC to determine whether IL-8 regulates angiogenesis, tumorigenicity, and metastasis in TCC of the bladder.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Human TCC cells of the 253J B-V line (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% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin (CMEM; Ref. 32).

Transfection and Selection of Tumor Cells Expressing IL-8. The tumor cells were plated onto 100-mm dishes at a density of 1 × 10^6/dish. The monolayers (60–70% confluent) were transfected with a full-length pcDNA3/sense IL-8, pcDNA3/AS-IL-8, or control pcDNA3 plasmids [EcoRI-EcoRI, 1.5 kb; a gift from Dr. K. Matsushima, University of Kanazawa, Kanazawa, Japan (21)] containing a drug-selectable marker for neomycin resistance and a strong cytomegalovirus early promoter using a stable mammalian transfection kit from Stratagene (La Jolla, CA). The cultures were placed in a 37°C incubator for 12 h and then washed and fed with CMEM. After 24 h, 500-1000 μg/ml G418 sulfate (Life Technologies, Inc., Gaithersburg, MD) were added. The CMEM/G418 medium was replaced every 3 days until individual, resistant colonies were isolated and established in culture as individual lines. All cell lines were maintained in CMEM/G418 and frozen after one to three in vitro passages. The expression of IL-8 in individual clones was identified by Northern blot analysis and ELISA. To avoid clonal variations, positive clones were then pooled for the in vitro and in vivo studies.

The 253-P and 253J B-V cells were transfected with pcDNA3/sense IL-8 and pcDNA3/AS IL-8, respectively, or with control pcDNA3 plasmids. Individual G418-resistant (500–1000 μg/ml) colonies were established as separate adherent cultures. We selected pooled sense-IL-8-transfected 253-P cells [253-P(II)-8], the highest IL-8-expressing 253-P clone [253-P(II)-8], and the lowest IL-8-expressing 253-J clone [253-J(II)-8 Low] and pooled AS-IL-8-transfected 253 B-V cells [253 B-V(AS IL-8)], the highest IL-8-expressing AS clone [253 B-V(AS IL-8 High)], and the lowest IL-8-expressing AS clone [253 B-V(AS IL-8 Low)], as indicated by the expression level of IL-8 mRNA and protein as determined by Northern blot analysis and ELISA, respectively.

Northern Blot Analysis. Polyadenylated mRNA was extracted directly from the tumors or from 10^6 cultured cells using the Fasttrack mRNA isolation kit (Invitrogen Co., San Diego, CA). The mRNA was electrophoresed onto a 1% agarose gel, electrotransferred to Genescreen nylon membranes (DuPont Co., Boston, MA), and cross-linked with a UV Stratalinker 1800 (Stratagene, La Jolla, CA) at 120,000 mJ/cm^2. Filters were washed twice at 65°C with 30 mM NaCl, 3 mM sodium citrate, and 0.1% SDS (w/v), then hybridized.

Received; 8/18/99; accepted 2/18/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by NIH Grants CA67914 and CA56973 and Core Grant CA16672.

2 To whom requests for reprints should be addressed, at Department of Cancer Biology, Box 173, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-8165; Fax: (713) 792-8747; E-mail: cdinney@mdanderson.org.

3 The abbreviations used are: TCC, transitional cell carcinoma; VEGF, vascular endothelial cell growth factor; bFGF, basic fibroblast growth factor; IL, interleukin; rfl, recombinant IL; MVD, microvessel density; AS, antisense; MMP-9, matrix metalloproteinase type 9; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ActD, actinomycin D; CAT, chloramphenicol acetyltransferase; RT-PCR, reverse transcription-PCR; CMEM, complete Eagle’s minimum essential medium; ISH, in situ hybridization.
The membranes were then hybridized and probed for IL-8, bFGF, VEGF, and MMP-9; the presence of GAPDH was used to control for loading. The cDNA probes used were: a 0.5-kb EcoRI cDNA fragment corresponding to human IL-8 (a gift of Dr. K. Matsushima, University of Kanazawa, Kanazawa, Japan; Ref. 21); a 1.4-kb cdNA fragment of bovine bFGF (33); a 204-kb fragment of human VEGF cdNA inserted in a pGEM-based construct (a gift of Dr. B. Berse, Harvard Medical School, Boston, MA; Ref. 34); a 1.0-kb cdNA fragment corresponding to human MMP-9 (29); and a 1.28-kb fragment from pR GAPDH cut with PstI (35). The insert was excised with BamHI and EcoRI. Each cdNA fragment was purified by agarose gel electrophoresis, recovered using GeneClean (BIO 101, Inc., La Jolla, CA), and radiolabeled by a random primer technique using a commercial kit (Boehringer Mannheim Corp., Indianapolis, IN) and [α-32P]deoxycytidine triphosphate (Amersham Corp., Arlington Heights, IL; Ref. 36). The steady-state expressions of IL-8, bFGF, VEGF, and MMP-9 mRNA transcripts were quantified by densitometry of autoradiographs with the use of the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA); each sample measurement was calculated as the ratio of the average areas of the specific mRNA transcripts to the 1.3-kb GAPDH mRNA transcript in the linear range of the film.

