[CANCER RESEARCH 60, 2290–2299, April 15, 2000]

Interleukin 8 Expression Regulates Tumorigenicity and Metastasis in Human Bladder Cancer[1]

Keiji Inoue, Joel W. Slaton, Sun Jin Kim, Paul Perrotte, Beryl Y. Eve, Menashe Bar-Eli, Robert Radinsky, and Colin P. N. Dinney[2]

Departments of Cancer Biology [K. I., J. W. S, S. J. K., B. Y. E., M. B-E., R. R., C. P. N. D.] and Urology [P. P., C. P. N. D.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

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 overexpresses IL-8 relative to the nontumorigenic and nonmetastatic 253J-P cell line. To determine whether IL-8 expression regulates tumorigenicity 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 overexpressed 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[3] 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 (4–6). Continued empiricism in the treatment of advanced TCC is unlikely to produce significant improvement over current therapy. Rather, a knowledge of the cellular and molecular properties of bladder cancer and of the tumor-host interactions that influence the dissemination of metastatic disease is essential for the design of more effective treatment.

Metastasis is a highly selective process involving multiple tumor-host interactions (4–7). A crucial step in metastasis is vascularization in and around the tumor (8–10). This process of angiogenesis is regulated by the balance between stimulatory and inhibitory factors released by the tumor and the microenvironment (11). Human bladder cancer produces a number of proangiogenic factors, including VEGF (12, 13), bFGF (14–16), midkine (17), thymidine phosphorylase (18), and IL-8 (19). MVD, a pathological surrogate for angiogenesis, correlates with stage and prognosis for patients with bladder cancer (20).

IL-8 was originally identified as a leukocyte chemoattractant (21, 22), but subsequent studies have shown that IL-8 induces angiogenesis (23, 24) and is expressed by melanoma (25–27), lung (28), prostate (29), gastric (30), and ovarian (31) cancers and by TCC of the bladder (19). Although IL-8 is expressed by TCC, its exact role in the process of angiogenesis and the progression of TCC has not been elucidated.

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 [*Eco*RI-*Eco*RI, 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 253J-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 253J-P cells [253J-P(IL-8)], the highest IL-8-expressing 253J-P clone [253J-P(IL-8 High)], and the lowest IL-8-expressing 253J-P clone [253J-P(IL-8 Low)] and pooled AS-IL-8-transfected 253J B-V cells [253J B-V(AS IL-8)], the highest IL-8-expressing AS clone [253J B-V(AS IL-8 High)], and the lowest IL-8-expressing AS clone [253J 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 10^8 cultured cells using the Fasttrack mRNA isolation kit (Invitrogen Co., San Diego, CA). The mRNA was electrophoresed onto 1% denatured formaldehyde 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).

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; rIL, 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 *Eco*RI 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 *Pst*I (35). The insert was excised with *Bam*HI and *Eco*RI. 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-^{32}P]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 (5×10^3) were seeded in a 96-well plate. Conditioned medium was removed after 24 h. The cells were then washed with 200 μl of HBSS, 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 (1×10^3) were seeded in a 96-well plate. Conditioned medium was removed after 24 h, the cells were washed with 200 μl of HBSS, 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-P(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(Neo) (26.9 h).

Collagenase Activity. To determine collagenase activity, electrophoresis of serum-free conditioned medium was performed as described previously (38). Cells (5×10^3) were seeded in six-well plates and grown to 60–70% confluence. The cells were washed with HBSS 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_r 72,000 or M_r 92,000 on the Coomassie blue background of the slab gel was considered positive for the presence of the enzymatic activity.

We next 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 mg) extracted from various cell lines was transcribed into cDNA using downstreaming 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′-ACA TTC CTG TGC AAG GTG G-3′ and AS 5′-CAG GGT GAA TCC GTA GCA GA-3′.

Invasion through Matrigel. Polyinylprolidone-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 (2×10^5) 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 chemoattractants. 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, Goletha, 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 ×200 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% confluent) 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 (2×10^6 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. 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 AAC CCT CTG CAC CC, 66% GC content (21); bFGF, CGG GAA GGC GCC GCT GCC GCC, 85.7% GC content (33); VEGF/VPF, TGG TGA TGT TGG ACT CTT CAG TGG GCU, 57.7% GC content (34); and MMP-9, CCG GTC CAC CTC GCT GGC GCT CCG GU, 80.0% GC content (29). The specificity of the oligonucleotide sequence was initially determined by a 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 (hyperbiotinylated) at the 3′ end via direct coupling, with the use of standard phosphoramidite chemical methods (Research Genetics, Huntsville, AL). The lyophilized 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 Autoalcohol (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 2× 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 for line 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 12 min. The samples were washed three times with PBS and incubated for 20 min at room temperature with a protein-blocking solution of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with a 1:100 dilution of rat monoclonal anti-CD31 antibody (PharMingen, San Diego, CA; Ref. 45), a 1:50 dilution of a rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA), a 1:500 dilution of rabbit polyclonal anti-bFGF antibody (Sigma Chemical Co., St. Louis, MO), a 1:500 dilution of rabbit polyclonal anti-VEGF/VPF antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or a 1:100 dilution of mouse monoclonal anti-MMP-9 antibody (Oncogene Research Products, Cambridge, MA). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of the secondary antibody:peroxidase-conjugated antirat IgG (H+L; Jackson ImmunoResearch Laboratory, Inc., West Grove, PA), antirabbit IgG, $F(ab)_2$ fragment (Jackson ImmunoResearch Laboratory, Inc.), or antimouse IgG1 (PharMingen). The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill's hematoxylin (Biogenex Laboratories, San Ramon, CA), and washed three times with PBS. The slides were mounted with Universal Mount mounting medium (Research Genetics).

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 Optotronics Tec 470 camera (Optotronics 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

