Interleukin 8 Expression Regulates Tumorigenicity and Metastases in Androgen-independent Prostate Cancer


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 metastatic PC-3M-LN4 cell line overexpresses IL-8 relative to the poorly metastatic PC-3P cell line. We evaluated whether IL-8 expression by human prostate cancer growing within the prostate of athymic nude mice regulates tumor angiogenesis, growth, and metastasis. PC-3P cells were transfected with the full-length sense IL-8 cDNA, whereas PC-3M-LN4 cells were transfected with the full-sequence antisense IL-8 cDNA. Control cells were transfected with the neomycin resistance gene (Neo). In vitro, sense-transfected PC-3P cells overexpressed IL-8-specific mRNA and protein, which resulted in up-regulation of matrix metalloproteinase 9 (MMP-9) mRNA, and collagenase activity, resulting in increased invasion through Matrigel. After antisense transfection of the PC-3M-LN4 cells, IL-8 and MMP-9 expression, collagenase activity, and invasion were markedly reduced relative to controls. After orthotopic implantation, the sense-transfected PC-3P cells were highly tumorigenic and metastatic, with significantly increased neovascularity and IL-8 expression compared with either PC-3P cells or controls. Antisense transfection significantly reduced the expression of IL-8 and MMP-9 and tumor-induced neovascularity, resulting in inhibition of tumorigenicity and metastasis. These results demonstrate that IL-8 expression regulates angiogenesis in prostate cancer, in part by induction of MMP-9 expression, and subsequently regulates the growth and metastasis of human prostate cancer.

INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer-related deaths among men in the United States (1). Although modest improvements in early detection and therapy have occurred (2, 3), most deaths from prostate cancer are caused by metastases that resist conventional androgen-deprivation therapy (4–6). Continued empiricism in the treatment of advanced prostate cancer is unlikely to produce significant improvement over current therapy. Rather, knowledge of the cellular and molecular properties of prostate 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 (7–11). A crucial step in metastasis is vascularization in and around the tumor (12, 13). The balance between stimulatory and inhibitory factors released by the tumor and the microenvironment regulates this process of angiogenesis (14–16). Human prostate cancer produces a number of proangiogenic factors, including VEGF (17, 18), bFGF (19, 20), and IL-8 (18, 21, 22). MVD, a pathological surrogate for angiogenesis, correlates with stage and prognosis for patients with prostate cancer (23).

IL-8 was originally identified as a leukocyte chemotactic factor (24, 25) but is now also known to be an autocrine growth factor for malignant melanoma (26) and keratinocytes (27). In addition, IL-8 displays mitogenic and morphogenic activity for endothelial cells (28) and regulates angiogenesis in lung cancer (29, 30) and melanoma (31, 32). IL-8 is expressed by prostate cancer (18, 21, 22). MVD, a pathological surrogate for angiogenesis, correlates with stage and prognosis for patients with prostate cancer (23).

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3 The abbreviations used are: VEGF, vascular endothelial cell growth factor; bFGF, basic fibroblast growth factor; IL, interleukin; rIL, recombinant IL; MMP, matrix metalloproteinase; MVD, microvessel density; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CEM, complete Eagle’s minimum essential medium; ISH, in situ hybridization; IHC, immunohistochemical staining; ActD, actinomycin D; CAT, chloramphenicol acetyltransferase; RT-PCR, reverse transcription-PCR.

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MMP-2 by human melanoma (32), we determined whether IL-8 also regulated MMP-9 expression by human prostate cancer.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Cells of the highly metastatic human prostate carcinoma cells line PC-3M-LN4 (39) and the poorly metastatic cell line PC-3P (40) were grown as monolayer cultures in RPMI 1640 supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, nonessential amino acids, and penicillin-streptomycin [complete RPMI (CRPMI); Ref. 39].

Transfection and Selection of PC-3P and PC-3M-LN4 Cells Expressing IL-8. Tumor cells were plated onto 100-mm dishes at a density of 1 × 10⁶/dish. The monolayers (60–70% confluent) were transfected with pcDNA3/sense IL-8, pcDNA3/antisense IL-8 [a gift from Dr. K. Matsushima (24)], or control pcDNA3.neo plasmids 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 modified complete MEM (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 of the lines were maintained in CMEM/G418 and frozen after one to three passages. To avoid clonal variations, six positive clones were then pooled for the in vitro and in vivo studies.