Assay for IL-8, bFGF, and VEGF. Viable cells (5 x 10^5) were seeded in a 96-well plate. Conditioned medium was removed after 24 h. The cells were then washed with 200 μl of HBBS, and 200 μl of 10% bovine serum supplemented by fresh MEM were added. Twenty-four h later, IL-8 and VEGF in cell-free culture supernatants and cell-associated bFGF in freeze-thaw cell lysates were determined using the commercial Quantine ELISA kit (R&D System, Minneapolis, MN). The protein concentration for each factor was then determined by comparing the absorbance with that of the standard. Results were expressed in terms of cell numbers (37).

Growth Curve. Viable cells (1 x 10^5) were seeded in a 96-well plate. Conditioned medium was removed after 24 h, the cells were then washed with 200 μl of HBBS, and 200 μl of fresh CMEM or CMEM/G418 conditioned medium were added. Every 24 h, the number of viable cells in each cell line was determined by absorbance comparison. The doubling times of each cell line were determined by plotting the absorbance on a semilogarithmic axis versus time (Cricket Software, Malvern, PA; Fig. 3). The doubling times of the 253J-P sense-IL-8 transfectants (IL-8, 35.0 h; IL-8 Low, 37.2 h; and IL-8 High, 37.6 h) were similar to those for both 253J-P (35.0 h) and 253J-Neo (35.4 h), and the doubling times of the 253J B-V AS-IL-8 transfectants (AS IL-8, 26.6 h; AS IL-8 Low, 27.3 h; and AS IL-8 High, 27.1 h) were similar to 253J B-V (26.0 h) and 253J B-V(No)(26.9 h).

Collagenase Activity. To determine collagenase activity, electrophoresis of serum-free conditioned medium was performed as described previously (38). Cells (5 x 10^5) were seeded in six-well plates and grown to 60–70% confluence. The cells were washed with HBBS and grown for 24 h in serum-free medium; the collagenase activity of the supernatant fluid was determined, and the remaining cells were counted to confirm the cell number. Identification of a transparent band at M, 72,000 or M, 92,000 on the Coomassie blue background of the slab gel was considered positive for the presence of the enzymatic activity.

Next we investigated whether the increase in MMP-9 activity is mediated by IL-8. Parental 253J-P cells were incubated in the presence of different doses of (0–20 μg/ml of human rIL-8), and the activity of MMP-9 was determined. Next, we investigated whether the increased activity of MMP-9 caused by rIL-8 was inhibited by neutralization with an anti-IL-8 antibody (100 μg/ml), with nonspecific IgG (100 μg/ml) serving as control.

PCR Analysis. RT-PCR analysis was performed as described previously (39). Briefly, total cellular RNA (1 μg) extracted from various cell lines was transcribed into cdNA using downstream primers IL-8 receptor type A and IL-8 receptor type B, respectively (Reverse Transcription System; Promega Corp., Madison, WI). The reverse transcription reaction was performed at 42°C for 50 min. PCR was performed with 40 cycles of denaturation (94°C for 1.5 min), annealing (58°C for 45 s), extension (72°C for 2.5 min), and 7 min of extension after completion of all cycles. Amplified fragments were analyzed on the 2% gel, and bands of expected sizes were confirmed by sequencing. The primer sequences used were as follows: IL-8 receptor type A, sense 5′-AGT TCT TGG CAC GTC ATC G-3′ and AS 5′-CTT GGA GGT ACC TCA ACA GC-3′; and IL-8 receptor type B, sense 5′-AAT TTC CTG TGC AAG GTG G-3′ and AS 5′-CAG GGT GAA TCC GTG CTA GA-3′.