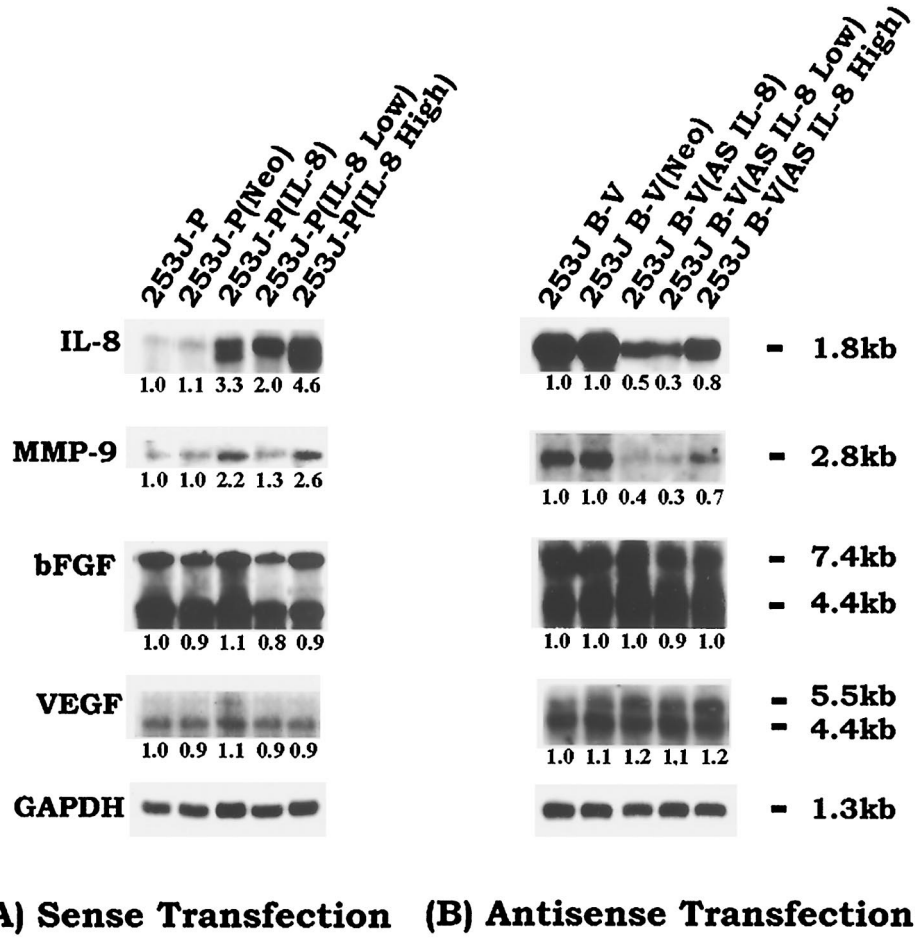

Fig. 1. Northern blot analysis of mRNA for IL-8, bFGF, VEGF, and MMP-9 in poorly tumorigenic human prostate cancer cell line 253J-P, Neo transfectant 253J-P(Neo), and sense-IL-8 transfectants (*IL-8*, *IL-8 Low*, and *IL-8 High*; *A*) and in highly metastatic human bladder cancer cell line 253J B-V, Neo transfectant 253J B-V(Neo), and AS-IL-8 transfectants (*AS IL-8*, *AS IL-8 Low*, and *AS IL-8 High*; *B*). The difference in expression is shown by the ratio of mRNA expression of transfectants to that of parental cells defined as 1.0. GAPDH served as control for loading. 253J-P(IL-8) and 253J-P(IL-8 High) cells overexpressed IL-8-specific mRNA, with 3.3- and 4.6-fold increases, whereas mRNA expression of IL-8 was decreased 2.0- and 3.3-fold, respectively. Moreover, the mRNA expression of MMP-9 was increased 2.2- and 2.6-fold in 253J-P(IL-8) and 253J-P(IL-8 High) cells, respectively, whereas it was markedly reduced by 40 and 30% in the 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) cells, respectively. This is one representative experiment of three.

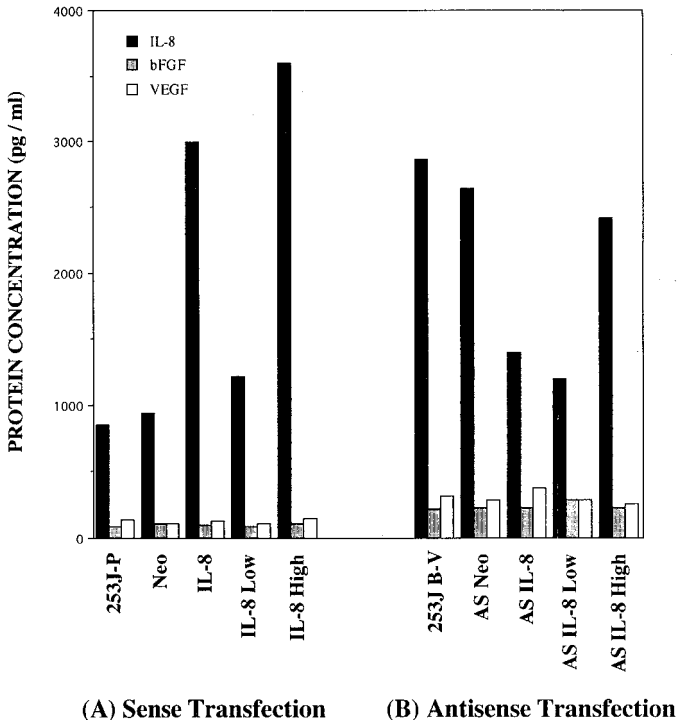

Fig. 2. Protein expression of IL-8, bFGF, and VEGF in 253J-P, 253J-P(Neo), and sense-IL-8 transfectants (*IL-8*, *IL-8 Low*, and *IL-8 High*; *A*) and in 253J B-V, 253J B-V(Neo), and AS-IL-8 transfectants (*AS IL-8*, *AS IL-8 Low*, and *AS IL-8 High*; *B*) was analyzed by ELISA. Cells (5×10^3/well) were cultured for 48 h in CMEM or CMEM/G418. Cell-free culture supernatant was analyzed for IL-8 and VEGF. Cell lysate was analyzed for bFGF. Protein expression of IL-8 in 253J-P(IL-8) and 253J-P(IL-8 High) cells was increased 3.5- and 4.3-fold, respectively, and protein expression in 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) cells decreased 2.1- and 2.4-fold, respectively.

program (Bioscan, Edmonds, WA). Three different areas in each sample were quantified to yield an average measurement of intensity of immunostaining. The results were presented as the number of cells for each cell line compared with the control, which was set to 100 (37).

MMP-9 mRNA Half-Life Studies. To determine the effect of IL-8 on MMP-9 mRNA stability, 253J-P, 253J B-V, 253J-P(Neo), 253J B-V(Neo), 253J-P(IL-8), and 253J B-V(AS IL-8) cells were incubated for 24 h. Further transcription in cells was then blocked by the addition of ActD (Calbiochem-Novabiotechnology, Inc., Lake Placid, NY; final concentration, 5 μg/ml). Total RNA was extracted from the cells 0, 0.5, 1, 2, 4, and 6 h after the addition of ActD, and Northern blot analysis was performed for MMP-9 mRNA expression. MMP-9 mRNA expression of each time point was compared with the control value (total RNA extracted from cells prior to ActD treatment was arbitrarily defined as 100%). The half-life of MMP-9 mRNA was determined by plotting relative MMP-9 mRNA expression levels on a semilogarithmic axis *versus* time (Cricket Software, Malvern, PA).

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 transfectant. 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.

RESULTS

Expression of IL-8, bFGF, VEGF, and MMP-9. Northern blot analyses for IL-8, bFGF, VEGF, and MMP-9 steady-state gene expression by the nontumorigenic human TCC cell line 253J-P; the sense-IL-8 transfectants 253J-P (IL-8), 253J-P(IL-8 High), and 253J-P(IL-8 Low); and the Neo-transfected 253J-P (Neo) are shown in Fig. 1*A*. Northern blot analyses for the highly metastatic human TCC cell line 253J B-V; its AS-IL-8 transfectants 253J B-V(AS IL-8), 253J B-V(AS IL-8 Low), and 253J B-V(AS IL-8 High); and the Neo-transfected 253J-BV(Neo) are shown in Fig. 1*B*. The level of expression is shown as the ratio of mRNA expression of the transfectants to that of both the corresponding parental and Neo transfectant cell lines. IL-8 expression was increased 3.3-, 4.6-, and 2.0-fold by the 253J-P(IL-8), 253J-P(IL-8 High), and the 253J-P(IL-8 Low) cell lines, respectively, whereas there was no change in the mRNA expression of bFGF or VEGF. The mRNA expression of IL-8 by 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) was decreased 2.0- and 3.3-fold, respectively, whereas there was no change in either bFGF or VEGF mRNA expression. IL-8, bFGF, and VEGF protein production by 253J-P, 253J B-V, and the transfected cell lines was evaluated by ELISA (Fig. 2). Changes in protein expression by the transfectants paralleled the changes seen in mRNA expression. Levels of IL-8 expression were 3.5- and 4.3-fold higher in the 253J-P(IL-8) and 253J-P(IL-8 High) cells, respectively, and decreased 2.1- and 2.4-fold by the 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) cell lines. bFGF and VEGF protein expression was unchanged.