The less tumorigenic and metastatic PC-3P cells and the highly tumorigenic, highly metastatic PC-3M-LN4 cells were transfected with pcDNA3/sense IL-8 or pcDNA3/antisense IL-8, respectively, or with control pcDNA3.neo. Individual G418-resistant (500–1000 μg/ml) colonies were established as separate adherent cultures. We selected pooled sense IL-8-transfected PC-3P cells [PC-3P(IL-8)], the highest IL-8-expressing clone [PC-3P(IL-8 High)], and the lowest IL-8-expressing clone [PC-3P(IL-8 Low)], and we selected pooled antisense IL-8-transfected PC-3LN4 cells [PC-3M-3LN4(AS IL-8)], the highest IL-8-expressing clone [PC-3M-3LN4(AS IL-8 High)], and the lowest IL-8-expressing clone [PC-3M-3LN4(AS IL-8 Low)], according to 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 10⁶ cultured cells using the Fasttrack mRNA isolation kit (Invitrogen, San Diego, CA). The mRNA was electrophoresed onto 1% denatured formaldehyde agarose gel, electrotransferred to Genescreen nylon membrane (DuPont, Boston, MA), and cross-linked with a UV Stratalinker 1800 (Stratagene) at 120,000 mJ/cm². Filters were washed twice at 65°C with 30 mM NaCl/3 mM sodium citrate-0.1% SDS (w/v). 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) a 0.5-kb EcoRI cDNA fragment corresponding to human IL-8 (a gift of Dr. K. Matsushima, Kanazawa, Japan; Ref. 24); (b) a 1.4-kb cDNA fragment of bovine bFGF (41); (c) a 204-kb fragment of human VEGF cDNA inserted in a PGEM-based construct (a gift Dr. B. Berse, Harvard Medical School, Boston, MA; Ref. 42); (d) a 1.0-kb cDNA fragment correspond-

ELISA for IL-8, bFGF, and VEGF. Viable cells (5 × 10⁶) 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 10% bovine serum supplemented by fresh MEM were added. Twenty-four hours 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 absorbance comparison to the standard curve. Results were expressed as numbers of cells (45).

Growth Curve. Viable cells (1 × 10⁴) were seeded in a 96-well plate. Conditioned medium was removed after 24 h, and the cells were washed with 200 μl of HBSS. Either 200 μl of fresh CRPMI medium or CMEM/G418 conditioned medium were added. Every 24 h, the numbers of viable cells in each cell line were determined by absorbance comparison. The doubling time of each cell line was determined by plotting the absorbance on a semilogarithmic axis versus time (Cricket Software, Malvern, PA). The doubling times of the PC-3P sense IL-8 transfectants (IL-8, 40.1 h; IL-8 Low, 38.1 h; IL-8 High, 41.2 h) were similar to those of PC-3P (38.6 h) and PC-3P(Neo) (39.1 h). The doubling times of the PC-3M-LN4 antisense IL-8-transfectants (AS IL-8, 22.0 h; AS IL-8 Low, 21.5 h; AS IL-8 High, 21.7 h) were similar to those of PC-3M-LN4 (22.0 h) and PC-3M-LN4(Neo) (22.6 h).

Collagenase Activity. To determine collagenase activity, electrophoresis of serum-free conditioned medium was performed as described previously (46). Cells (5 × 10⁴) 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 supernatant fluid was collected to determine collagenase activity, and the remaining cells were counted to confirm the cell number. Collected samples were centrifuged to concentrate using MICROCON microconcentrators (Amicon, Inc., Beverly, MA). Thirty μl in each sample with 10 μl loading buffer (10% SDS) were electrophoresed on 20% SDS-polyacrylamide gels containing 1 ng/ml gelatin. After electrophoresis, gels were washed in 2.5% Triton X-100 to remove SDS and allow proteins to renature. Then gels were immersed in incubation buffer containing 1 Triton X-100, 50 mM Tris (pH 7.5), 5 mM CaCl₂, and 1 mM ZnCl₂ for 24 h at 37°C. The zymograms were stained with 0.1% (w/v) Coomassie Blue R-250 (Sigma) and destained in 40% methanol-10% acetic acid. Identification
of a transparent band at $M_r$ 92,000 on the Coomassie blue background of the slab gel was considered positive for the presence of the enzymatic activity. The collagenase activity was quantified using densitometry of Image Quant software program (Molecular Dynamics, Sunnyvale, CA).

To determine whether the increase in MMP-9 activity is mediated by IL-8, we incubated parental PC-3P cells in the presence of different doses (0–20 μg/ml) of human rIL-8, and the activity of MMP-9 was determined. We then determined the increased activity of MMP-9 by rIL-8 was inhibited by neutralization by using an anti-IL-8 antibody (100 μg/ml), with a nonspecific IgG (100 μg/ml) as a control.

**PCR Analysis.** RT-PCR analysis was performed as described previously (47). Briefly, total cellular RNA (1 mg) extracted from various cell lines was transcribed into cDNA using downstreaming primers IL-8 receptors type A and B, respectively (Reverse Transcription System; Promega). 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), and 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: for IL-8 receptor type A, sense 5'-AGT TCT TGG CAC GTC ATC G-3' and antisense 5'-CTT GGA GGT ACC TCA ACA GC-3'; and for IL-8 receptor type B, sense 5'-ACA TTC CTG TGC AAG GTG G-3' and antisense 5'-CAG GGT GAA TCC GTA GCA GA-3'.