Invasion through Matrigel. Polynylpoliodone-free polycarbonate filters (8-μm pore size; Nucleopore; Becton Dickinson Labware, Franklin Lakes, NJ) were coated with a mixture of basement membrane components (Matrigel; 25 μg/filter) and placed in modified Boyden chambers. The cells (2 x 10^5) were released from their culture dishes by short exposure to EDTA (1 mM, centrifuged, resuspended in 0.1% BSA/DMEM, and placed in the upper compartment of the Boyden chamber. Fibroblast-conditioned medium was placed in the lower compartment as a source of chemotactants. After incubation for 6 h at 37°C, the cells on the lower surface of the filter were stained with Diff-Quick (American Scientific Products, McGaw Park, IL) and quantified with a cooled charge-coupled device Optotronics Tec 470 camera (Optotronics Engineering, Goleta, CA) linked to a computer and digital printer (Sony Corp., Tokyo, Japan). The results were expressed as the average number of cells in the five highest spots identified within a single x200 field on the lower surface of the filter (40).

Animals. Male athymic BALB/c nude mice were obtained from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in a laminar airflow cabinet under specific pathogen-free conditions and used at 8–12 weeks of age. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the NIH.

Orthotopic Implantation of Tumor Cells. Cultured 253J-P, 253J B-V, Neo-transfected, and sense- and AS-IL-8-transfected cells (60–70% confluency) were prepared for injection as described previously (32). Mice were anesthetized with methoxyflurane. For orthotopic implantation, a lower midline incision was made, and viable tumor cells (2 x 10^5 in 0.05 ml of HBBS) were implanted into the bladder wall. The formation of a bulia 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. The mice were killed and necropsied 6 weeks after implantation of tumor cells. The primary tumors were removed and weighed, and the presence of metastases (in lymph nodes and lung) was determined grossly and microscopically. The bladders were then either quickly frozen in liquid nitrogen for mRNA extraction, fixed in 10% buffered formalin, placed in OCT compound (Miles Laboratories, Elkhart, IN), or mechanically dissociated and put into tissue culture.

In Situ mRNA Hybridization Analysis. Specific AS oligonucleotide DNA probes were designed complementary to the mRNA transcripts based on published reports of the cdNA sequence: IL-8, CTC CAC ACC CCT CTG CAC CC, 66% GC content (21); bFGF, CGG GAA GCC GCT GCC GCC, 85.7% GC content (33); VEGF/PVF, TGG TGA TGT TGG ACT CTT CAG TGG GCC, 57.7% GC content (34); and MMP-9, CGC GTC CAC CTC GCT GGC GTC CGG GU, 80.0% GC content (29). The specificity of the oligonucleotide sequence was initially determined by the Gene Bank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (Genetics Computer Group, Madison, WI), based on the FastA algorithm; these sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each of the sequences was also confirmed by Northern blot analysis (41). A poly d(T)20 oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiontilylated) at the 3′ end via direct coupling, with the use of standard phosphoramidite chemical methods (Research Genetics, Huntsville, AL). The lophiydized probes were reconstituted in a stock solution at 1 μg/μl in 10 mM Tris (pH 7.6) and 1 mM EDTA. Immediately before use, the stock solution was diluted with probe dilution (Research Genetics).

ISH mRNA hybridization was performed as described previously with minor modifications (42, 43). ISH was performed using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 44). Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific; Refs. 42 and 43). The slides were placed in the Microprobe slide holder, dewaxed, and rehydrated with Autodewaxer and Autoprocichol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was performed for 45 min at 45°C, and the samples were then washed three times with 2x SSC for 2 min at 45°C. The samples were 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. If necessary, samples were incubated a second time with fresh chromogen substrate to enhance a weak reaction. A red stain indicated a positive reaction. To control for endogenous alkaline phosphatase, the sample was treated in the absence of the biotinylated probe, using chromogen alone.

**Quantification of Color Reaction.** Stained sections were examined in a 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 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 based on this threshold. The integrated absorbance of the selected fields was determined based on its equivalence to the mean log inverse gray value multiplied by the area of the field. The samples were not counterstained; therefore, the absorbance was attributable solely to the product of the ISH reaction. Three different fields in each sample were quantified to derive an average value. The intensity of staining was determined by comparison with the integrated absorbance of poly d(T)₂₀. The results were presented as the number of each cells in each field compared with the control, which was set to 100 (37).