Because IL-8 regulates protease activity by human melanoma, we

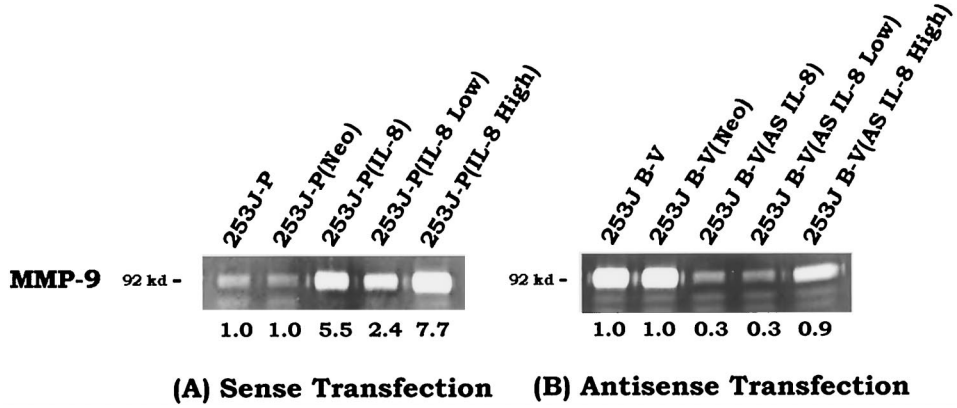

Fig. 3. Gelatinolytic activity of conditioned medium of 253J-P, 253J-P(Neo), and sense-IL-8 transfectants (*IL-8*, *IL-8 Low*, and *IL-8 High*; *A*) and in 253J B-V, 253J B-V(Neo), and AS-IL-8 transfectants (*AS IL-8*, *AS IL-8 Low*, and *AS IL-8 High*; *B*). CMEM was used as internal control. The difference in expression is expressed as the ratio of gelatinolytic activity of transfectants to that of parental cells (defined as 1.0) after normalizing supernatant volume to cell number. The collagenase activity of 253J-P(IL-8) and 253J-P(IL-8 High) cells was increased 5.5- and 7.7-fold, respectively, and the collagenase activity of 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) clone cells was decreased 3.0- and 3.6-fold, respectively. This is one representative experiment of three.

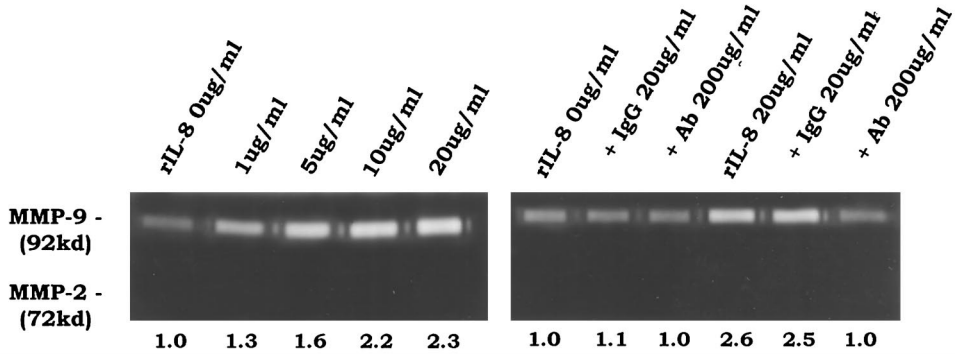

Fig. 4. Regulation of MMP-9 activity by IL-8. Parental 253J-P cells were incubated in the presence of different doses of human rIL-8 (0–20 μg/ml), and the activity of MMP-9 was determined. The results shown in Fig. 4*A* indicate that IL-8 caused a dose-dependent increase in the activity of MMP-9. Moreover, the increased activity of MMP-9 by rIL-8 was inhibited by neutralization with an anti-IL-8 antibody (100 μg/ml; *B*). This is one representative experiment of three.

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 253J 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.

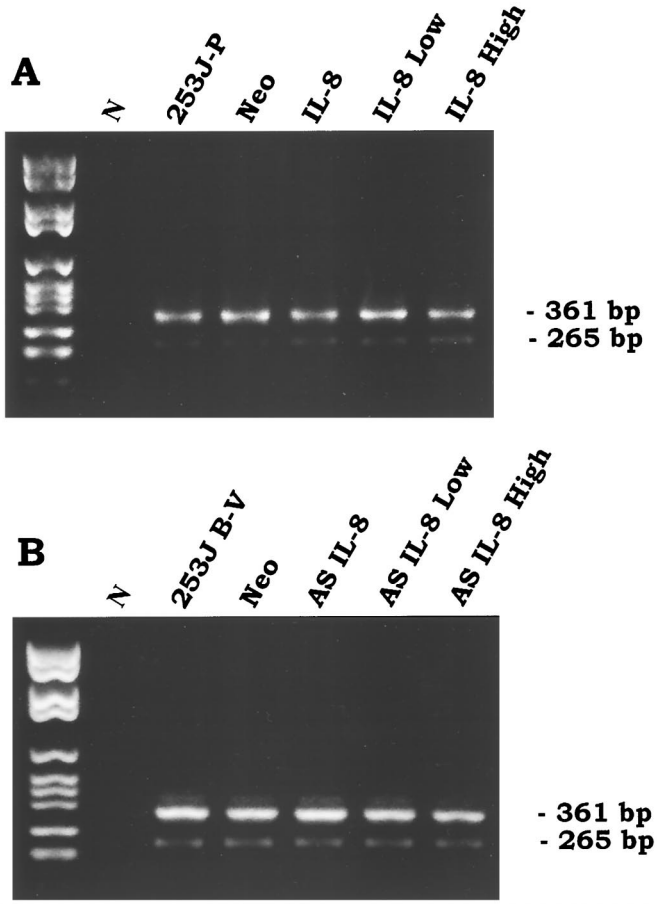

Fig. 5. RT-PCR analysis was performed by primer sequences sense 5′-AGT TCT TGG CAC GTC ATC G-3′and AS 5′-CTT GGA GGT ACC TCA ACA GC-3′ for IL-8 receptor type A and sense 5′-ACA TTC CTG TGC AAG GTG G-3′ and AS 5′-CAG GGT GAA TCC GTA GCA GA-3′ for IL-8 receptor type B and performed without reverse transcriptase as a negative control (*N*). RT-PCR analysis revealed that 253J-P, 253J-P(Neo), and sense-IL-8 transfectants (*IL-8*, *IL-8 Low*, and *IL-8 High*; Fig. 4*A*) and also 253J B-V, 253J B-V(Neo), and AS-IL-8 transfectants (*AS IL-8*, *AS IL-8 Low*, and *AS IL-8 High*; Fig. 4*B*) expressed mRNA for both types of IL-8 receptors. This is one representative experiment of two.