**Invasion Assay through Matrigel.** Polynylprolidone-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 mg/filter) and placed in modified Boyden chambers. The cells (2 × 10^5) were released from their culture dishes by short exposure to EDTA (1 mmol/l), centrifuged, resuspended in 0.1% BSA-DMEM, and placed in the upper compartment of the Boyden chamber. Fibroblast-conditioned medium in the lower compartment served as a chemoattractant. 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, McGraw Park, IL) and quantified with a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering, Goletha, CA) linked to a computer and digital printer (Sony Corporation, 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 (48).
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 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 PC-3P, PC-3M-LN4, Neo, sense, and antisense IL-8 transfected cells (60–70% confluent) were prepared for injection as described previously (39, 49). Mice were anesthetized with methoxyflurane. For orthotopic implantation, a lower midline incision was made, and viable tumor cells (2 \times 10^6/40 \mu l) in HBSS were implanted into the dorsal prostate lobes using a 30-gauge needle with a 1-ml disposable syringe and a calibrated push button-controlled dispensing device (Hamilton Syringe Company, Reno, NV). Formation of a bulla indicated a satisfactory injection. The prostate was returned to the abdominal cavity, and the abdominal wall was closed with a single layer of metal clips. Mice were killed 6 weeks after implantation of tumor cells. The primary tumors were removed and weighed, and the presence of metastases (in the lymph nodes) was determined grossly and microscopically. The prostates 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.

ISH Analysis. Specific antisense 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% guanosine cytosine (GC) content (21); bFGF (CGG GAA GGC GCC GCT GCC), 85.7% GC content (41); VEGF/VPF (TGG TGA TGT TGG ACT CTT CAG TGG GCU), 57.7% GC content (42); and MMP-9 (CCG GTC CAC CTC GCT GGC GCT CCG GU), 80.0% GC content (21). 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 (50). 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 to a stock solution at 1 mg/ml in 10 mmol/l Tris (pH 7.6) and 1 mmol/l EDTA. Immediately before use, the stock solution was diluted with probe dilution (Research Genetics, Huntsville, AL).

In situ mRNA hybridization was performed as described previously with minor modifications, (51, 52) using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 53). Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific; Refs. 51 and 52). 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× sodium saline chloride (Research Genetics) for 2 min at 45°C. The samples were incubated with alkaline phosphatase-labeled avidin for 1 min, and incubated with a chromogen substrate for 15 min at 45°C. Additional incubation with fresh chromogen substrate was performed if necessary to enhance a weak reaction in this assay; a red staining indicated a

Fig. 3  Gelatinolytic activity of conditioned medium of PC-3P, Neo transfectant PC-3P(Neo), and sense IL-8 transfectants PC-3P(IL-8), PC-3P(IL-8 Low), and PC-3P(IL-8 High) (A) and of PC-3M-LN4, Neo transfectant PC-3M-LN4(Neo), and antisense IL-8 transfectants PC-3M-LN4(AS IL-8), PC-3M-LN4(AS IL-8 Low), and PC-3M-LN4(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). The collagenase activity of PC-3P(IL-8) and PC-3P(IL-8 High) cells was increased 6.0- and 7.0-fold, respectively, and that of PC-3M-LN4(AS IL-8) and PC-3M-LN4(AS IL-8 Low) cells was decreased 2.5-to 5.0-fold.

Fig. 4  Regulation of MMP-9 activity by IL-8. We next analyzed whether the increase in MMP-9 activity is mediated by IL-8. Parental PC-3P 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 at left indicate that IL-8 caused an increase in the activity of MMP-9 in a dose-dependent manner. Moreover, the increased activity of MMP-9 by rIL-8 was inhibited by neutralization with anti-IL-8 antibody (100 μg/ml) (right).
positive reaction. Control for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and the use of 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., Tokyo, Japan). The images were analyzed using the Optimas image analysis software (version 4.10; Bioscan, 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 each field 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 cells for each cell line compared with the control, which was set to 100 (45).

**IHC.** 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, antigen retrieval was performed with pepsin for 12 min, and 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 the appropriate dilution (1:100) of rat monoclonal anti-CD31 antibody (PharMingen, San Diego, CA; Ref. 54), 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 anti-rat IgG (H+L) (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA), antirabbit IgG, F(ab)₂ fragment (Jackson ImmunoResearch Laboratory, Inc.), or antimouse IgG1 (PharMingen, San Diego, CA). 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 again washed three times with PBS. The slides were mounted with Universal Mount mounting medium (Research Genetics).

