Expression of the Disintegrin Metalloprotease, ADAM-10, in Prostate Cancer and Its Regulation by Dihydrotestosterone, Insulin-Like Growth Factor I, and Epidermal Growth Factor in the Prostate Cancer Cell Model LNCaP

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ABSTRACT

Purpose: The disintegrin metalloprotease ADAM-10 is a multidomain metalloprotease that is potentially significant in tumor progression due to its extracellular matrix-degrading properties. Previously, ADAM-10 mRNA was detected in prostate cancer (PCa) cell lines; however, the presence of ADAM-10 protein and its cellular localization, regulation, and role have yet to be described. We hypothesized that ADAM-10 mRNA and protein may be regulated by growth factors such as 5α-dihydrotestosterone, insulin-like growth factor I, and epidermal growth factor, known modulators of PCa cell growth and invasion.

Experimental Design: ADAM-10 expression was analyzed by in situ hybridization and immunohistochemistry in prostate tissues obtained from 23 patients with prostate disease. ADAM-10 regulation was assessed using quantitative reverse transcription-PCR and Western blot analysis in the PCa cell line LNCaP.

Results: ADAM-10 expression was localized to the secretory cells of prostate glands, with additional basal cell expression in benign glands. ADAM-10 protein was predominantly membrane bound in cancer glands but showed marked nuclear localization in cancer glands. By Western blot, the 100-kDa proform and the 60-kDa active form of ADAM-10 were synergistically up-regulated in LNCaP cells treated with insulin-like growth factor I plus 5α-dihydrotestosterone. Epidermal growth factor also up-regulated both ADAM-10 mRNA and protein.

Conclusions: This study describes for the first time the expression, regulation, and cellular localization of ADAM-10 protein in PCa. The regulation and membrane localization of ADAM-10 support our hypothesis that ADAM-10 has a role in extracellular matrix maintenance and cell invasion, although the potential role of nuclear ADAM-10 is not yet known.

INTRODUCTION

The disintegrin metalloproteases ADAMs, like the matrix metalloproteinases (MMPs), are members of the metzincin (zinc-dependent metalloprotease) superfamily. To date, more than 30 ADAMs have been characterized (1), some of which are involved in diverse biological functions such as fertilization, neurogenesis (2, 3), and the ectodomain shedding of growth factors such as amyloid precursor protein and tumor necrosis factor α (4, 5). The ADAMs and MMPs share a homologous metalloprotease catalytic motif (HEXGHNLGXXH), which is responsible for zinc-dependent protease activity (6). In particular, ADAM-10 has been shown to have substrate specificity overlap with the MMPs, particularly with respect to the degradation of extracellular matrix (ECM) components. For example, purified bovine ADAM-10 (synonym, MADM) cleaves native type IV collagen (7), a major component of the basement membrane and the ECM. Thus, like the MMPs in aggressive cancer cell types (8), ADAM-10 displays potential ECM remodeling capabilities. However, relatively little is known about the association and roles of ADAM-10 in cancer. To address this, the present study has examined the expression and hormonal regulation of ADAM-10 in prostate cancer (PCa).

Although many cancers of the prostate are slow-growing and may remain organ-confined for many years, a significant number become highly aggressive and generate primary metastases to the bone and lymph nodes. Growth of both the normal prostate and PCa cells is regulated by key growth factors including androgens, insulin-like growth factor (IGF)-I, and epidermal growth factor (EGF) (9). Hence, these three growth factors are key promoters of PCa development. Because members of the MMP family have been shown to be responsive to similar growth factors involved in cancer progression (10, 11), and given the evidence for functional homology between the ADAMs and MMPs, we hypothesized that the ADAMs may be regulated in a manner similar to that of the MMPs.