**Immunohistochemical Analysis.** For immunohistochemical analysis, frozen tissue sections (8-μm thick) were fixed with cold acetone. Tissue sections (5-μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with PBS, and antigen retrieval was performed with pepsin for 12 min; endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 15 min. If necessary, samples were incubated a second time with fresh chromogen substrate to enhance a weak reaction. A red stain was attributable solely to the product of the ISH reaction. Three different fields in each sample were quantified to derive an average value. The intensity of staining was determined by comparison with the integrated absorbance of poly d(T)₂₀. The results were presented as the number of each cells in each field compared with the control, which was set to 100 (37).

**Quantification of MVD.** MVD was determined by light microscopy after immunostaining of sections with anti-CD31 antibodies according to the procedure of Weidner et al. (46). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. The tissue was recorded using a cooled charge-coupled device camera (Optronics Tec 470 camera (Optronics Engineering) linked to a computer and digital printer (Sony Corp.). The density of microvessels was expressed as the average number of the five highest areas identified within a single ×200 field.

**Quantification of Intensity of Immunostaining.** The intensity of immunostaining of IL-8, bFGF, VEGF, and MMP-9 was quantified in three different areas of each sample by an image analyzer using the Optimas software.
IL-8 regulates tumorigenicity in bladder cancer

CAT Assay. Using the FuGENE 6 protocol (Boehringer Mannheim Corp.), we transfected the basic CAT expression vector with no promoter/enhancer sequences (pCAT-basic) or a control plasmid with SV40 promoter and enhancer (pCAT-control; Promega) into 253J-P cells, sense-transfected cells, 253J-BV cells, AS-transfected cells, and each Neo transfec tant. One copy of the full-sequence, human 570-bp MMP-9 promoter (a gift of Dr. Seiki Motoharu, University of Tokyo, Tokyo, Japan) was ligated upstream of the basic CAT expression vector. We transfected 5 × 10^3 cells/well of six-well tissue culture dishes with 2.5 μg of the reporter CAT constructs and 2.5 μg of a β-actin expression plasmid. After 48 h, extracts were prepared from all plates, normalized for β-actin activity, and assayed for CAT activity (47) according to the methods of Hudson et al. (48), described previously. The CAT assay was quantified by densitometry of autoradiographs with the use of the Image Quant software program (Molecular Dynamics, Sunnyvale, CA) and was evaluated as the ratio of acetylated species to all species.

Statistical Analysis. The statistical differences in vessel counts and staining intensity for IL-8, bFGF, VEGF, and MMP-9 of bladder tumors were analyzed by the Mann-Whitney U test. The incidences of tumor and metastasis were statistically analyzed by Fisher’s exact test. A value of P < 0.05 was considered significant.
first evaluated whether the expression of MMP-9 was altered in the 253J-P and 253J B-V cells after transfection with sense or AS IL-8 transcripts. Fig. 1 shows that MMP-9 mRNA expression was increased 2.2- and 2.6-fold by 253J-P(IL-8) and 253J-P(IL-8 High) cells, respectively, and reduced 2.5- and 3.3-fold by 253J B-V(AS IL-8) and 253 J B-V(AS IL-8 Low), respectively, after transfection with IL-8 sense or AS transcripts. These results show that IL-8 regulates MMP-9 expression by the 253J-P and 253J B-V human TCC cells.

Collagenase Activity. To demonstrate that MMP-9 expressed by the transfected cells is biologically active, collagenase activity was determined by zymography after normalizing the volume of supernatant for cell number. The results shown in Fig. 4A indicate that IL-8 caused an increase in the activity of MMP-9. Moreover, the increased activity of MMP-9 by rIL-8 was inhibited by neutralization with an anti-IL-8 antibody (Fig. 4B).

RT-PCR Analysis. RT-PCR analysis revealed that 253J-P, 253J-P(Neo), and AS-IL-8-transfected 253J-P and also 253J B-V, 253J B-V(Neo), and AS-IL-8-transfected 253J B-V express mRNA for both types of IL-8 receptors (Fig. 5).