Quantification of Microvessel Density. Microvessel density was determined by light microscopy after immunostaining frozen sections with anti-CD31 antibodies as described by Weidner et al. (55). 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 CCD Optotronics Tec 470 camera (Optotronics Engineering, Goletha, CA) 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 quantitated in each sample by an image analyzer using the Optimas software program (Bioscan). Three different areas in each sample were quantified to yield an average measurement. The results were presented as the number of cells for each cell line compared with the control, which was set to 100 (45).

MMP-9 mRNA Half-Life Studies. To determine the effect of IL-8 on MMP-9 mRNA stability, PC-3P, PC-3P(Neo), and PC-3P(IL-8) cells and PC-3M-LN4, PC-3M-LN4(Neo), and...
PC-3M-LN4(AS IL-8) cells were incubated for 24 h. Further transcription in the cells was then blocked by the addition of ActD (Calbiochem-Novabiotherapy, Inc., Lake Placid NY; final concentration, 5 μg/ml). Total RNA was extracted from the cells at 0, 1, 2, and 4 h after the addition of ActD, and MMP-9 mRNA expression was determined by Northern blot analysis. 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).

**CAT Assay.** Using the FuGENE 6 protocol (Boehringer Mannheim Corp.), we transfected with the basic CAT expression vector with no promoter/enhancer sequences (pCAT-basic) or a control plasmid with SV40 promoter and enhancer (pCAT-control; Promega Corp., Madison, WI) into PC-3P cells, sense-transfected PC-3P cells, PC-3M-LN4 cells, antisense-transfected PC-3M-LN4 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 in a six-well tissue culture dish 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 (56), as Hudson et al. (57) described previously. Each assay was repeated twice; there was <10% variation in transfection efficiency. The CAT assay was quantified by densitometry of autoradiographs with the use of the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA) and was evaluated as the ratio of acetylated species to all species.

**Statistical Analysis.** The Mann-Whitney U test analyzed the statistical differences in vessel counts and staining intensity for IL-8, bFGF, VEGF, and MMP-9 of prostate tumors. The incidences of tumor and metastasis were statistically analyzed by χ² test. A value of P < 0.05 was considered significant.

**RESULTS**

**In Vitro Expression of IL-8, bFGF, VEGF, and MMP-9.**

Northern blot analysis for IL-8, bFGF, VEGF, and MMP-9 steady-state gene expression by PC-3P, PC-3P(IL-8), PC-3P(IL-8 Low), PC-3P(IL-8 High), and PC-3P(Neo) is shown in Fig. 1A, and that by PC-3M-LN4, PC-3M-LN4(AS IL-8), PC-3M-LN4(AS IL-8 Low), PC-3M-LN4(AS IL-8 High), and PC-3M-LN4(Neo) is shown in Fig. 1B. The level of expression is shown as the ratio of mRNA expression by the transfectants to that by the corresponding parental and Neo transfected cell lines (which in both cases was equivalent for all three factors). IL-8 mRNA expression levels were increased 10.4- and 15.1-fold, respectively, compared with either PC-3P or PC-3P(Neo) and decreased 2.5- and 10.0-fold by PC-3M-LN4(AS IL-8) and PC-3M-LN4(AS IL-8 Low) cells, respectively, compared with either PC-3M-LN4 or PC-3M-LN4(Neo).
ELISA (Fig. 2). Changes in protein expression by the transfectants paralleled the changes seen in mRNA expression. IL-8 expression levels were 3.0- and 6.0-fold higher in the PC-3P(IL-8) and PC-3P(IL-8 High) cells, respectively, than in the PC-3P parental cell line. PC-3M-LN4(AS IL-8) and PC-3M-LN4(AS IL-8 Low) cell lines were only one-quarter and one-tenth of those in the parental PC-3M-LN4 cells, respectively. bFGF and VEGF protein expression levels were unchanged in all cell lines after transfection.

Metalloproteinase Expression after IL-8 Transfection.
Because IL-8 regulates protease activity by human melanoma, we evaluated whether MMP-9 expression was altered in the PC-3P and PC-3M-LN4 cells by transfection with sense or antisense IL-8 transcripts. Fig. 1 shows that MMP-9 mRNA expression levels were 3.0- and 6.0-fold higher in PC-3P(IL-8) and PC-3P(IL-8 High) cells, respectively, compared with their controls, but the values for both PC-3M-LN4(AS IL-8) and PC-3M-LN4(AS IL-8 Low) cells were only one-third of those in the parental cell lines. These results demonstrate that IL-8 regulates MMP-9 mRNA expression by the PC-3P and PC-3M-LN4 human prostate cancer cells. Our results are consistent with reports that IL-8 regulates MMP expression by malignant melanoma (31, 32).

