Gene Expression of Angiogenic Factors Correlates with Metastatic Potential of Prostate Cancer Cells

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ABSTRACT

We hypothesize that expression of proangiogenic genes correlates with the metastatic potential of prostate cancer cells. LNCaP, DU-145, and PC-3 are prostate cancer cell lines with low, moderate, and high metastatic potential, respectively, as we demonstrated by their capacity to invade an extracellular matrix, an established tumor invasion assay. The constitutive gene expression of the proangiogenic factors, vascular endothelial growth factor, intercellular adhesion molecule-1, inter leukin-8, and transforming growth factor-β2, was significantly greater in the more metastatic DU-145 and PC-3 cells as compared with LNCaP cells. Matrix metalloproteinase (MMP)-9 is thought to contribute to the invasive phenotype of tumor cells. PC-3 cells showed increased expression of MMP-9 and membrane type 4-MMP as compared with LNCaP and DU-145. Tissue inhibitors of metalloproteinase 1 and 4 gene expression were elevated in DU-145 and PC-3 cells, but paradoxically, LNCaP cells had undetectable levels of these genes. We transfected and overexpressed MMP-9 in poorly metastatic LNCaP cells and measured their invasive activity. Transient expression of human MMP-9 in LNCaP cells produced a 3–5-fold increase in MMP-9 activity with a comparable increase in invasiveness. Antisense ablation of the expression of MMP-9 in DU-145 and PC-3 cells produced concomitant inhibition of the gene expression of the proangiogenic factors, vascular endothelial growth factor, and intercellular adhesion molecule-1 (ICAM-1). Treatment of DU-145 and PC-3 cells with a selective chemical inhibitor of MMP-9 proteinase activity also inhibited their invasive activity. These results support our hypothesis that metastatic potential of prostate cancer cells correlates with expression of proangiogenic factors.

INTRODUCTION

In general, tumor growth and metastasis is dependent on angiogenesis, which is controlled by a variety of angiogenic factors (1–3). Angiogenesis occurs through a series of interrelated steps, including endothelial cell proliferation, motility of endothelial cells through the extracellular matrix toward angiogenic stimuli, and capillary differentiation (4). Newly formed blood vessels facilitate the metastatic dissemination of cancer cells. In prostate cancer, angiogenesis correlates with disease stage and metastasis (5). A variety of growth promoting factors such as vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), tumor necrosis factor-α, platelet derived growth factor, interleukin-8 (IL-8), and other proteins and peptides can induce angiogenesis (6). A variety of tissue extracts as well as several characterized cytokines, including IFNs IL-12 and matrix glycoproteins, have been reported to inhibit angiogenesis (7).

The matrix metalloproteinases (MMPs) are comprised of 23 zinc-dependent proteolytic enzymes that are involved in the degradation of the extracellular matrix and have been associated with malignancy (8). These enzymes are secreted or localized at the cell surface, indicating that their substrates are extracellular proteins, some of which are associated with tumor cell invasion of the basement membrane and stroma, blood vessel penetration, and metastasis (9). A subset of MMPs known as membrane type (MT)-MMPs also may be involved in tumor invasion. MMP enzyme activity is regulated at multiple levels (10). MMP genes are transcriptionally responsive to wide variety of oncogenes, growth factors, cytokines, and hormones (9). When secreted, these enzymes are membrane bound and remain dormant until they are activated proteolytically (9). The role of MMPs in tumor invasion and metastasis was proposed by Lio1ta and colleagues (11) in 1980. Since then much additional data implicating MMPs in cancer and other diseases has accumulated. There is general correlation between the stage of tumor progression and level of MMP expression. Also a positive correlation was found between MMP-2 expression and Gleason score in prostate cancer patients (12). Varied but altered gene expression of MMP-7 and MMP-9 and reduced tissue inhibitor of metalloproteinase (TIMP) expression was observed in benign prostatic hyperplasia and prostate cancer (13). In a recent study, differential expression of MMPs in primary cell cultures, including benign and malignant prostate cell lines, was reported (14). All MMPs can be inhibited by a family of four different TIMP proteins (9). MMPs and TIMPs are always in a state of dynamic balance, and alterations in the MMP to TIMP ratio occur with tumor metastasis (15). Currently, there is a dearth of information on the association of the expression of angiogenic factors, MMPs, and TIMPs with the metastatic potential of prostate cancer cells. The present investigation addresses this issue.

MATERIALS AND METHODS

Prostate Cancer Cell Lines. The human prostate cancer cell lines PC-3, LNCaP, and DU-145 were obtained from the American Type Culture Collection (Manassas, VA) and normal prostate epithelial cells were obtained from Clonetics (Walkersville, MD). DU-145 cells are less invasive in vitro assays and exhibit relatively low potential for metastasis in vivo compared with the more malignant PC-3 cells. LNCaP cells grow readily in tissue culture as well as in nude mice at the site of inoculation. Cell cultures were maintained in RPMI 1640 supplemented with nonessential amino acids, l-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), sodium pyruvate, Earle’s balanced salt solution, 10% fetal bovine serum, and penicillin and streptomycin (Flow Labs, Rockville, MD). Approximately 3 × 10⁵ cells/60-mm dish were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 48 h. At the end of incubation, the supernates were harvested and stored at −70°C.