Collagenase Activity. To demonstrate that MMP-9 expressed by the transfected cells is biologically active, collagenase activity was determined by zymogram after normalizing the volume of supernatant for cell number (Fig. 3). By densitometry, the collagenase activity of 253J-P(IL-8) and 253J-P(IL-8 High) was increased 5.5- and 7.7-fold, respectively, compared with 253J-P and 252J-P(Neo) (Fig. 4*A*), whereas that of 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) was decreased 3.0- and 3.6-fold, respectively, compared with either 253J B-V or 252J B-V(Neo) (Fig. 4*B*).

We next analyzed whether the increase in MMP-9 activity is mediated by IL-8 (Fig. 4). To that end, parental 253J-P cells were incubated in the presence of different doses of human rIL-8, and the activity of MMP-9 was determined by zymography after normalizing the volume of supernatant for cell number. The results shown in Fig. 4*A* 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 anti-IL-8 antibody (Fig. 4*B*).

RT-PCR Analysis. RT-PCR analysis revealed that 253J-P, 253J-P(Neo), and sense-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. 6*A*). 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. 6*B*).

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. 7*A*), 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. 7*B*),

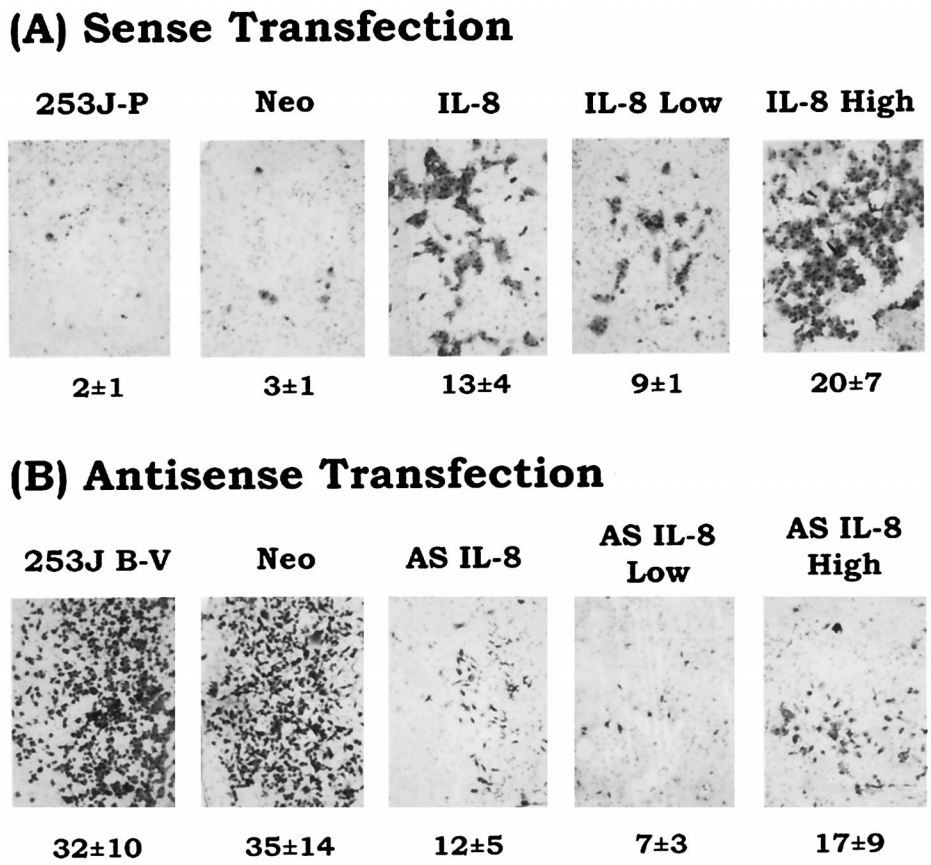

Fig. 6. Migration of cells (2×10^5) of sense transfectants (*A*) and AS transfectants (*B*) through Matrigel-coated filters into fibroblast-conditioned medium as a source of chemoattractants was expressed as the average number of cells in the five highest spots identified within a single ×200 field on the lower surface of the filter. 253J-P(IL-8) and 253J-P(IL-8 High) cells exhibited an increased ability to penetrate through Matrigel-coated filters, with a 6.5- and 10.0-fold increase in the number of migrated cells, respectively ($P < 0.005$), whereas it was markedly reduced in 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) cells relative to controls, with 63 and 78% reduction ($P < 0.005$), respectively. This is one representative experiment of three.

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

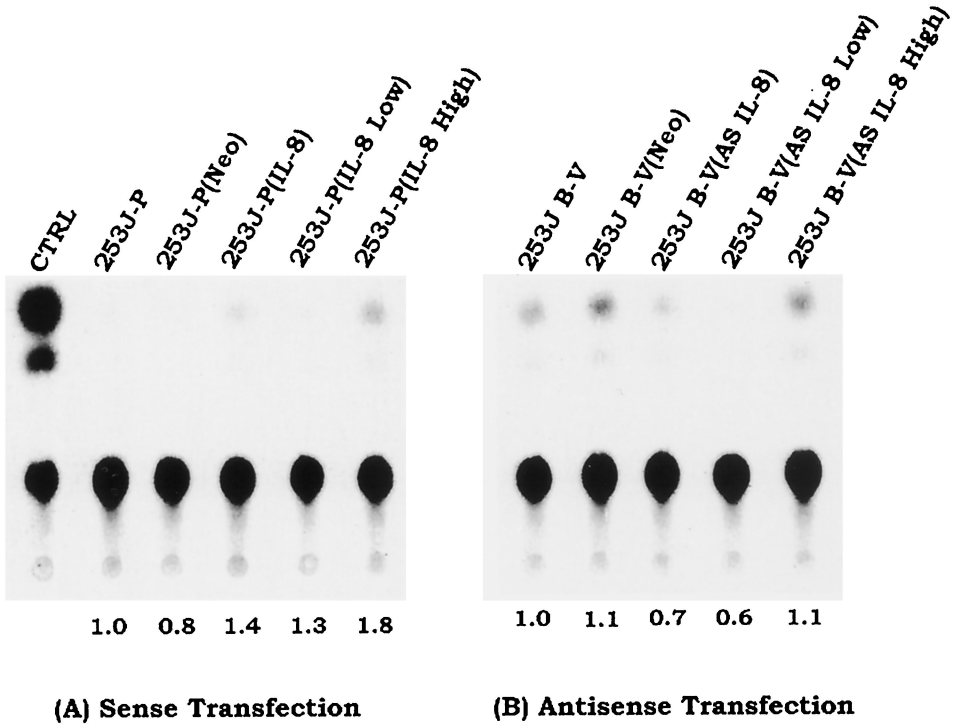

Fig. 7. Effect of IL-8 expression on CAT activity driven by the MMP-9 promoter in sense-IL-8 (*A*) and AS-IL-8 (*B*) transfectants. The CAT activity was evaluated as the ratio of acetylated species to all species. The difference in expression is shown by the ratio of the CAT activity of transfectants to that of parental cells defined as 1.0. CAT activity in 253J-P(IL-8) and 253J-P(IL-8 High) cells was increased 1.4- and 1.8-fold, respectively, and CAT activity in 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) cells was decreased 1.4- and 1.7-fold, respectively. This is one representative experiment of two.