Table 1
Tumorigenicity and production of spontaneous metastases after orthotopic implantation of PC-3P, PC-3P(Neo), and sense IL-8 transfectants and of PC-3M-LN4, PCM-LN4 (AS Neo), and antisense IL-8 transfectant in prostate of nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumorigenicity</th>
<th>Lymph node metastasis</th>
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<tbody>
<tr>
<td></td>
<td>Incidence</td>
<td>Median prostate weight (range) (mg)</td>
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<tr>
<td>Sense transfection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-3P</td>
<td>4/7</td>
<td>55 (24–480)</td>
</tr>
<tr>
<td>PC-3P(Neo)</td>
<td>6/9</td>
<td>120 (27–443)</td>
</tr>
<tr>
<td>PC-3P(IL-8)</td>
<td>8/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,270 (258–1,850)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>PC-3P(IL-8 Low)</td>
<td>5/8</td>
<td>155 (25–720)</td>
</tr>
<tr>
<td>PC-3P(IL-8 High)</td>
<td>9/9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,975 (355–2,915)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Antisense transfection</td>
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<tr>
<td>PC-3M-LN4</td>
<td>5/5</td>
<td>1,921 (250–3,359)</td>
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<tr>
<td>PC-3M-LN4(AS Neo)</td>
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<td>PC-3M-LN4(AS IL-8)</td>
<td>2/8&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>(33, 36)&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>PC-3M-LN4(AS IL-8 Low)</td>
<td>2/8&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>(31, 39)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PC-3M-LN4(AS IL-8 High)</td>
<td>6/9</td>
<td>39 (30–525)&lt;sup&gt;f&lt;/sup&gt;</td>
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<sup>a</sup> P < 0.05 against PC-3P and PC-3PNeo (<sup>x</sup><sup>2</sup> test).
<sup>b</sup> P < 0.005 against PC-3P and PC-3P(Neo) (Mann-Whitney statistical comparison).
<sup>c</sup> P < 0.05.
<sup>d</sup> Explants grown in culture; no detectable tumor by immunohistochemistry.
<sup>e</sup> P < 0.0005 against PC-3M-LN4 and PC-3M-LN4(AS Neo) (Mann-Whitney statistical comparison).
<sup>f</sup> P < 0.0005 against PC-3M-LN4 and PC-3M-LN4(AS Neo) (<sup>x</sup><sup>2</sup> test).

Table 2
The mRNA expression level, protein expression level, and MVD in prostate tumor with PC-3P, PC-3P(Neo), and sense IL-8 transfectants and with PC-3M-LN4, PC-3M-LN4(AS Neo), and antisense IL-8 transfectants

<table>
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<tr>
<th>Cell line</th>
<th>mRNA expression index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protein expression index&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Microvessel density&lt;sup&gt;c&lt;/sup&gt; (per ×200 field)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-8</td>
<td>bFGF</td>
<td>VEGF</td>
</tr>
<tr>
<td>Sense transfection</td>
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<td></td>
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<tr>
<td>PC-3P</td>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PC-3P(Neo)</td>
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<td>98</td>
<td>99</td>
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<tr>
<td>PC-3P(IL-8)</td>
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<td>99</td>
<td>94</td>
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<tr>
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<tr>
<td>PC-3P(IL-8 High)</td>
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<td>96</td>
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<tr>
<td>Antisense transfection</td>
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<tr>
<td>PC-3M-LN4</td>
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<td>100</td>
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<tr>
<td>PC-3M-LN4(AS Neo)</td>
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<td>97</td>
</tr>
<tr>
<td>PC-3M-LN4(AS IL-8 High)</td>
<td>58</td>
<td>98</td>
<td>94</td>
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</table>

<sup>a</sup> The intensity of the cytoplasmic color reaction was quantified by an image analyzer and compared with maximal intensity of poly d(T) color reaction in each sample. The results were presented as the number of calls for each line with PC-3P and PC-3M-LN4 defined as 100.
<sup>b</sup> The intensity of the cytoplasmic immunostaining was quantified by an image analyzer in three different areas of each sample to yield an average measurement and compared with the intensity of the normal epithelial cells of prostate glands and adjusted to the intensity of the cells of the tumors with parental cell line defined as 100.
<sup>c</sup> MVD was expressed as an average number of five highest areas identified within a single ×200 field.
<sup>d</sup> P < 0.005 against PC-3P and PC-3P(Neo).
<sup>e</sup> P < 0.005 against PC-3M-LN4 and PC-3M-LN4(AS Neo) (Mann-Whitney statistical comparison).
<sup>f</sup> MVD was expressed as an average number of five highest areas identified within a single ×200 field.
<sup>g</sup> P < 0.005 against PC-3M-LN4 and PC-3M-LN4 and PC-3M-LN4(As Neo) (Mann-Whitney statistical comparison).
Fig. 8 ISH in PC-3P, Neo transfectant PC-3P(Neo), and sense IL-8 transfectants PC-3P(Neo) and sense IL-8 transfectants PC-3P(IL-8), PC-3P(IL-8 Low), and PC-3P(IL-8 High) (A), and in PC-3M-LN4, Neo transfectant PC-3M-LN4(Neo), and antisense IL-8 transfectants PC-3M-LN4(AS IL-8), PC-3M-LN4(AS IL-8 Low), and PC-3M-LN4(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 2.5- and 3.0-fold in the tumor of PC-3P(IL-8) and PC-3P(IL-8 High) relative to PC-3P or PC-3P(Neo), respectively. The tumor of PC-3M-LN4(AS IL-8 High) showed a 40% reduction in the mRNA expression of IL-8 and a 30% reduction in the mRNA expression of MMP-9 relative to that of either parental PC-3M-LN4 or PC-3M-LN4(Neo).
raphy after normalizing the volume of supernatant for cell number (Fig. 3). By densitometry, the collagenase activity of PC-3P(IL-8) and PC-3P(IL-8 High) cells was increased 6.0- and 7.0-fold, compared with either PC-3P or PC-3P(Neo) (Fig. 3A), respectively, whereas that of PC-3M-LN4(AS IL-8) and PC-3M-LN4(AS IL-8 Low) was decreased 2.5- and 5.0-fold compared with either PC-3M-LN4 or PC-3M-LN4(Neo), respectively (Fig. 3B).