RNA Extraction and Reverse Transcription-PCR Amplification. Cytoplasmic RNA was extracted from cells by an acid guanidinium thiocyanate/phenol/chloroform method described by Chomczynski and Sacchi (16) and was subjected to reverse transcription-PCR using a Perkin-Elmer kit. First-strand cDNA was derived by reverse transcription from 1.0 μg of total RNA by use of random hexamers and 50 units of murine leukemia virus reverse transcriptase in a tube containing 5 mM MgCl₂, 1 mM of each deoxynucleoside triphosphate (A, T, G, C), 50 mM KCl, 10 mM Tris buffer (pH 8.3), and 20 units
of RNase inhibitor. The mixture was incubated at 45°C for 35 min, heated to 95°C for 5 min, and placed on ice until used for PCR. The cDNA obtained was PCR amplified with specific sense and antisense primers from highly conserved sequences of the genes of interest along with a housekeeping gene, β-actin (see Supplementary Data). PCR was performed for 30 cycles under the following conditions: denaturation for 1 min at 94°C; annealing for 1 min at 60°C; and extension for 2 min at 72°C, with the final extension lasting 7 min. Amplimers were separated by electrophoresis on 1.2% agarose gel. Resultant bands were visualized with UV light, size determined, and the image densities of PCR products were quantified using a scanning densitometer and compared with the density of coamplified β-actin to determine the ratio of expression. All bands were normalized to the constitutive expression of the housekeeping gene. Values are expressed as intensity of the band based on the absorbance.

**Real-Time Quantitative PCR.** The relative abundance of each mRNA species was assessed using the 5′-fluorogenic nucleic acid assay to perform real-time quantitative PCR (17). To provide precise quantification of the initial target in each PCR reaction, the amplification plot is examined at 2 points during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above background and determining the time point at which each sample’s amplification plot reaches the threshold cycle number (Ct). All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene, β-actin. For each mRNA sample, a difference in Ct values (∆Ct) was calculated by taking the mean Ct of duplicate tubes and subtracting the mean Ct of the duplicate tubes for the reference (β-actin) RNA measured on an aliquot from the same reverse transcription reaction. This calculation assumes that all PCR reactions are working at 100% efficiency. All PCR efficiencies were found to be >95%; therefore, this assumption introduces minimal error into the calculations.

**E.L.I.S.A. and Biochemical Assays.** VEGF, ICAM-1, IL-8, IFN-γ, and TGF-β2 levels in the culture supernates were quantified using highly sensitive and specific E.L.I.S.A kits obtained from Biosource, Inc. (Camarillo, CA). MMP-9 Activity Assay system was measured using the Biotrak MMP-9 and specific ELISA kits obtained from Biosource, Inc. (Camarillo, CA). TGF-β levels in the culture supernates were quantified using highly sensitive and specific ELISA kits obtained from Biosource, Inc. (Camarillo, CA). MMP-9 Activity Assay system was measured using the Biotrak MMP-9 Activity Assay system (Amersham Biosciences Corp., Piscataway, NJ). The assay performance and the inter- and intra-assay variations were within the manufacturer’s limits. Protein was assayed by the Bio-Rad Protein Assay Kit II from Bio-Rad (Hercules, CA).

**Northern Blot Analysis.** Total RNA was isolated using Trizol reagent (Ambion, Austin, TX). RNA (20 μg) was separated on a formaldehyde gel containing 1% agarose, electrophoresed into nylon membranes (Micron Separations, Westboro, MA), and UV cross-linked to the membrane using a Stratalinker Model 1800 (Stratagene, La Jolla, CA) at 120,000 μm2/cm2. Membranes were washed at 55°C with 30 mm sodium citrate and 0.1% (v/v) SDS. The cDNA probes for MMP-9, MT-1-MMP, TIMP-1, and β-actin were radio-labeled using a PRIME-it II kit from Strategenie. After prehybridization using Ultraspeed (Ambion) for 60°C; annealing for 1 min at 68°C, the 32P-labeled specific probe was hybridized at 68°C overnight. After hybridization, the blot was washed twice with 2× saline-sodium phosphate-EDTA, 0.1% SDS for 15 min at 42°C, then twice with 0.1× saline-sodium phosphate-EDTA, 0.1% SDS for 15 min at 68°C. Blots were exposed to KODAK Biomax-MS film overnight at −20°C and the autoradiograph obtained. Bands of interest were quantified using a scanning densitometer. The results were integrated and normalized to the housekeeping gene, β-actin.

**Western Blot Analysis.** LNCaP, DU-145, and PC-3 cells were cultured for 48 h and washed with PBS. Total protein was extracted using Mammalian Protein Extraction Reagent (M-PER; Pierce, Rockford, IL), and an equal amount of protein from each cell line was loaded per lane and separated on a 7.5% SDS-Tris glycine PAGE gel. Gels were electroblotted onto nitrocellulose membranes (Novex, San Diego, CA) and blocked overnight by incubating with 1× Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% nonfat dry milk. Membranes were probed with 2 μg/ml affinity purified, polyclonal antibodies produced in goats against MMP-9, MT-1-MMP, TIMP-1, and β-Actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After incubation with the respective primary antibodies, the membranes were incubated for 2 h at room temperature with biotin-conjugated, donkey anti-goat IgG antibody (Santa Cruz Biotechnology, Inc.). After three washes with 1× Tris-buffered saline of 10 min each, blots were incubated for another 30 min with a streptavidin-alkaline phosphatase conjugate (Life Technologies, Inc.) followed by colorimetric detection using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate reagent (Roche, Indianapolis, IN).