Invasion Assay through Matrigel. We analyzed whether the activation of MMP-9 in the IL-8-transfected cells correlated with an increase in penetration through the basement membrane, an important step in the process of tumor invasion and metastasis. 253J-P(IL-8) and 253J-P(IL-8 High) cells exhibited increased ability to penetrate through Matrigel-coated filters, with a 6.5- and 10.0-fold increase, respectively, compared with either 253J-P or 253J-P(Neo) (P < 0.005; Fig. 6A). Invasion by 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) was reduced 63 and 78%, respectively, compared with invasion by 253J B-V or 253J B-V(Neo) (P < 0.005; Fig. 6B).

Stability of MMP-9 mRNA. To determine the mechanism by which IL-8 enhanced the expression of MMP-9 mRNA, the stability of MMP-9 mRNA was investigated by examining its half-life. The half-life of MMP-9 mRNA in 253J-P(IL-8) and 253J B-V(AS IL-8) cells was similar to that in 253J-P or 253J-P(Neo), and 253J B-V or 253J B-V(Neo), respectively (data not shown).

CAT Activity. The full sequence MMP-9 promoter was linked upstream of the CAT reporter gene and transfected into sense-IL-8 transfected, AS-IL-8-transfected, each Neo-transfected, and each parental cell to examine the effect of IL-8 expression on MMP-9 transcription. Forty-eight h after transfection, cell extracts were prepared, and equivalent amounts of extracts exhibiting the same β-actin activity were tested for CAT activity. CAT activity driven by the MMP-9 promoter in 253J-P(IL-8) and 253J-P(IL-8 High) was increased 1.4- and 1.8-fold, respectively (Fig. 7A), compared with either 253J-P or 253J-P(Neo), and decreased 1.4- and 1.7-fold by 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low), respectively (Fig. 7B),
compared with either 253J B-V and 253J B-V(Neo). CAT activity driven by the SV-40 promoter was the same in both cell populations and served as an additional internal control for transfection efficiency.

**Tumorigenicity and Production of Metastasis.** To evaluate whether IL-8 expression regulates tumorigenicity and metastases of TCC, we implanted 253J-P, 253J B-V, Neo-transfected, and IL-8-transfected cells into the bladders of athymic nude mice and evaluated tumor growth and metastasis 6 and 12 weeks later (Table 1). The 253J-P(IL-8) and 253J-P(IL-8 High) cells formed tumors that were palpable at 6 weeks and demonstrated a significant enhancement of
DISCUSSION

Tumor growth and metastasis depend upon the ability of the tumor to induce its own blood supply (8–10). This process, angiogenesis,
depends on the outcome between stimulatory and inhibitory regulation by the tumor and its microenvironment (10, 11). Human TCC expresses a number of angiogenesis factors including VEGF (12, 13), bFGF (14–16), midkine (17), thymidine phosphorylase (18), and IL-8 (19). IL-8 is expressed by a number of human malignancies, and its expression correlates with the metastatic potential of that tumor (22–26). Direct evidence for the role of IL-8 in tumor growth and metastasis was provided by Luca et al. (27), who enforced expression of IL-8 in the SB-2 melanoma cell line and increased its tumorigenic and metastatic potential. Recently, Moore et al. (49) demonstrated that...
IL-8 regulated the growth of the human prostate cancer PC-3. They reported that neutralizing antibodies to IL-8 reduced the angiogenic activity of PC-3 homogenates and inhibited tumor growth after ectopic implantation in SCID mice, suggesting that the growth inhibition seen after treatment with IL-8 neutralizing antibodies is secondary to inhibition of tumor-induced angiogenesis.

The present study confirms and expands upon these studies. Our goal was to evaluate whether the level of expression of IL-8 directly regulates tumor-induced neovascularization and subsequent tumor growth and metastasis of human TCCs growing within the bladder of athymic nude mice. We enforced IL-8 expression by transfecting the poorly tumorigenic and nonmetastatic human TCC cell line 253J-P (which expresses relatively low levels of IL-8) with the sense-IL-8 construct and were able to establish several cell lines that overexpressed IL-8. Two of these cell lines, 253J-P(IL-8) and 253J-P(IL-8 High), demonstrated enhanced tumorigenicity and spontaneous lymph node metastasis compared with the 253J-P or 253J-P(Neo) controls. Conversely, after AS-IL-8 transfection, we were able to reduce IL-8 expression by the highly tumorigenic and metastatic 253J B-V cell line (which expresses relatively high levels of IL-8) and establish cell lines that were significantly less tumorigenic (lower incidence and smaller tumors) and metastatic than 253J B-V or 253J B-V(Neo) controls. Because neither bFGF nor VEGF expression was altered by IL-8 transfection, we conclude that these effects are independent of the activity of these angiogenesis factors. Furthermore, because IL-8 transfection did not affect in vitro proliferation of 253J-P or 253J B-V, the effects on growth and metastasis are independent of proliferation, although the cells do have both type A (CXCR1; Refs. 50 and 51) and type B (CXCR2; Refs. 50 and 51) IL-8 receptors. Therefore, our results provide direct evidence for the involvement of IL-8 in the induction of in vivo angiogenesis and in the subsequent growth and metastasis of TCC. These results are similar to previous reports in which transfection with VEGF or bFGF increased MVD and enhanced tumor growth and metastasis of melanoma and breast cancer (52–54).