We next analyzed whether the increase in MMP-9 activity is mediated by IL-8 (Fig. 4). To that end, parental PC-3P 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. 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 anti-IL-8 antibody (Fig. 4B).

**RT-PCR Analysis.** RT-PCR analysis revealed that PC-3P, PC-3P(Neo), and sense IL-8 transfectants (IL-8, IL-8 Low, and IL-8 High; Fig. 5A), as well as PC-3M-LN4, PC-3M-LN4(Neo), and antisense IL-8 transfectants (AS IL-8, AS IL-8 Low, and AS IL-8 High; Fig. 5B), express mRNA for both IL-8 receptors.

**Invasion Assay through Matrigel.** We next analyzed whether the expression of MMP-9 and collagenase activity by the IL-8-transfected cells correlated with invasion through the basement membrane. PC-3P(IL-8) and PC-3P(IL-8 High) cells exhibited increased invasion through Matrigel-coated filters, with 3.0- and 4.0-fold increases, compared with either PC-3P or PC-3P(Neo) (P < 0.005), respectively (Fig. 6A). Invasion by PC-3M-LN4(AS IL-8) and PC-3M-LN4(AS IL-8 Low) was 80 and 85% lower, compared with invasion by PC-3M-LN4 or PC-3M-LN4(Neo) (P < 0.005), respectively (Fig. 6B).

**CAT Activity.** The full-sequence MMP-9 promoter was linked upstream of the CAT reporter gene and transfected into PC-3P, PC-3M-LN4, sense IL-8 transfected, antisense IL-8 transfected, and the Neo-transfected cells to examine the effect of IL-8 expression on MMP-9 transcription. Forty-eight hours 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 MMP-9 promoter in PC-3P(IL-8) and PC-3P(IL-8 High) was increased 1.5- and 2.9-fold (Fig. 7A) compared with either PC-3P or PC-3P(Neo), respectively, and decreased 2.5- and 10.0-fold by PC-3M-LN4(AS IL-8) and PC-3M-LN4(AS IL-8 Low) (Fig. 7B) compared with either PC-3M-LN4 or PC-3M-LN4(Neo), respectively. CAT activity driven by the SV40 promoter was the same in both cell populations and served as an additional internal control for transfection efficiency. Next, the stability of MMP-9 mRNA was investigated by examining its half-life. The half-life of MMP-9 mRNA of PC-3P(IL-8) was similar to that of PC-3P or PC-3P(Neo), and that of PC-3M-LN4(AS IL-8) was similar to that of PC-3M-LN4 or PC-3M-LN4(Neo) (data not shown).