**MMP-9 Gene Ablation by Antisense Oligonucleotides (ASODNs).** DU-145 and PC-3 cells at 70–80% confluence on 60-mm plastic dishes were transfected with 5′-phosphorothioate-modified MMP-9 ASODN targeted to bases 1221–1240 of MMP-9 mRNA (GenBank accession no. NM-029934) or nonsense oligonucleotide sequences predicted by Laser Gene software (DNASTAR, Inc., Madison WI). ASODNs and nonsense oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The oligodeoxynucleotides were modified by phosphorothioation for stability. Oligonucleotides were diluted in 1 ml of serum-free OptiMEM I (Life Technologies, Inc.) and were mixed and equilibrated for 20 min with Lipofectamine (Invitrogen, Inc., Carlsbad, CA). Final concentration of oligonucleotide ranged from 20 to 400 μM. Cells were treated with the oligonucleotide mixture for 3 h at 37°C. Serum was added to a final concentration of 10%, and the cells were used 48 h later.

**Cell Invasion Assay.** The invasive activity of prostate cancer cells in vitro was tested using a Quantitative Cell Invasion Assay kit (Chemicon International, Inc., Temecula, CA). The invasion assay was performed using a 24-well tissue culture plate with 12 cell culture inserts. The insert contains a porous (8 μm) polycarbonate membrane precoated with basement membrane proteins derived from the Engelbreth-Holm-Swarm mouse tumor. To prepare the fibroblast culture conditioned medium used as a chemotractant in the lower well of the invasion chamber, fibroblasts were seeded in a 175-cm2 flask in MEM supplemented with 15% fetal bovine serum. At ~90% confluency, the medium was removed, and the cells were washed once with serum-free MEM supplemented with 50 μg/ml ascorbic acid. Cells were then incubated for 24 h with 30 μl of serum-free MEM containing 50 μg/ml ascorbic acid. The latter conditioned medium was sterile filtered, divided into 5-ml aliquots, and frozen at −70°C. Conditioned medium was thawed and prewarmed to 37°C before use. The gel layers were rehydrated for 2 h with 500 μl of serum-free medium. A suspension of 105 cells in 500 μl of serum-free media was added to the top of each membrane and incubated for 48 h at 37°C in a 95% air, 5% CO2 incubator. The medium was discarded, and noninvading cells were removed from the upper surface of the gel with a cotton swab. Cells that migrated through the porous membrane and adhered to the lower surface were stained and counted. To confirm the cell count data, we also used another method using 10% acetic acid (200 μl/well) to extract the stain from the cells and measure absorbance at 560 nm in a spectrophotometer. A positive correlation was observed between the increase in absorbance and the increase in number of invasive cells (correlation coefficient, r = 0.92). In separate experiments a specific inhibitor of MMP-9 activity (MMP-9 Inhibitor I; Calbiochem, La Jolla, CA) was added to the top chamber to a final concentration of 1 nm along with the cells.

**Construction of Expression Vectors and Transfection.** A cDNA encoding the full human MMP-9 sequence (clone 4054882; Invitrogen, Inc.) was cloned into the expression plasmid, pCDNA6. LNCaP cells were seeded into 6-well plates at a density of 1.4 × 105 cells/well. After 24 h, each well was treated with a mixture containing 2 μg of plasmid DNA and 3 μl of Lipofectin reagent (Invitrogen, Inc.). The cells were harvested 36 h after transfection.

**Statistical Analysis.** The results presented are means ± SE, and statistical significance between groups was determined by Student’s t test. Differences were considered significant at P < 0.05.

**RESULTS**

**Gene Expression of Pro- and Antiangiogenic Cytokines by Prostate Cancer Cells.** Three prostate cancer cell lines with different metastatic potentials were used. On s.c. injection into the prostate gland of nude mice, PC-3 cells are highly metastatic, whereas DU-145 and LNCaP cells are moderately and poorly metastatic respectively. Our studies were undertaken to examine the constitutive expression of growth factors known to regulate angiogenesis and correlate these results with the known metastatic potential of the respective prostate cancer cells. Cell lines were cultured for 48 h, and RNA was extracted and amplified by PCR. Our results illustrated in Fig. 1A show the constitutive level of gene expression of the proangiogenic growth factors. Proangiogenic growth factor VEGF is significantly higher in PC-3 and DU-145 cells as compared with poorly metastatic LNCaP.
cells. The PCR products of β-actin and VEGF migrated in agarose gels as 543 and 568 bp, respectively, as predicted. VEGF expression by DU-145 and PC-3 cells, respectively, was 3- and 5-fold greater than LNCaP cells (Fig. 1C, a). Expression of other proangiogenic factors such as TGF-β2, IL-8, and ICAM-1 also was considerably greater in the highly metastatic cell line, PC-3, and in the moderately metastatic DU-145 cells when compared with LNCaP cells (Fig. 1A, rows 3–5, and Fig. 1C, b–d). However the constitutive gene expres-
sion of IFN-γ, the PCR product of which migrated as a 241-bp band in agarose gel (Fig. 1A, row 6) was highest in poorly metastatic LNCaP cells (Fig. 1C, e), and this was significantly ($P < 0.001$) greater than the constitutive gene expression of IFN-γ in DU-145 (Fig. 1A, row 6, Lane 3) and PC-3 (Fig. 1A, row 6, Lane 4) cells.

**Gene Expression of MMPs and TIMPs by Prostate Cancer Cells with Different Metastatic Potential.** Because MMPs are involved in angiogenesis and tumor metastasis, we compared the constitutive levels of MMP gene expression in prostate cancer cell lines with different metastatic potentials. Our results are shown in Fig. 1, B and D. A significantly higher level of MMP-9 gene expression was seen in PC-3 cells, which have a greater metastatic potential compared with DU-145 and LNCaP cells with lower metastatic potentials (Fig. 1B, row 2). MT1-MMP was expressed at a low level in all three cell lines when compared with MT4-MMP; however, higher levels were observed in DU-145 and PC-3 cell lines. MT4-MMP was expressed at the same level in PC-3 and DU-145 and showed nearly a 3-fold increase in both cell lines when compared with LNCaP cells (Fig. 1B, row 3, and Fig. 1D, c).