Table 1 *Tumorigenicity and production of spontaneous metastases after orthotopic implantation of 253J-P, Neo transfectant, and sense-IL-8 transfectants and of 253J B-V, Neo transfectant, and AS-IL-8 transfectant in the bladder wall of athymic nude mice*

	Tumorigenicity		
Cell line	Incidence	Median bladder weight (range, mg)	Lymph node metastasis incidence
Sense transfection[c]			
253J-P(Neo)	0/9		0/9
253J-P(IL-8)	3/10	124 (0–303)	0/10
253J-P(IL-8 Low)	0/8		0/8
253J-P(IL-8 High)	3/9	161 (0–364)	0/9
AS transfection[c]			
253J BV	8/8	188 (112–284)	4/8
253J BV(AS Neo)	9/9	237 (88–516)	8/9
253J BV(AS IL-8)	5/8[a]	40 (0–321)	0/8[a]
253J BV(AS IL-8:Low)	4/8[a]	40 (0–72)[b]	0/8[a]
253J BV(AS IL-8 High)	9/10	105 (0–333)	3/10

[a] $P < 0.05$ against 253J B-V and 253J B-V(AS Neo) (χ^2 test).
[b] $P < 0.01$ against 253J B-V and 253J B-V(AS Neo) (Mann-Whitney statistical comparison).
[c] One representative experiment of three.

tumorigenicity compared with either 253J-P or 253J-P(Neo). No tumors were detected either grossly or microscopically in mice implanted with 253J-P or 253J-P(Neo) at 6 or 12 weeks. Three of 10 animals implanted with 253J-P(IL-8) developed tumors with a median weight of 124 mg (range, 25–303 mg; $P < 0.05$ compared with both controls); and three of nine animals implanted with 253J-P(IL-8 High) developed tumors with a median weight of 161 mg (range, 26–364 mg; $P < 0.05$ compared with either control). None of the mice implanted with 253J-P(IL-8 Low) developed tumors by 6 or 12 weeks. By 12 weeks, lymph node metastasis were observed in the mice implanted with 253J-P(IL-8) and 253J-P(IL-8 High) cells.

The effect of AS IL-8 transfection on the tumorigenicity and metastatic potential of the highly tumorigenic and metastatic cell line 253J B-V was also evaluated (Table 1). By 6 weeks, tumors were palpable in all mice implanted with 253J B-V (median weight, 188 mg; range, 112–284 mg) and 253J B-V(Neo) (median weight, 237 mg; range, 88–516 mg). Tumors also were detected in 9 of 10 mice implanted with 253J B-V(IL-8 High) (median weight, 105 mg; range, 26–333 mg). In contrast, tumorigenicity was significantly reduced after implantation of the 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) cells. Only five of eight mice implanted with 253J B-V(AS IL-8) developed tumors at 6 weeks, and these tumors were significantly smaller than the 253J B-V or 253J B-V(Neo), [median weight, 40 mg; range, 25–321 mg; $P < 0.01$ compared with 253J B-V and 253J B-V(Neo)]. Four of eight mice implanted with 253J B-V(AS IL-8 Low) developed tumors by 6 weeks, and these tumors were also significantly smaller than either 253J B-V or 253J B-V [median weight, 40 mg; range, 27–72 mg; $P < 0.01$ compared with 253J B-V or 253J B-V(Neo)]. Furthermore, no spontaneous lymph node metastases were detected in mice implanted with either 253J B-V(AS IL-8) or 252J B-V(AS IL-8 Low) at 6 weeks [$P < 0.05$, compared with either 253J B-V (four of eight) or 253J B-V(Neo) (eight of nine)]. Therefore, IL-8 expression regulates both the tumorigenicity and metastasis of human TCC.

Expression of IL-8, bFGF, VEGF, and MMP-9. The *in vivo* expressions of IL-8, bFGF, VEGF, and MMP-9 mRNA and protein were evaluated by ISH (Table 2; Fig. 8) and immunohistochemical staining (Table 2; Fig. 9), respectively, and correlated directly with the *in vitro* expression of these factors. Both the 253J-P(IL-8) and 253J-P(IL-8 High) tumors expressed equal amounts of IL-8 and MMP-9 mRNA and protein. Both IL-8 and MMP-9 expression was down-regulated in the AS IL-8-transfected tumors. IL-8 mRNA expression was down-regulated 30 and 55%, and MMP-9 mRNA was down-regulated 29 and 32% in the 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) tumors, respectively. IL-8 protein expression was likewise down-regulated 28 and 40%, and MMP-9 protein was down-regulated 30 and 37% by 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low), respectively. No difference was observed in the *in vivo* expression of bFGF or VEGF mRNA or protein after transfection with IL-8 sense or AS transcripts.

MVD. Tumor-induced neovascularization (MVD) was determined by immunohistochemical staining using anti-CD31 antibodies (Table 2; Fig. 9). The number of $CD31^+$ microvessels counted per $\times 200$ field in the 253J-P(IL-8) and 253J-P(IL-8 High) tumors was 65 ± 21 and 74 ± 25, respectively. Because the 253J-P and 253J-P(Neo) cells did not develop tumors, we could not evaluate the effect of the overexpression of IL-8 on tumor-induced neovascularization. However, AS-IL-8 transfection significantly inhibited tumor-induced neovascularization of the 253J B-V cells. The MVD was significantly reduced from 116 ± 41 and 134 ± 50 in the 253J B-V and 253J B-V(Neo) tumors, respectively, to 65 ± 12 and 47 ± 13 in the 253J B-V(AS IL-8) and 253J B-V(AS IL-8 High) bladder tumors ($P < 0.005$; Table 2; Fig. 9). Tumor-induced neovascularization correlates directly with the IL-8 expression of the tumors and tumor growth and metastasis.

DISCUSSION

Tumor growth and metastasis depend upon the ability of the tumor to induce its own blood supply (8–10). This process, angiogenesis,

Table 2 *The mRNA expression level, protein expression level, and MVD in bladder tumor with sense-IL-8 transfectants (A) and with 253J B-V, AS-IL-8 transfectants, and Neo transfectant (B)*

	mRNA expression index[a]				Protein expression index[b]				
Cell line	IL-8	bFGF	VEGF	MMP-9	IL-8	bFGF	VEGF	MMP-9	MVD[c] (per $\times 200$ field)
Sense transfection[e]									
253J-P(IL-8)	100	100	100	100	100	100	100	100	65 ± 41
253J-P(IL-8 High)	124	98	96	127	126	93	100	132	74 ± 25
AS transfection[e]									
253J B-V	100	100	100	100	100	100	100	100	116 ± 41
253J B-V(AS Neo)	104	100	100	102	108	96	100	100	134 ± 50
253J B-V(AS IL-8)	69	102	105	71	72	104	106	70	65 ± 12[d]
253J B-V(AS IL-8 Low)	45	105	105	68	60	96	106	63	47 ± 13[d]
253J B-V(AS IL-8 High)	73	104	100	90	80	100	106	78	86 ± 20

[a] The intensity of the cytoplasmic color reaction was quantified by an image analyzer and compared with the maximal intensity of the poly d(T) color reaction in each sample. The results are presented as the number of cells for each line with sense-IL-8-transfected 253J-P(IL-8) and 253J B-V defined as 100.
[b] The intensity of the cytoplasmic immunostaining was quantified by an image analyzer in three different areas of each sample to yield an average measurement. The results are presented as the number of cells for each cell line, with sense-IL-8-transfected 253J-P(IL-8) and 253J B-V defined as 100.
[c] MVD was expressed as an average number of five highest areas identified within a single $\times 200$ field.
[d] $P < 0.005$ against 253J B-V and 253J B-V(AS Neo) (Mann-Whitney statistical comparison).
[e] One representative experiment of three.