**Tumorigenicity and Production of Metastasis.** To evaluate whether IL-8 expression regulates tumorigenicity and metastasis of androgen-independent prostate cancer, we implanted PC-3P, PC-3M-LN4, Neo-transfected, and the IL-8 sense- and antisense-transfected cells into the prostate of athymic nude mice and evaluated tumor growth and metastasis 6 weeks later (Table 1). The PC-3P(IL-8) and PC-3P(IL-8 High) tumors were larger than the PC-3P and PC-3P(Neo) tumors [mean weight (range; P): 1270 mg (258–1850 mg; P < 0.005) and 1975 mg (355–2915 mg; P < 0.005) versus 55 mg (24–480 mg) and 120 mg (27–443 mg)], respectively. The incidences of spontaneous lymph node metastasis, as well as tumor burden within the metastatic lymph nodes, were significantly greater for the PC-3P(IL-8) and PC-3P(IL-8 High) cell lines than for the PC-3P and PC-3P(Neo) [incidences: 8 of 8, 9 of 9, 4 of 7, 4 of 9, respectively (P < 0.05); mean lymph node weight (range): 93 mg (36–124 mg), 131 mg (55–188 mg), 20 mg (16–28 mg), and 24 mg (15–32 mg), respectively; P < 0.005]. Conversely, the tumorigenicity of PC-3M-LN4 was significantly inhibited when IL-8 expression was reduced by antisense IL-8 transfection. Only two of eight mice implanted with PC-3M-LN4(AS IL-8) (tumor weights, 33 and 36 mg) and PC-3M-LN4(AS IL-8 Low) (tumor weights, 31 and 39 mg) developed tumors at 6 weeks (P < 0.01) compared with the mice implanted with PC-3M-LN4 and PC-3M-LN4(Neo). These tumors were not apparent histologically but grew out as tissue explants in culture. Tumorigenicity was also inhibited in PC-3M-LN4(AS IL-8 High), but this difference did not reach statistical significance. There was a significant reduction in spontaneous lymph node metastases at 6 weeks in mice implanted with PC-3M-LN4(AS IL-8), PC-3M-LN4(AS IL-8 Low), or PC-3M-LN4(AS IL-8 High) (no mice developed metastasis) compared with mice implanted either PC-3M-LN4 or PC-3M-LN4(Neo) (all mice developed metastasis; P < 0.0005). Therefore, IL-8 expression by PC-3P and PC-3M-LN4 regulates both tumorigenicity and metastasis in androgen-independent prostate cancer.
**In Vivo Expression of IL-8, bFGF, VEGF, and MMP-9.** IL-8, bFGF, VEGF, and MMP-9 mRNA and protein were evaluated by ISH (Table 2; Fig. 8) and IHC (Table 2; Fig. 9), respectively. The mRNA and protein expressions of IL-8 and MMP-9 were increased 2.5- and 3.0-fold in the PC-3P(II-8) and PC-3P(II-8 High) tumors, respectively, relative to either PC-3P or PC-3P(Neo). The PC-3M-LN4(AS IL-8 High) tumors showed a 42% reduction in the mRNA and protein expression of IL-8 and a 33% reduction in the mRNA and protein expression of MMP-9 relative to either PC-3M-LN4 or PC-3M-LN4(Neo) tumors. There was no change in the mRNA and protein expression of bFGF or VEGF in the IL-8-transfected tumors.

**Tumor Angiogenesis.** Tumor-induced neovascularization (as indicated by MVD) was determined by IHC using anti-CD31 antibodies (Table 2; Fig. 9). The numbers of CD31+ microvessels counted per ×200 field were 40 ± 9 and 45 ± 6 in PC-3P and PC-3P(Neo), respectively, compared with 80 ± 13 and 91 ± 18 in PC-3P(II-8) and PC-3P(II-8 High), respectively (P < 0.005). Conversely, antisense IL-8 transfection of PC-3M-LN4 significantly decreased MVD from 100 ± 20 and 104 ± 23 in the PC-3M-LN4 and PC-3M-LN4(Neo) tumors, respectively, to 47 ± 14 in the PC-3M-LN4(AS IL-8 High) tumors (P < 0.005; Table 2; Fig. 9). Because the PC-3M-LN4(II-8) and PC-3M-LN4(AS IL-8 Low) cells grew only as explants in tissue culture, we could not evaluate MVD. These studies indicate that tumor-induced neovascularization correlates directly with IL-8 expression, tumorigenicity, and metastasis.

**DISCUSSION**

Prostate cancer growth and metastasis depend upon the ability of the cancer to induce its own blood supply (18, 21, 23). This process of angiogenesis depends on the outcome between stimulatory and inhibitory regulation by the tumor and its microenvironment (14–16). Human prostate cancer express a number of angiogenesis factors including VEGF (17, 18, 58), bFGF (19, 20), and IL-8 (18, 21, 22, 33). The metastatic potential of the LNCaP prostate cancer cell line correlates with VEGF (58), and that of the PC-3 lineage with bFGF and IL-8 expression (19, 21). Recently, Moore et al. (33) provided direct evidence that IL-8 regulates the growth of 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 provides direct evidence for the role of IL-8 in regulating tumor-induced neovascularization and subsequent growth of human prostate cancer implanted within the prostate of athymic nude mice. Our results are similar to those reported by Luca et al. (32), who enforced the expression of IL-8 in the SB-2 melanoma cell line by sense transfection and demonstrated that IL-8 regulated tumorigenicity in human melanoma.