The metastatic potential of bladder cancer depends upon the expression of several metastasis-related genes, such as IL-8, that regulate endothelial cell proliferation and capillary morphogenesis (55), and other genes, such as MMP-9, that regulate the degradation of the extracellular matrix (56–61). The local production of MMP-9 or other proteases such as plasminogen activator by bladder cancer cells or stroma facilitates the local degradation of the extracellular matrix and results in tumor invasion and subsequent metastasis (56–61). The proteolytic effect of MMPs facilitates the migration of endothelial cells through the altered extracellular matrix toward the source of the angiogenic stimulus; in this manner, MMPs are an integral component of the angiogenesis pathway. The highly metastatic 253J B-V cell line expresses high levels of the metalloproteinase MMP-9 compared with the nonmetastatic 253J-P cell line. Recently, Luca et al. (27) reported that IL-8 induces MMP-2 activity by malignant melanoma cells. The up-regulation of collagenase activity by IL-8 was considered to be an important mechanism to explain the associated increase in metastatic ability. Similarly, we found that the activity of MMP-9 by the TCC cells directly correlated with their expression of IL-8. Moreover, when we altered the expression of IL-8 by sense or AS transfection, we observed a corresponding change in MMP-9 expression and activity. The MMP-9 induced by transfection is biologically active, because it induced collagenase activity and increased cellular invasion through Matrigel; when it was reduced after AS transfection, both collagenase activity and invasion through Matrigel were decreased. When the 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) cells were implanted in vivo, the expression of MMP-9 was reduced within the tumors. These tumors were smaller than their controls, which may reflect relative growth inhibition secondary to the inability to induce as robust a microcirculation, and were nonmetastatic, attributable perhaps to a reduction in MMP-9 activity.

It is likely that IL-8 regulates MMP-9 expression at the transcriptional level. To investigate this mechanism, we evaluated MMP-9 mRNA stability and the level of gene transcription of MMP-9 in IL-8 transfectants and control cells. Although the expression of MMP-9 mRNA varied among the IL-8 transfectants and controls, the stability of MMP-9 mRNA was not changed by transfection with sense-IL-8 or AS-IL-8. However, CAT activity driven by the MMP-9 promoter was up-regulated in IL-8 sense transfectants and down-regulated after AS transfection. It is well established that bFGF regulates MMP activity in TCC (62). Because bFGF levels are not affected by IL-8 transfection, in our cells the regulation of MMP-9 transcription is independent of bFGF and likely attributable to the level of IL-8. These results are in keeping with the report of Luca et al. (27), who found that IL-8 regulated MMP-2 gene transcription.

In summary, our present study demonstrates that IL-8 regulates angiogenesis, tumorigenesis, and metastasis by human TCC, which may be mediated in part by regulating the expression and activity of MMP-9.