Examination of the gene expression of TIMPs showed major differences among the cell lines. TIMP-3 was generally expressed at a lower level compared with TIMP-1 and TIMP-4 in all three cell lines. Among the cell lines, LNCaP showed higher expression of TIMP-3 compared with DU-145 and PC-3, both of the latter showed comparable expression of TIMP-3. TIMP-1 and TIMP-4 showed similar increased patterns of expression in PC-3 and DU-145 cells compared with undetectable levels in LNCaP cells. There was an almost 3-fold increase of TIMP-1 and TIMP-4 expression in DU-145 and PC-3 cells compared with LNCaP cells (Fig. 1B, rows 5 and 7, and Fig. 1D, d and f).

To determine whether the patterns of gene expression of angiogenic factors also are reflected at the protein level, equal aliquots of culture supernatants from the same number (3 × 10⁶) of cells was assayed using a sandwich ELISA procedure. The results of these studies are depicted in Fig. 1E. DU-145 and PC-3 cells secreted VEGF at levels that were 2- and 4-fold higher, respectively, compared with LNCaP cells (Fig. 1E, a). TGF-β2 levels in supernates from PC-3 (404 ± 27 pg/ml) and DU-145 (385 ± 25 pg/ml) cells were significantly ($P < 0.001$ for both) higher when compared with LNCaP cells (4.0 ± 0.8; Fig. 1E, b). Because angiogenesis and metastasis also are associated with IL-8 and ICAM-1 expression, we measured the amounts of these products secreted into culture supernatants. The concentration of IL-8 in the supernates of PC-3 cells after 48 h of culture was 185 ± 18 pg/ml, which was significantly higher ($P < 0.001$) than LNCaP cells (25 ± 20 pg/ml) but not significantly different from the amount secreted by DU-145 cells (175 ± 18 pg/ml). Furthermore, secreted ICAM-1 proteins also were significantly higher in the supernates of DU-145 and PC-3 cells, 24.0 ± 5.0 and 38.0 ± 7.0 pg/ml, respectively ($P < 0.001$ for both), when compared with the supernates of LNCaP cells (4.0 ± 0.8 pg/ml). In contrast, LNCaP cells secreted significantly more IFN-γ (~2-fold more) than the highly metastatic PC-3 cells (Fig. 1E, e). The observed differences were not caused by variation in viability or rate of cell growth.

**Quantitative Analysis of Gene Expression by Prostate Cancer Cell Lines and Normal Prostate Epithelial Cells Using Real-Time PCR.** We extended and confirmed the gene expression results above using quantitative, real-time reverse transcription-PCR and further included normal prostate epithelial cells to compare with the prostate cancer cell lines (Fig. 2). When compared with normal prostate epithelial cells, in general, there was consistently higher expression of the genes for a wide range of proangiogenic factors by prostate cancer cell lines. Moreover expression of these genes positively correlated with the metastatic potential of the cell line (i.e., proangiogenic gene expression by PC-3 > DU-145 > LNCaP). As expected for normal cells, the normal prostate epithelial cells did not show significant expression of the IFN-γ gene. However, there was an inverse trend between IFN-γ expression and the metastatic potential of the cell line (i.e., LNCaP > DU-145 > PC-3).

**Northern and Western Blot Analyses for Expression of MMPs and TIMPs in Prostate Cancer Cells.** Northern blot analyses were carried out for selected genes to more rigorously confirm the gene expression data as determined by reverse transcription-PCR (Fig. 3). For this purpose, three diverse but related genes were selected: MMP-9, MT-1-MMP, and TIMP-1. When the mean densitometric value of the RNA bands for each gene normalized to the level of control 28S rRNA were compared, there was a 4-, 8-, and 3-fold increase in the expression of MMP-9, MT1-MMP, and TIMP-1 genes, respectively, by the highly metastatic PC-3 cells in comparison to the minimally metastatic LNCaP cells (Fig. 3B). Levels of β-actin mRNA were similar in all three cell lines, demonstrating that the Northern blot results were specific for the genes of interest and were not due to a general increase in total RNA.

Western blot analysis of cell extracts was carried out to determine whether increased mRNA expression, as observed, correlated with increased translation of the gene product. The results depicted in Fig. 4 show that the translated protein products of these genes were increased in relation to the metastatic potential of the cell line. The products of the MMP-9, MT1-MMP, and TIMP-1 genes were detected as predicted as 101-, 68-, and 22-Kd fragments, respectively, by the specific antibodies used (Fig. 4A). All bands were analyzed by densitometry and the values for MMP-9 were normalized to the values of the constitutively synthesized β-actin (Fig. 4B). We found the greatest levels of MMP-9, MT1-MMP, and TIMP-1 proteins in the highly metastatic PC-3 cells, whereas the minimally metastatic LNCaP cells manifested the lowest levels of these products. DU-145 cells produced intermediate levels. Thus, the levels of these proteins correlated with their metastatic potential.