We enforced IL-8 expression by transfecting the poorly tumorigenic and poorly metastatic human prostate cancer cell line PC-3P (which expresses relatively low levels of IL-8) with the sense IL-8 construct and were able to establish several cell lines that overexpress IL-8 relative to the original PC-3P cell line. These sense IL-8-transfected cells demonstrated enhanced tumor growth and metastasis compared with the PC-3P or PC-3P(Neo) cells and demonstrated enhanced tumor-induced neovascularization, growth within the prostate, and spontaneous metastasis to the lymph nodes. Conversely, after antisense IL-8 transfection, we were able to reduce IL-8 expression by the highly tumorigenic and metastatic PC-3M-LN4 cell line (which expresses relatively high levels of IL-8) and to inhibit tumor-induced neovascularization, growth within the prostate, and metastasis. 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. Because IL-8 transfection did not affect in vitro proliferation of PC-3P or PC-3M-LN4, the effects on growth and metastasis are independent of proliferation, although the cells do have both type A (CXCR1; Refs. 59 and 60) and type B (CXCR2; Refs. 59 and 60) of the IL-8 receptors. Therefore, our results provide evidence for the involvement of IL-8 in the induction of in vivo angiogenesis and in the subsequent growth and metastasis of prostate cancer. 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 (61–63). However, because of the differences in tumor size between control and IL-8 sense and antisense transfectants, the difference in tumor-induced angiogenesis and in metastatic potential may reflect tumor size.

The metastatic potential of prostate cancer depends upon the expression of several metastasis-related genes, such as IL-8, that regulate endothelial cell proliferation and capillary morphogenesis (28), and other genes, such as MMP-9, that regulate the degradation of the extracellular matrix (35, 64). The local production of MMP-9 or other proteases, such as plasminogen activator, by prostate cancer cells or stroma facilitates the local degradation of the extracellular matrix and results in tumor invasion and subsequent metastasis (35, 64–66). 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 PC-3M-LN4 expresses high levels of MMP-9 compared with the poorly metastatic PC-3P cell line. Recently, Luca et al. (32) reported that IL-8 regulates MMP-2 activity by malignant melanoma cells. They transfected the melanoma cell line SB-2 with the sense IL-8 transcript and up-regulated MMP-2 expression and collagenase activity. They considered this up-regulation of collagenase activity to be an important mechanism that explained the associated increase in metastatic ability demonstrated by the sense-transfected SB-2 cells. Similarly, we found that the activity of both MMP-9 by human prostate cancer cells directly correlated with their expression of IL-8. Moreover, when we altered the expression of IL-8 by sense or antisense transfection, we observed a corresponding change in MMP-9 expression and activity both in vitro and in vivo. The MMP-9 induced by sense transfection was biologically active, because it increased collagenase activity and increased cellular invasion through Matrigel. When MMP-9 activity was reduced after antisense transfection, both collagenase activity and invasion through Matrigel decreased. The altered local growth of the antisense-transfected tumors may reflect a relative growth inhibition secondary to the inability to induce a robust microcircu-
loration, whereas the loss of metastatic potential may be attributable to both a decrease in the tumor-induced neovascularization by IL-8 and a reduction in invasion attributable to the reduction in MMP-9 activity. Conversely, increased IL-8 expression by the sense IL-8-transfected prostate cancer cells may explain their enhanced tumorigenicity, whereas both increased IL-8 and MMP expression may contribute to their increased metastatic potential. Because these experiments were conducted in athymic nude mice, they do not address the well-established role of IL-8 in tumor immunity (25).

MMP-9 expression is regulated by both transcriptional and posttranscriptional events. Whereas bFGF and VEGF regulate the transcription of MMP-9 through activation of Ets-1 and Ets-2 binding sites in the promoter (67, 68), transforming growth factor-β1 up-regulates MMP-9 by increasing mRNA stability (37). IL-8 probably regulates MMP-9 expression at the level of transcription. We evaluated MMP-9 mRNA stability and the level of gene transcription of MMP-9 in IL-8 transfected and control cells. Although the expression of MMP-9 mRNA varied among the IL-8 transfecants and controls, the stability of MMP-9 mRNA was not changed by transfection with sense or antisense IL-8. However, CAT activity driven by the MMP-9 promoter was up-regulated in IL-8 sense transfectants and down-regulated after antisense transfection. bFGF regulates MMP-9 expression in human bladder cancer. Because bFGF levels were not affected by IL-8 transfection, the regulation of MMP-9 transcription in PC-3P and PC-3M-LN4 cells is independent of bFGF and likely regulated by IL-8. These results are in keeping with the report of Luca et al. (32), who found that IL-8 regulated MMP-2 gene transcription.

In summary, our present study demonstrates that IL-8 regulates angiogenesis, tumorigenesis, MMP-9 expression, and metastasis by androgen-independent human prostate cancer. This effect may be mediated, in part, by the regulation of the expression and activity of MMP-9.

REFERENCES


Clinical Cancer Research

Interleukin 8 Expression Regulates Tumorigenicity and Metastases in Androgen-independent Prostate Cancer

Keiji Inoue, Joel W. Slaton, Beryl Y. Eve, et al.


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