Previously, we described the mRNA expression of five ADAMs in several PCa cell lines (12). In the same study, we found that the androgen 5α-dihydrotestosterone (DHT) up-regulated the levels of ADAM-10 mRNA in the androgen-sensitive PCa cell line LNCaP. We now report regulation and cellular localization of expression of this ADAM, at the protein level.
level, in prostate tissue sections and in the PCa cell line LNCaP. Our findings suggest IGF-I and DHT together or EGF alone increases ADAM-10 protein expression. Furthermore, in situ hybridization and immunohistochemistry identified the glandular epithelial cells as the predominant ADAM-10-expressing cell type in the prostate. No major difference in intensity was observed, but a definitive shift in localization from the cell membrane to the nucleus occurred as the pathology progressed from a benign to a cancerous state.

MATERIALS AND METHODS

Antibodies

The rabbit anti-ADAM-10/KUZBANIAN/MADM polyclonal antibody, purchased from Chemicon International (Temecula, CA), was raised against a COOH-terminal peptide corresponding to amino acids 732–748 of human ADAM-10. The anti-β-tubulin monoclonal antibody was purchased from Lab Vision (Fremont, CA). The anti-proliferating cell nuclear antigen monoclonal antibody was purchased from Zymed (San Francisco, CA).

Tissue and Sample Preparation

Archival paraffin-embedded prostate tissue blocks from 17 patients with PCa [14 patients with medium-grade disease (Gleason score of 6–7), 3 patients with high-grade disease (Gleason score of 8–10)], and 6 patients with benign prostatic hyperplasia were obtained from Royal Brisbane Hospital (Queensland, Australia). Ethics approval was obtained from institutional ethics committees. Tissue blocks were sectioned (3–5 μm) and mounted on 3-aminopropyl triethoxysilane-coated slides. Sections were then dewaxed and rehydrated through xylene, graded ethanol (100%, 90%, and 70%), and water.

In Situ Hybridization

cRNA Probe Preparation. An ADAM-10 cDNA template was generated by subcloning a 236-bp ADAM-10 reverse transcription-PCR product from LNCaP RNA. PCR was carried out using a forward (5’-TGGATGGTGGCCTCATTG-GTGA-3’) and a reverse (5’-TGCGATTAGCGTCATTGTC-3’) primer under the following conditions: 94°C for 4 min; followed by 40 cycles of 94°C for 1 min, 55°C for 30 s (annealing), and 72°C for 30 s. The PCR product was ligated into a pGEM-T Easy vector (Promega, Madison, WI), and the insert was confirmed by sequencing. Both antisense and sense (control) cRNA transcripts were generated from each strand of the cloned sequence using T7 or SP6 polymerases, respectively, using templates linearized by SauI and SacII, respectively. cRNA probes were labeled with digoxigenin using the digoxigenin RNA labeling kit protocol (Roche, Mannheim, Germany) according to the manufacturer’s instructions.

In Situ Hybridization Procedure. Tissue sections were deparaffinized through several washes in xylene, graded alcohols, and diethylpyrocarbonate-treated water. To increase the accessibility of the target RNA, the tissues were treated by hydrolysis with 0.2 M HCl (20 min at room temperature) followed by 0.3% Triton X-100 in PBS (15 min at room temperature) and proteolysis with proteinase K (10 μg/ml; 15 min, 37°C). Protease activity was subsequently inhibited by incubation in 0.1 M glycine (2 × 5 min at room temperature). Neutral buffered formalin (10%, 30 min at room temperature) was added to re-fix the tissues. A further dehydration in 70%, 90%, and 100% ethanol was performed. The slides were dried for hybridization. Slides were immersed overnight (at 50°C) in hybridization solution [10% dextran sulfate, 1 × Denhardt’s solution, 50% formamide, 4 × SSC (pH 7.0)], 500 μg/ml herring sperm DNA, and 200 μg/ml tRNA with a 150 ng/ml final concentration of digoxigenin-RNA probe.