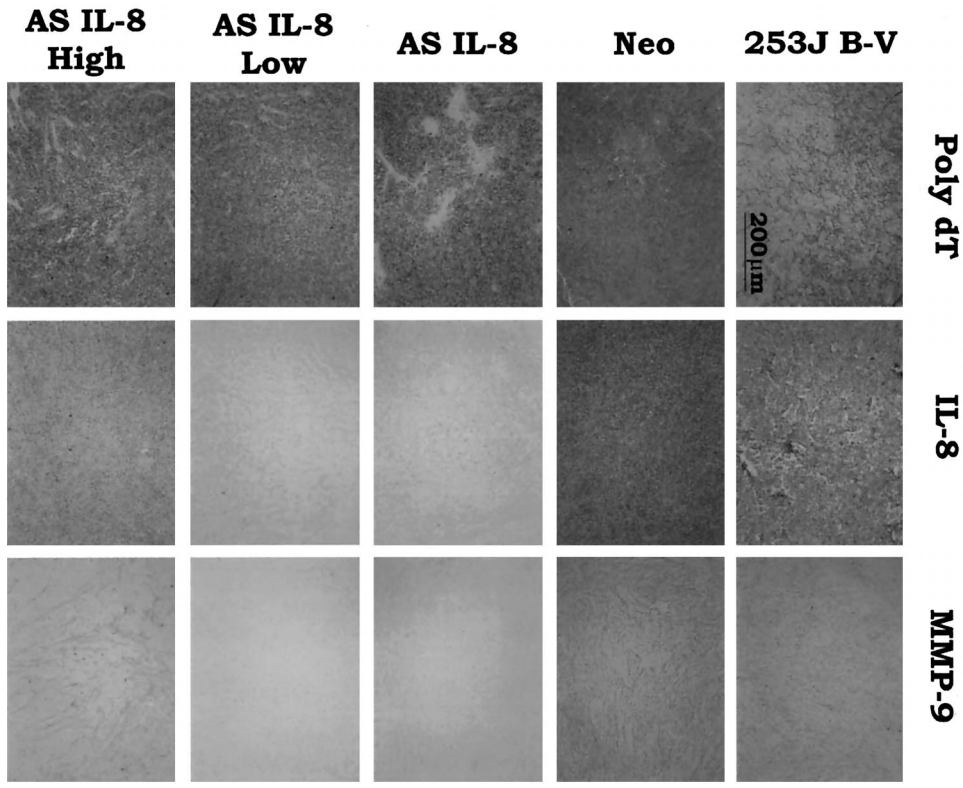

Fig. 8. ISH in 253J-P, 253J-P(Neo), sense-IL-8 transfectants (*IL-8*, *IL-8 Low*, and *IL-8 High*; *A*), and in 253J B-V, 253J B-V(Neo), and AS-IL-8 transfectants (*AS IL-8*, *AS IL-8 Low*, and *AS IL-8 High*; *B*). The intensity of staining was determined by comparison with the integrated absorbance of poly $d(T)_{20}$, which was set to 100. The mRNA expression of IL-8 and MMP-9 was increased 1.3-fold in the tumor of 253J-P(IL-8 High) relative to 253J-P(IL-8). The tumor of 253J B-V(AS IL-8) showed ~30% reduction in the mRNA expression of IL-8 and MMP-9 relative to that of either parental 253J B-V or 253J B-V(Neo). This is one representative experiment of three.

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

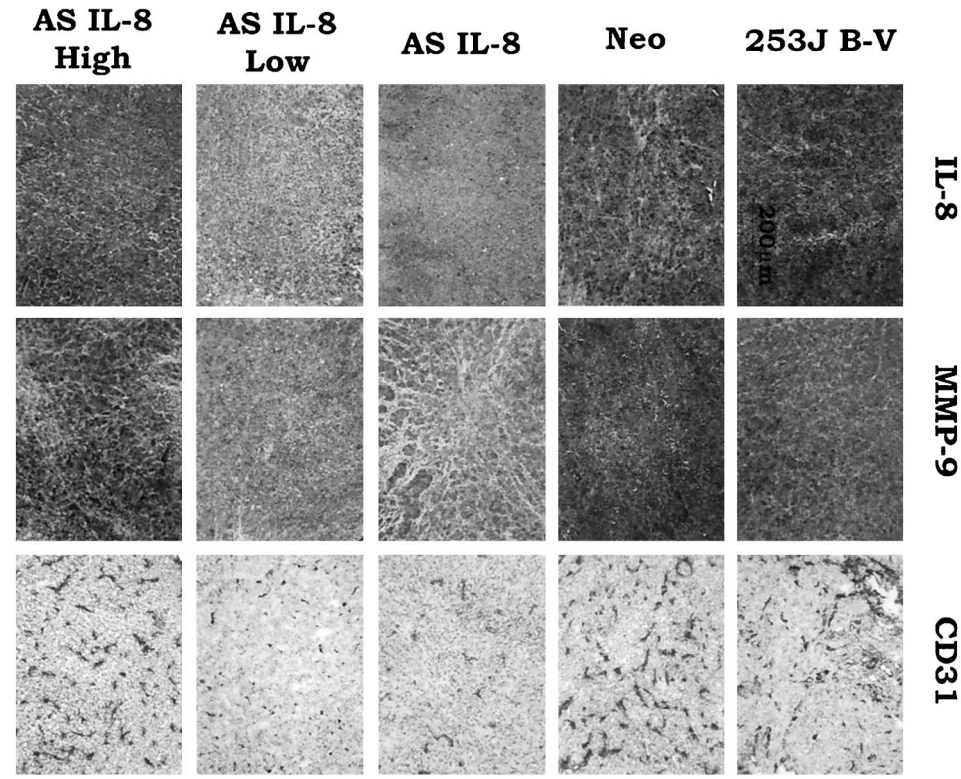

Fig. 9. Immunohistochemistry and MVD in 253J-P, 253J-P(Neo), and sense-IL-8 transfectants (*IL-8*, *IL-8 Low*, and *IL-8 High*; *A*) and in 253J B-V, 253J B-V(Neo), and AS-IL-8 transfectants (*AS IL-8*, *AS IL-8 Low*, and *AS IL-8 High*; *B*). Three different areas in each sample were quantified to yield an average measurement of intensity of immunostaining. The density of microvessels was expressed as an average number of five highest areas identified within a single ×200 field. The protein expression of IL-8 and MMP-9 was increased 1.3-fold in the tumor of 253J-P(IL-8 High) relative to 253J-P(IL-8). The tumor of 253J B-V(AS IL-8) showed 30% reduction in the protein expression of IL-8 and MMP-9 relative to that of either parental 253J B-V or 253J B-V(Neo). The number of $CD31^+$ microvessels counted per ×200 field in bladder tumors of 253J-P(IL-8) and 253J-P(IL-8 High) cells was 65 ± 21 and 74 ± 25, respectively. The number of $CD31^+$ microvessels was reduced from 116 ± 41 or 134 ± 50 in the tumors of parental 253J B-V or 253J B-V(Neo), respectively, as control to 65 ± 12 and 47 ± 13 in the bladder tumor of 253J B-V(AS IL-8) and 253J B-V(AS IL-8 Low) cells, respectively ($P < 0.005$). This is one representative experiment of three.