**Effect of MMP-9 Gene Ablation on the Expression of Genes for Proangiogenic Factors by Prostate Cancer Cells.** MMP-9 expression is associated with higher grade and more aggressive tumors (2, 18). In the present study, we found that MMP-9 gene expression was increased 3-fold in the more metastatic DU-145 and PC-3 cell lines as compared with LNCaP cells. Thus, we examined the effect of MMP-9 gene ablation on expression of genes that potentially may contribute to the metastatic potential and angiogenesis of prostate cancer cell lines.
gene ablation using specific ASODN on the subsequent expression of genes for proangiogenic factors by prostate cancer cells. To eliminate the possibility that MMP-9 ASODN causes nonspecific, toxic effects on prostate cancer cells, we determined the viability of PC-3 cells cultured with MMP-9 ASODN for 48 h. PC-3 cells treated with MMP-9 ASODN at concentrations ranging from 20 to 400 nM showed viabilities comparable with the 92% viability of untreated control cultures as determined by the trypan blue dye exclusion assay. Lack of toxicity of MMP-9 ASODN was confirmed by demonstrating that it did not affect the constitutive expression of the housekeeping gene, β-actin. As shown in Fig. 5A, only specific ASODN, and neither Lipofectamine alone (i.e., without ASODN or missense oligonucleotide), significantly inhibited MMP-9 gene expression by DU-145 and PC-3 cells in a dose-dependent manner. Complete ablation of MMP-9 expression by DU-145 cells was achieved at a concentration of 80 nM ASODN, whereas the more malignant PC-3 cells required a higher concentration (200 nM) of ASODN. Concomitant decreases in the gene expression of the proangiogenic growth factors, VEGF and ICAM-1, also were observed with antisense suppression of MMP-9 expression in both DU-145 and PC-3 cells in a dose-dependent manner (Fig. 5A and B, rows 3 and 4). In MMP-9 ASODN-transfected PC-3 cells, there was also a dose-dependent decrease in total MMP-9 levels (Fig. 6A) and activity (Fig. 6B) as determined by MMP-9 activity measurement. Moreover, only specific MMP-9 ASODN but not missense oligonucleotide significantly (P < 0.01) decreased the synthesis and secretion of VEGF and ICAM-1 by MMP-9-transfected PC-3 cells (Fig. 6C) in a dose-dependent manner. These results strongly support our premise that MMP-9 promotes angiogenesis by regulating the production of proangiogenic factors such as VEGF and ICAM-1.

Invasive Activity of Prostate Cancer Cells. To demonstrate that our gene and protein expression findings correlate with the biological activity of the prostate cancer cell lines used, we examined the ability of these cells to invade a gel layer composed of basement membrane proteins, a well-established in vitro model of tumor invasiveness. We found that only the prostate cancer cell lines invaded the basement membrane in comparison with normal prostate epithelial cells that were not invasive at all. Moreover the highly metastatic prostate cancer cell line, PC-3, showed the greatest levels of invasiveness followed by DU-145 cells and LNCaP cells in an order consistent with
their known metastatic potential (Fig. 7 and Table 1). PC-3 cells were ~20-fold more invasive and DU-145 were ~9-fold more invasive, respectively, in comparison to LNCaP cells.

A selective inhibitor of MMP-9 proteinase activity, MMP-9 Inhibitor I (Calbiochem), was added at a concentration of 1 nM to the cell invasion assay to determine the role of MMP-9 in tumor invasiveness. As shown in Fig. 7C, inhibition of MMP-9 enzymatic activity resulted in a remarkable decrease in invasiveness of both PC-3 and DU-145 cells. Treatment of PC-3 cells with MMP-9 inhibitor decreased invasiveness from 287.4 to 88.7 cells/filter (70% inhibition, \( P < 0.0001 \)), whereas DU-145 cells decreased from 158.8 cells/filter to 24.25 cells/filter (85% inhibition, \( P < 0.0001 \)).

**Effect of Transfection of MMP-9 Gene on Invasiveness of LNCaP Cells.** To confirm the role of MMP-9 in invasive activity of prostate tumors, LNCaP cells with low invasive activity in our experimental assay were transiently transfected with either a pcDNA6-MMP-9 expression plasmid or with the vector alone as described in “Materials and Methods.” On the basis of the expression of a cotransfected β-galactosidase reporter gene, at least 80% of the LNCaP cells took up the exogenous plasmid DNA. In three separate experiments, LNCaP cells transfected with the gene for MMP-9 secreted 3–5-fold more MMP-9 into the culture media after 36 h of incubation than control LNCaP cells treated with the vector alone (Table 2). As the MMP-9 transfected, LNCaP cells functionally overexpress this en-
Fig. 7. Invasive activity of normal prostate epithelium (NP) and prostate cancer cell lines. Cells were applied to the upper surface of a filter coated with basement membrane proteins using a cell invasion assay as described in “Materials Methods.” After incubation for 48 h, the upper surface of the filter was scrubbed free of cells and protein, the filter was fixed and stained and the lower surface was photographed. A, representative filters photographed: Lanes 1–4 are NP, LNCaP, DU-145, and PC-3; Lanes 5 and 6 show DU-145 and PC-3 cells treated with MMP-9-specific inhibitor. B, representative photographs at a magnification of ×100, Lanes 1–4 are NP, LNCaP, DU-145, and PC-3; Lanes 5 and 6 show DU-145 and PC-3 cells treated with MMP-9-specific inhibitor. C, quantitative values of the total number of cells invading through the filter.