REFERENCES

a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induc-
tion of MDNCF mRNA by interleukin 1 and tumor necrosis factor. J. Exp. Med., 167:
22. Matsushima, K., Baldwin, E. T., and Mukaida, N. Interleukin-8 and MCAF: novel
V. M., Eliner, S. G., and Strieter, R. M. Interleukin-8 as a macrophage-derived
mediator of angiogenesis [see comments]. Science (Washington DC), 258: 1798–
P. J., and Eliner, S. G. Interleukin-8. A crucial factor that induces neovascularization.
25. Schadendorf, O., Moller, A., Agermannis, B., Worm, M., Sticherling, M., and
Czarnetzki, B. IL-8 produced by human malignant melanoma cells in vitro is an
essential autocrine growth factor. J. Immunol., 151: 2667–2675, 1993; erratum 153:
of interleukin 8 correlates with the metastatic potential of human melanoma cells
27. Luca, M., Huang, S., Gershovnoul, J. E., Singh, R. K., Reich, R., and Bar-Eli,
M. Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity
28. Arenberg, D. A., Kunkel, S. L., Polverini, P. J., Glass, M., Burdick, M. D., and
Strieter, R. M. Inhibition of interleukin-8 reduces tumorigenesis of human non-small
and Fidler, I. J. Correlation of metastasis-related gene expression with metastatic
potential in human prostate carcinoma cells implanted in nude mice using an in situ
30. Kitadai, Y., Haruma, K., Sumii, K., Yamamoto, S., Ue, T., Yokozaki, H., Yasui, W.,
Ohmoto, Y., Kajiyama, G., Fidler, I. J., and Tahara, E. Expression of interleukin-8
correlates with vascularization in human gastric carcinomas. Am. J. Pathol., 152: 93–100,
1998.
31. Yoneda, J., Kuniyasu, H., Crispens, M. A., Price, J. E., Bucana, C. D., and Fidler,
I. J. Expression of angiogenesis-related genes and progression of human ovarian carcino-
Fan, D., Bucana, C. D., Fidler, I. J., and Kittion, J. J. Isolation and characterization of
metastatic variants from human transitional cell carcinoma passed by orthotopic
permeability factor (vascular endothelial growth factor) gene is expressed differen-
35. Fort, P., Marty, L., Piechaczek, M., El Sabrouty, S., Dani, C., Jeanpault, E., and
Blanchard, J. M. Various rat adult tissues express only one major mRNA species from the
glyceroldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res., 13:
36. Feinberg, A. P., and Vogelstein, B. A technique for radiolabeling DNA restriction
37. Perrote, P., Matsushima, T., Inoue, K., Kuniyasu, H., Ue, B. Y., Hicklin, D. J.,
Radinsky, R., and Dinney, C. P. Anti-epidermal growth factor receptor antibody C225
specifically inhibits angiogenesis in human transitional cell carcinoma growing orthotopically
and Lotan, R. Inhibition by retinoic acid of type IV collagenolysis and invasion
of interleukin 8 correlates with the metastatic potential of human melanoma cells
41. Weidner, N., Semple, J. P., Welch, W. L., and Folkman, J. Tumor angiogenesis and
metastasis-correlation in invasive breast carcinoma. N. Engl. J. Med., 324: 1–8,
42. Bucana, C. D., Radinsky, R., Lichtarge, O., Singh, R. K., and Fidler, I. J. Expression
of basic fibroblast growth factor is required for angiogenesis in vivo. Cancer Res., 56:
43. Murphy, P. M., and Tiffany, H. L. Cloning of complementary DNA encoding a
44. Radinsky, R., Ellis, L. M., Sanchez, R., Brigati, D. J., and Fidler, I. J. Rapid colorimetric
in situ mRNA hybridization technique using inhibited peroxidase-conjugated
oligonucleotide probes for analysis of mdr1 in mouse colon carcinoma cells. J. His-
45. Vecchi, A., Garlanda, C., Lampugnani, M. G., Matutecci, C., Stoppacciario, A.,
for endothelial cells of mouse blood vessels. Their application in the identification of
H. F. Expression of vascular permeability factor vascular endothelial growth factor by
melanoma cells increases tumor growth, angiogenesis, and experimental metastasis.
47. Morris, B. C., Amsel, A., Tognazzi, K., Yeo, K. T., Manseau, E. J., and Dvorak,
H. F. Requirement for matrix metalloproteinase-2 (MMP-2a) expression in human prostate cancer.
H. F. Requirement for matrix metalloproteinase-2 (MMP-2a) expression in human prostate cancer.
49. Jones, J. R., and Fidler, I. J. Rapid colorimetric in situ mRNA hybridization technique
for analysis of epidermal growth factor receptor in paraffin-embedded surgical specimens
Interleukin 8 Expression Regulates Tumorigenicity and Metastasis in Human Bladder Cancer

Keiji Inoue, Joel W. Slaton, Sun Jin Kim, et al.

*Cancer Res* 2000;60:2290-2299.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/8/2290

Cited articles
This article cites 58 articles, 25 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/8/2290.full.html#ref-list-1

Citing articles
This article has been cited by 25 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/8/2290.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.