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

1. Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. Cancer Statistics. CA Cancer J. Clin., *49:* 8–31, 1999.
2. Skinner, D. G., and Lieskovksy, G. Management of invasive and high grade bladder cancer. *In:* D. Skinner and G. Lieskovsky (eds.), Genitourinary Cancer, pp. 295–312. Philadelphia: W. B. Saunders, 1988.
3. Caldwell, W. L. Carcinoma of the urinary bladder. J. Am. Med. Assoc., *16:* 1643–1645, 1974.
4. Killion, J. J., and Fidler, I. J. The biology of tumor metastasis. Semin. Oncol., *16:* 106–115, 1989.
5. Fidler, I. J., and Hart, R. I. Biological diversity in metastatic neoplasms: origins and implications. Science (Washington DC), *217:* 998–1003, 1982.
6. Fidler, I. J., and Balch, C. M. The biology of cancer metastasis and implications for therapy. Curr. Probl. Surg., *24:* 129–209, 1987.
7. Fidler, I. J. Critical factors in the biology of human cancer metastasis: twenty-eighth G. H. A. Clowes Memorial Award Lecture. Cancer Res., *50:* 6130–6138, 1990.
8. Folkman, J. What is the evidence that tumors are angiogenesis dependent? J. Natl. Cancer Inst., *82:* 4–6, 1990.
9. Folkman, J. How is blood vessel growth regulated in normal and neoplastic tissue? G. H. A. Clowes Memorial Award Lecture. Cancer Res., *46:* 467–473, 1986.
10. Folkman, J., and Shing, Y. Angiogenesis. J. Biol. Chem., *267:* 10931–10934, 1992.
11. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell, *64:* 327–336, 1991.
12. Crew, J. P., O'Brien, T., Bradburn, M., Fuggle, S., Bicknell, R., Cranston, D., and Harris, A. L. Vascular endothelial growth factor is a predictor of relapse and stage progression in superficial bladder cancer. Cancer Res., *57:* 5281–5285, 1997.
13. O'Brien, T., Cranston, D., Fuggle, S., Bicknell, R., and Harris, A. L. Different angiogenic pathways characterize superficial and invasive bladder cancer. Cancer Res., *55:* 510–513, 1995.
14. Allen, L. E., and Maher, P. A. Expression of basic fibroblast growth factor and its receptor in an invasive bladder carcinoma cell line. J. Cell. Physiol., *155:* 368–375, 1993.
15. Nguyen, M., Watanabe, H., and Budson, A. E. Elevated levels of the angiogenic peptide basic fibroblast growth factor in urine of bladder cancer patients. J. Natl. Cancer Inst., *85:* 241–242, 1993.
16. O'Brien, T., Cranston, D., Fuggle, S., Bicknell, R., and Harris, A. L. Two mechanisms of basic fibroblast growth factor-induced angiogenesis in bladder cancer. Cancer Res., *57:* 136–140, 1997.
17. O'Brien, T., Cranston, D., Fuggle, S., Bicknell, R., and Harris, A. L. The angiogenic factor midkine is expressed in bladder cancer, and overexpression correlates with a poor outcome in patients with invasive cancers. Cancer Res., *56:* 2515–2518, 1996.
18. Kubota, Y., Miura, T., Moriyama, M., Noguchi, S., Matsuzaki, J., Takebayashi, S., and Hosaka, M. Thymidine phosphorylase activity in human bladder cancer: difference between superficial and invasive cancer. Clin. Cancer Res., *3:* 973–976, 1997.
19. Tachibana, M., Miyakawa, A., Nakashima, J., Murai, M., Nakamura, K., Kubo, A., and Hata, J. I. Constitutive production of multiple cytokines and a human chorionic gonadotrophin β-subunit by a human bladder cancer cell line (KU-19-19): possible demonstration of totipotential differentiation. Br. J. Cancer, *76:* 163–174, 1997.
20. Bochner, B. H., Cote, R. J., and Weidner, N. Angiogenesis in bladder cancer: relationship between microvessel density and tumor prognosis. J. Natl. Cancer Inst., *87:* 1603–1612, 1995.
21. Matsushima, K., Morishita, K., Yoshimura, T., Lavu, S., Kobayashi, Y., Lew, W., Appella, E., Kung, H. F., Leonard, E. J., and Oppenheim, J. J. Molecular cloning of