Table 1 Invasiveness of prostate cancer cell lines and normal prostate epithelial cells

<table>
<thead>
<tr>
<th>Prostate cells</th>
<th>Mean number of invading cells ± SE</th>
<th>Invasive index as a factor of control cells</th>
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<tbody>
<tr>
<td>Normal epithelium</td>
<td>1.7 ± 0.3</td>
<td>1</td>
</tr>
<tr>
<td>LNCaP</td>
<td>14.4 ± 3.2</td>
<td>8.5</td>
</tr>
<tr>
<td>DU-145</td>
<td>128.4 ± 16.6</td>
<td>75.5</td>
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<tr>
<td>PC-3</td>
<td>287.4 ± 18.4</td>
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DISCUSSION

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is required for tumor growth and metastasis (1, 2) and is regulated by both pro- and antiangiogenic signals generated by the tumor and associated stroma. When the balance of these signals favors angiogenesis, endothelial cell proliferation, migration, and tubule formation occurs followed by formation of tumor neovasculature. Recently, a number of factors regulating angiogenesis have been identified (4). Metastasis is dependent, in part, on the expression of proangiogenic factors. The ratio of the concentrations of activator and inhibitor molecules alter the balance between pro- and antiangiogenic signals (19). The present study seeks to correlate the constitutive expression of genes associated with angiogenesis with the known metastatic potential of three different prostate cancer cell lines. Herein we demonstrate that both gene expression and protein synthesis of VEGF, ICAM-1, and IL-8 were significantly higher in the more metastatic PC-3 and DU-145 cells as compared with poorly metastatic LNCaP cells. VEGF is expressed at low levels by stromal cells of the normal prostate (20). VEGF expression by prostate cancer cells was far greater than by cells from benign prostate hyperplasia (21). VEGF expression is also known to enhance the tumorigenicity of human prostate cancer cells (22, 23). The role of VEGF in prostate cancer progression has been confirmed using anti-VEGF antibodies for treatment (24, 25). In all cases treated with anti-VEGF antibody, suppression of angiogenesis, tumor growth, and metastasis were observed, even in well-established tumors. Thus, the 2–3-fold increase in the expression of the VEGF gene by the more metastatic PC-3 and DU-145 prostate cancer cell lines described in the present study, coupled with increased production of VEGF protein by these cells, supports our hypothesis that the metastatic potential of prostate cancer cells correlates with their capacity to induce and support tumor angiogenesis. In addition to increased expression of VEGF, we observed that similar increases in the expression of TGF-β2, IL-8, and ICAM-1 correlated with the malignant potential of prostate cancer.

Table 2 MMP-9 secreted levels in LNCaP cells after transient transfection with the pcDNA6-MMP-9 expression plasmid

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pcDNA6 ng/ml/mg protein</th>
<th>pcDNA6-MMP-9 ng/ml/mg protein</th>
<th>% of MMP-9 relative to control</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>2.1 ± 0.5</td>
<td>7.0 ± 0.8</td>
<td>333</td>
</tr>
<tr>
<td>2</td>
<td>2.8 ± 0.2</td>
<td>14.0 ± 0.6</td>
<td>500</td>
</tr>
<tr>
<td>3</td>
<td>2.3 ± 0.3</td>
<td>11.8 ± 1.2</td>
<td>434</td>
</tr>
</tbody>
</table>

MMP, matrix metalloproteinase.

b MMP-9 secretion is the mean ± SE for n = 3 in LNCaP cell culture media 36 h after transfection with human MMP-9 cDNA (LNCaP-MMP-9) or with expression plasmid alone (LNCaP-pcDNA6.1). Values are normalized for the recovery of total protein secreted. Experiments 1–3 correspond to experiments 1–3 in Fig 8.
that the effect of TGF-β is biphasic; at lower concentrations, it promotes VEGF expression and basic fibroblast growth factor-induced epithelial tubule formation, although at higher concentrations, it inhibits these activities. TGF-β overproduction also favors malignancy by suppressing the cellular and humoral immunity of the host, promoting angiogenesis, stimulating stromal matrix formation, and enhancing prostate cancer cell motility and metastasis (32).

The present study demonstrates that increased gene expression and synthesis of ICAM-1 correlates with increased metastatic potential of prostate cancer cells. ICAM-1 provides a structural and functional interface between epithelial cells and the extracellular environment (33). Herein we also show that gene expression and synthesis of IFN-γ was significantly decreased in the moderately metastatic DU-145 and highly metastatic PC-3 prostate cancer cells compared with the poorly metastatic LNCaP cells. This observation is consistent with published evidence that IFN-γ is an inhibitor of angiogenesis (7, 34).

Liotta et al. (2) proposed a three-step model of cancer metastasis. The dissolution of basement membrane components such as type IV collagen, laminin, fibronectin, and proteoglycans, a critical step in this process is achieved through various matrix-degrading enzymes, including MMPs. MMPs are a growing family of proteases that have the common ability to degrade extracellular matrix components such as collagen and play a significant role in metastasis (35, 36). Several studies have shown an association of increased production of MMP-2, MMP-3, MMP-7, and MMP-9 with progression of prostate cancer. Other studies, analyzing primary cultures of human prostate tumor tissues and epithelial cells, have shown that malignant prostate cells secrete high levels of MMP-2 and MMP-9 and low levels of their inhibitors, TIMP-1 and TIMP-2 (37, 38). Wood et al. (39) reported that levels of MMP-9 are low in normal prostate and organ-confined tumors with a Gleason sum of ≤5, whereas MMP-9 is significantly expressed in high, 8–10, Gleason sum tissues. In agreement with these observations are the results of the present study, showing that constitutive expression of MMP-9 and MT-MMP-1 and MT-MMP-4 are consistently higher in the more aggressive DU-145 and PC-3 cells compared with LNCaP cells. MT1-MMP-deficient mice have a defect in angiogenesis (40), and a recent article suggests a novel link between MT-MMP activity and VEGF signaling (18). Thus, the increased constitutive expression of MMP-9, MT1-MMP, and MT4-MMP can be correlated with prostate cancer cells with greater metastatic potential. Wilson (41) reviewed the role of proteases in the development, function, and pathology of the prostate gland. Results from our study are concordant with many of his findings.

To additionally clarify the interaction between MMPs and TIMPs in the metastatic potential of prostate cancer cells, we investigated the expression of three different TIMPs, which are natural inhibitors of MMP activity by our three prostate cancer cell lines. Inhibition of MMP activity by TIMPs preserves the extracellular matrix, thereby inhibiting tumor invasion. Thus, the equilibrium between the activities of MMPs and TIMPs in tumor tissue may be an important factor regulating tumor invasion and metastasis (42, 43). Our study shows constitutive expression of TIMP-1, TIMP-3, and TIMP-4 in all three prostate cell lines analyzed. Inhibition of MMP activity by TIMPs preserves the extracellular matrix, thereby inhibiting tumor invasion. Thus, the equilibrium between the activities of MMPs and TIMPs in tumor tissue may be an important factor regulating tumor invasion and metastasis (42, 43). Our study shows constitutive expression of TIMP-1, TIMP-3, and TIMP-4 in all three prostate cell lines analyzed. Inhibition of MMP activity by TIMPs preserves the extracellular matrix, thereby inhibiting tumor invasion. Thus, the equilibrium between the activities of MMPs and TIMPs in tumor tissue may be an important factor regulating tumor invasion and metastasis (42, 43). Our study shows constitutive expression of TIMP-1, TIMP-3, and TIMP-4 in all three prostate cell lines analyzed. Inhibition of MMP activity by TIMPs preserves the extracellular matrix, thereby inhibiting tumor invasion. Thus, the equilibrium between the activities of MMPs and TIMPs in tumor tissue may be an important factor regulating tumor invasion and metastasis (42, 43). Our study shows constitutive expression of TIMP-1, TIMP-3, and TIMP-4 in all three prostate cell lines analyzed. Inhibition of MMP activity by TIMPs preserves the extracellular matrix, thereby inhibiting tumor invasion. Thus, the equilibrium between the activities of MMPs and TIMPs in tumor tissue may be an important factor regulating tumor invasion and metastasis (42, 43). Our study shows constitutive expression of TIMP-1, TIMP-3, and TIMP-4 in all three prostate cell lines analyzed. Inhibition of MMP activity by TIMPs preserves the extracellular matrix, thereby inhibiting tumor invasion. Thus, the equilibrium between the activities of MMPs and TIMPs in tumor tissue may be an important factor regulating tumor invasion and metastasis (42, 43). Our study shows constitutive expression of TIMP-1, TIMP-3, and TIMP-4 in all three prostate cell lines analyzed. Inhibition of MMP activity by TIMPs preserves the extracellular matrix, thereby inhibiting tumor invasion. Thus, the equilibrium between the activities of MMPs and TIMPs in tumor tissue may be an important factor regulating tumor invasion and metastasis (42, 43). Our study shows constitutive expression of TIMP-1, TIMP-3, and TIMP-4 in all three prostate cell lines analyzed. Inhibition of MMP activity by TIMPs preserves the extracellular matrix, thereby inhibiting tumor invasion. Thus, the equilibrium between the activities of MMPs and TIMPs in tumor tissue may be an important factor regulating tumor invasion and metastasis (42, 43).
tual, bladder (45), and gastric (46) tumors. Moreover, TIMP-1 has been reported to promote the growth of proerythroblasts and various types of cancer cells (47). Thus, the increased expression of TIMP-1 in DU-145 and PC-3, as reported herein, may be caused by the increased levels of TGF-β, an inflammatory cytokine, also observed in these cell lines, which, in turn, may facilitate their metastatic potential. Regarding TIMP-3, we observed its expression to be the inverse of TIMP-1 in all three cell lines. TIMP-3 is known to bind firmly to the endothelial cell membrane (48) and initiate apoptosis (49, 50). The differential expression of TIMP-3 by our prostate cancer cell lines also may correlate with predisposition to apoptosis, thereby decreasing malignant and metastatic potential.

The effects of ablation of MMP-9 in prostate cancer cells at the level of gene expression were examined. We found that ASODN for MMP-9 not only inhibited specific gene expression but also had an extensive indirect effect suppressing the expression and production of the proangiogenic factors, ICAM-1 and VEGF. These observations are consistent with Huang et al. (51) who showed that tumors in MMP-9-/- nude mice had lower levels of VEGF than tumors from MMP-9 +/- nude mice. This decrease in VEGF also was associated with a decrease in macrophage infiltration into the tumors. Moreover, MMP-9 is known to promote the migration and invasion of cancer cells through blood vessels by mediating the proteolytic degradation of type IV collagen in the basement membrane (2). Furthermore, experimental metastases were suppressed in MMP-9-deficient mice (52). MMP-9 and one of its indirect activators, urokinase type plasminogen activator, were reported to be required for the intravasation of tumor cells in a chick embryo metastasis model (53). Additionally, MMP-9 contributes to carcinogenesis in pancreatic islets and in skin epithelium by facilitating angiogenesis (18, 54). The extent of angiogenesis is determined by the balance between positive and negative regulatory molecules produced by tumor cells. MMP-9-deficient mice manifest decreased experimental metastasis and angiogenesis (52).

Taken together with our observation of decreased production of the proangiogenic factors, VEGF and ICAM-1, in MMP-9 ASODN-ablated prostate cancer cells supports our hypothesis that metastatic potential correlates with angiogenic activity.