a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor. J. Exp. Med., *167:* 1883–1893, 1988.
22. Matsushima, K., Baldwin, E. T., and Mukaida, N. Interleukin-8 and MCAF: novel leukocyte recruitment and activating cytokines. Chem. Immunol., *51:* 236–265, 1992.
23. Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G., and Strieter, R. M. Interleukin-8 as a macrophage-derived mediator of angiogenesis [see comments]. Science (Washington DC), *258:* 1798–1801, 1992.
24. Strieter, R. M., Kunkel, S. L., Elner, V. M., Martonyi, C. L., Koch, A. E., Polverini, P. J., and Elner, S. G. Interleukin-8. A corneal factor that induces neovascularization. Am. J. Pathol., *141:* 1279–1284, 1992.
25. Schadendorf, O., Moller, A., Algermissen, B., Worm, M., Sticherling, M., and Czarnetzki, B. M. IL-8 produced by human malignant melanoma cells *in vitro* is an essential autocrine growth factor. J. Immunol., *151:* 2667–2675, 1993; erratum *153:* 3360, 1994.
26. Singh, R. K., Gutman, M., Radinsky, R., Bucana, C. D., and Fidler, I. J. Expression of interleukin 8 correlates with the metastatic potential of human melanoma cells in nude mice. Cancer Res., *54:* 3242–3247, 1994.
27. Luca, M., Huang, S., Gershenwald, J. E., Singh, R. K., Reich, R., and Bar-Eli, M. Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. Am. J. Pathol., *151:* 1105–1113, 1997.
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 cell lung cancer in SCID mice. J. Clin. Investig., *97:* 2792–2802, 1996.
29. Greene, G. F., Kitadai, Y., Pettaway, C. A., von Eschenbach, A., Bucana, C. D., 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* messenger RNA hybridization technique. Am. J. Pathol., *150:* 1571–1582, 1997.
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 vascularity 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 carcinomas in nude mice. J. Natl. Cancer Inst., *90:* 447–454, 1998.
32. Dinney, C. P., Fishbeck, R., Singh, R. K., Eve, B., Pathak, S., Brown, N., Xie, B., Fan, D., Bucana, C. D., Fidler, I. J., and Killion, J. J. Isolation and characterization of metastatic variants from human transitional cell carcinoma passaged by orthotopic implantation in athymic nude mice. J. Urol., *154:* 1532–1538, 1995.
33. Rogelj, S., Weinberg, R. A., Fanning, P., and Klagsbrun, M. Basic fibroblast growth factor fused to a signal peptide transforms cells. Nature (Lond.), *331:* 173–175, 1988.
34. Berse, B., Brown, L. F., Van de Water, L., Dvorak, H. F., and Senger, D. R. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. Mol. Biol. Cell, *3:* 211–220, 1992.
35. Fort, P., Marty, L., Piechaczyk, M., El Sabrouty, S., Dani, C., Jeanteur, P., and Blanchard, J. M. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res., *13:* 1431–1442, 1985.
36. Feinberg, A. P., and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem., *132:* 6–13, 1983.
37. Perrotte, P., Matsumoto, T., Inoue, K., Kuniyasu, H., Eve, B. Y., Hicklin, D. J., Radinsky, R., and Dinney, C. P. Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. Clin. Cancer Res., *5:* 257–265, 1999.
38. Nakajima, M., Lotan, D., Baig, M. M., Carralero, R. M., Wood, W. R., Hendrix, M. J., and Lotan, R. Inhibition by retinoic acid of type IV collagenolysis and invasion through reconstituted basement membrane by metastatic rat mammary adenocarcinoma cells. Cancer Res., *49:* 1698–1706, 1989.
39. Wei, Q., Guan, Y., Cheng, L., Radinsky, R., Bar-Eli, M., Tsan, R., Li, L., and Legerski, R. J. Expression of five selected human mismatch repair genes simultaneously detected in normal and cancer cell lines by a nonradioactive multiplex reverse transcription-polymerase chain reaction. Pathobiology, *65:* 293–300, 1997.
40. Repesh, L. A. A new *in vitro* assay for quantitating tumor cell invasion. Invasion Metastasis, *9:* 192–208, 1989.
41. Kitadai, Y., Bucana, C. D., Ellis, L. M., Anzai, H., Tahara, E., and Fidler, I. J. *In situ* mRNA hybridization technique for analysis of metastasis-related genes in human colon carcinoma cells. Am. J. Pathol., *147:* 1238–1247, 1995.
42. Bucana, C. D., Radinsky, R., Dong, Z., Sanchez, R., Brigati, D. J., and Fidler, I. J. A rapid colorimetric *in situ* mRNA hybridization technique using hyperbiotinylated oligonucleotide probes for analysis of mdr1 in mouse colon carcinoma cells. J. Histochem. Cytochem., *41:* 499–506, 1993.
43. Radinsky, R., Bucana, C. D., Ellis, L. M., Sanchez, R., Cleary, K. R., Brigati, D. J., and Fidler, I. J. A rapid colorimetric *in situ* messenger RNA hybridization technique for analysis of epidermal growth factor receptor in paraffin-embedded surgical specimens of human colon carcinomas. Cancer Res., *53:* 937–943, 1993.
44. Reed, J. A., Manahan, L. J., Park, C. S., and Brigati, D. J. Complete one-hour immunocytochemistry based on capillary action. Biotechniques, *13:* 434–443, 1992.
45. Vecchi, A., Garlanda, C., Lampugnani, M. G., Resnati, M., Matteucci, C., Stoppacciaro, A., Schnurch, H., Risau, W., Ruco, L., Mantovani, A., *et al*. Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium. Eur. J. Cell Biol., *63:* 247–254, 1994.
46. Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis–correlation in invasive breast carcinoma. N. Engl. J. Med., *324:* 1–8, 1991.
47. Gorman, C. M., Moffat, L. F., and Howard, B. H. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol., *2:* 1044–1051, 1982.
48. Hudson, J. M., Frade, R., and Bar, E. M. Wild-type p53 regulates its own transcription in a cell-type specific manner. DNA Cell Biol., *14:* 759–766, 1995.
49. Moore, B. B., Arenberg, D. A., Stoy, K., Morgan, T., Addison, C. L., Morris, S. B., Glass, M., Wilke, C., Xue, Y. Y., Sitterding, S., Kunkel, S. L., Burdick, M. D., and Strieter, R. M. Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells. Am. J. Pathol., *154:* 1503–1512, 1999.
50. Holmes, W. E., Lee, J., Kuang, W. J., Rice, G. C., and Wood, W. I. Structure and functional expression of a human interleukin-8 receptor. Science (Washington DC), *253:* 1278–1280, 1991.
51. Murphy, P. M., and Tiffany, H. L. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. Science (Washington DC), *253:* 1280–1283, 1991.
52. Singh, R., Reich, R., Radinsky, R., Berry, K., Dave, B., and Fidler, I. Expression of basic fibroblast growth factor is necessary but insufficient for production of metastasis. Int. J. Oncol., *10:* 23–31, 1996.
53. Claffey, K. P., Brown, L. F., Tognazzi, K., Yeo, K. T., Manseau, E. J., and Dvorak, H. F. Expression of vascular permeability factor/vascular endothelial growth factor by melanoma cells increases tumor growth, angiogenesis, and experimental metastasis. Cancer Res., *56:* 172–181, 1996.
54. McLeskey, S. W., Tobias, C. A., Vezza, P. R., Filie, A. C., Kern, F. G., and Hanfelt, J. Tumor growth of FGF or VEGF transfected MCF-7 breast carcinoma cells correlates with density of specific microvessels independent of the transfected angiogenic factor. Am. J. Pathol., *153:* 1993–2006, 1998.
55. Kumar, R., Yoneda, J., Bucana, C. D., and Fidler, I. J. Regulation of distinct steps of angiogenesis by different angiogenic molecules. Int. J. Oncol., *12:* 749–757, 1998.
56. Liotta, L. A., Thorgeirsson, U. P., and Garbisa, S. Role of collagenases in tumor cell invasion. Cancer Metastasis Rev., *1:* 277–288, 1982.
57. Stetler-Stevenson, W. Type IV collagenases in tumor invasion and metastasis. Cancer Metastasis Rev., *9:* 289–303, 1990.
58. Hamdy, F. C., Fadlon, E. J., Cottam, D., Lawry, J., Thurrell, W., Silcocks, P. B., Anderson, J. B., Williams, J. L., and Rees, R. C. Matrix metalloproteinase 9 expression in primary human prostatic adenocarcinoma and benign prostatic hyperplasia. Br. J. Cancer, *69:* 177–182, 1994.
59. Sehgal, G., Hua, J., Bernhard, E. J., Sehgal, I., Thompson, T. C., and Muschel, R. J. Requirement for matrix metalloproteinase-9 (gelatinase B) expression in metastasis by murine prostate carcinoma. Am. J. Pathol., *152:* 591–596, 1998.
60. Festuccia, C., Dolo, V., Guerra, F., Violini, S., Muzi, P., Pavan, A., and Bologna, M. Plasminogen activator system modulates invasive capacity and proliferation in prostatic tumor cells. Clin. Exp. Metastasis, *16:* 513–528, 1998.
61. Stearns, M. E., and Stearns, M. Immunohistochemical studies of activated matrix metalloproteinase-2 (MMP-2a) expression in human prostate cancer. Oncol. Res., *8:* 63–67, 1996.
62. Miyake, H., Hoshimura, K., Hara, I., Eto, H., Arakawa, S., and Kamidono, S. Basic fibroblast growth factor regulates matrix metalloproteinases production and *in vitro* invasiveness in human bladder cancer cell lines. J. Urol., *157:* 2351–2355, 1997.