Invasion through the extracellular matrix is an important step in tumor metastasis. Proteolytic enzymes such as MMPs and collagenases form minute holes in the sheath-like basement membrane surrounding blood vessels to allow cancer cells to invade (55). Our objective for testing the three cell lines in the cell invasion assay was 2-fold: (a) to investigate whether differences in gene expression as revealed in the present study lead to differences in the invasive potential of the cells and (b) to test whether inhibition of MMP-9 gene expression and function produces a concomitant decrease in the invasive potential of the tumor cells. In this study, DU-145 and PC-3 cell lines were more invasive de novo than LNCaP cells, consistent with their known metastatic potential. Normal prostate epithelial cells were not invasive at all. Of the tumor cell lines used in this study, only LNCaP cells express lower levels of proangiogenic factors and did not express MMP-9 and its natural inhibitor TIMP-1. Although the invasive capacity of LNCaP cells is low, there are some discrepancies in the literature. Hoosein et al. (56) reported an extremely low invasive capacity for LNCaP cells, compared with the more aggressive PC-3 cell line. In contrast, Lanniado et al. (57) showed a narrow range of variation between these two prostate cancer cell lines, whereas Daihya et al. (58) reported a higher invasive capacity for LNCaP cells. Keer et al. (59) reported that PC-3 cells had a considerably higher invasive capacity compared with DU-145 cells in agreement with the present study. Explanations for these differences include, variations within cell lines and/or the loss of certain adhesion molecules over time and passage number and also differences in the expression of MMP activity as shown in the present study. Our data additionally demonstrate that the MMP-9-positive cell lines, DU-145 and PC-3, manifest increased invasive potential that could be reversed by treatment of the cells with a specific inhibitor of MMP-9. Thus, these studies demonstrate the direct relationship between the expression of MMP-9 and the expression of proangiogenic factors by prostate cancer cells that, in turn, is associated with increased invasiveness and metastatic potential. We also observed a correlation between increased constitutive expression of MMP-9 and increased invasive potential of prostate cancer cells. We extended that observation by overexpressing the gene for MMP-9 in prostate cancer cells. Transient transfection of poorly metastatic LNCaP cells with human MMP-9 cDNA significantly increased both gene expression and invasiveness in an in vitro model. Invasiveness of cells through a gel of basement membrane correlates with metastatic potential in vivo (55, 60).

The fundamental question of how an increase in the gene expression of MMP results in increased metastatic potential of prostate cancer cells may be explained by conflicting theories about their mechanisms of metastasis. The first mechanism is based on hemodynamics, where prostate cancer cells enter the circulation to reach lumbar vertebrae (bone metastasis) under increased intra-abdominal pressure (Bateson’s hypothesis). The second mechanism of extraprostastic spread of tumors is explained by Paget’s seed and soil hypothesis (61), which states that tumor metastasis is dependent on both the tumor cells (seed) and the microenvironment of the organ (soil). Some studies supporting the seed and soil hypothesis as a mechanism for prostate cancer metastasis also describe the tumor cells’ potential to establish a bi-directional communication or inductive interaction (62). These inductive interactions likely result in the secretion of both tumor cell- and host cell-derived factors that support tumor growth, angiogenesis, and metastasis. Such factors include diffusible growth factors that support osteoblast growth, capillary bed formation, and inducers of matrix-degrading enzymes (e.g., VEGF, ICAMs, and MMPs) that are involved in matrix remodeling (56, 63). In this study, we observed increased expression and production of proangiogenic factors in DU-145 and PC-3 cells simultaneously with an increase in the gene expression of certain MMPs. Thus, it is reasonable to conclude that DU-145 and PC-3 cells may have an increased likelihood of bi-directional communication. However, this premise is questioned by the observation of Sato et al. (64) who showed that a prostate cancer cell line producing lower amounts of angiogenic and metastatic factors, LNCaP, that is poorly metastatic on s.c. implantation into nude mice increases its metastatic potential upon orthotopic implantation into the prostate. Thus, the poor ability of LNCaP to disseminate from a s.c. site of injection may not reflect the actual metastatic potential of the cells and speaks to the seed and soil hypothesis. This observation highlights the importance of the host organ and tumor cell interaction in tumor progression but does not offer a mechanism for this effect. However, the elegant studies of Fukumare et al. (65) conclude that the natural host microenvironment (orthotopic site) has different effects on tumor angiogenesis and microcirculation compared with s.c. tissues that are not the site of origin or metastasis of prostate cancer. Specifically, they observed an increase in production of VEGF and vascular permeability factor by prostate tumor cells implanted at the orthotopic site as compared with s.c. implantation. Furthermore, Stephenson et al. (66) showed that upon intraprostatic injection, distant metastases were accompanied by increased production of MMPs by the tumor cells. Therefore, it is possible that upon orthotopic implantation, the tumor cells acquire new factor(s) involved in modulating gene expression and/or production of angiogenic factors. It is our view that metastasis of LNCaP cells to lymph nodes as observed by Sato et al. (64) upon orthotopic implantation could be due to an increase in production of certain
angiogenic factors at the orthotopic site, which emphasizes the importance of differences in the production of angiogenic factors as an essential requirement for metastasis.

In summary, our data provide direct evidence for a link between the metastatic potential of prostate cancer cells and their expression of several factors modulating angiogenesis. Prostate cancer tumors are highly dependent upon angiogenesis. The differential expression of genes and their protein products associated with angiogenesis and metastasis as described in this study may lead to the design of novel antiangiogenic therapies targeting specific genes controlling prostate tumor metastasis, notably MMP-9.

ACKNOWLEDGMENTS

We thank Dr. Kurapati V.K. Rao, Department of Chemical Carcinogenesis, Cancer Research Institute (Mumbai, India) on sabbatical at SUNY Buffalo for critical reading of the manuscript.

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