Washes of increasing stringency were performed [4 × SSC, 15 min at room temperature; 4 × SSC, 2 × 20 min at 50°C, 2 × SSC, 3 × 20 min at 50°C; 1 × SSC, 10 min room temperature; 0.5 × SSC, 10 min room temperature; 0.1 × SSC, 10 min room temperature]. Specific binding of the probe to the target RNA was detected immunologically by anti-digoxigenin alkaline phosphatase antibody using nitroblue tetrazolium chloride/X-phosphate 5-bromo-4-chloro-3-indolyl-phosphate (Roche) as the chromogenic substrate solution. The sections were then counterstained with 1% nuclear fast red and mounted in permanent aquamount (Dako, Carpinteria, CA). Chromogenic markers and bright-field microscopy were used for in situ localization of ADAM-10 mRNA.

Immunohistochemistry

Procedure. H&E was used for routine histological examination. For ADAM-10 staining, antigen retrieval was performed by heat pressure-cooking the sections in 0.01 M citrate buffer (pH 6.0) for 3 min. The sections were incubated in 3% methanolic peroxide (13 min at room temperature) to block the endogenous peroxidase activity present in the samples. Nonspecific sites were blocked by incubation with 10% Blotto for 1 h at room temperature. The optimal antibody dilution (0.7 μg/ml) was applied and incubated overnight at 4°C. The immunoreactive sites were detected using the Envision system (Dako). The location of the antigen was revealed by addition of the diaminobenzidine substrate-chromogen solution (5–10 min at room temperature), yielding a brown deposit. The nuclei were counterstained with hematoxylin.

As a negative control for the immunostaining, the primary antiserum was omitted from the staining schedule. ADAM-10 primary antibody was also preabsorbed with a purified blocking peptide (Chemicon) to detect nonspecific binding.

Scoring. All tissue sections were analyzed in a single run to allow direct comparison of staining intensities. Microscopic analysis was performed in a blind fashion, several times, on different days. Two variables in immunostaining with the ADAM-10 antibody were assessed: (a) location (pattern); and (b) intensity of staining. Intensity on prostate tissue was scored as negative (−), weak (+), moderate (2+), strong (3+), and very strong (4+). Weak intensity staining was defined as staining that could be detected at ×10 objective magnification. Very strong intensity was the strongest color obtained, and moderate staining and strong staining were the two intensities detected between very strong intensity and weak intensity staining. The staining pattern was defined as uniform or heterogeneous, depending on the variability of intensity within or between glands, for benign or cancerous epithelium.
Cell Culture

Characterization of ADAM-10 mRNA and Protein Expression. The PCA cell line LNCaP was obtained from American Type Culture Collection (Manassas, VA) and cultured in standard T25 and T75 culture flasks (Medos, Brisbane, Australia) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum/gentamicin, in an atmosphere of 95% air/5% CO2 at 37°C. Routine tests for Mycoplasma infection were found to be negative.

Cell Proliferation Assay Using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Dye Method. A 90% confluent cell culture of LNCaP was treated with trypsin/versine and seeded into a 96-well tissue culture plate (Medos) at 10,000 cells/well in 200 μl of RPMI 1640/10% heat-inactivated fetal bovine serum/phenol red-free/gentamicin medium. Cells were allowed to attach for 24 h, washed with 200 μl of warm (37°C) PBS (tissue culture grade; Oxoid, West Heidelberg, Australia), and serum starved for 24 h in serum-free RPMI 1640/phenol red-free/0.01% BSA/gentamicin medium. Test medium containing DHT (Sigma, St. Louis, MO) at concentrations of 0 (control) 1.0, and 10 nM ± 50 ng/ml human recombinant IGF-I (Grootep, Adelaide, Australia), 50 ng/ml IGF-I alone, or human recombinant EGF (Grootep) alone (0 control), 5, 10, and 50 ng/ml) was added and replaced every 24 h for a period of 96 h. A total of 16 wells/concentration were used. Each replacement of medium included a warm wash with 200 μl of PBS. The MTT dye method was carried out as described previously (12). Briefly, a standard curve was constructed by seeding doubling increases in LNCaP cell number into a 96-well tissue culture plate (Medos) in phenol red-free/10% heat-inactivated fetal bovine serum/RPMI 1640. The cells were allowed to plate down for 6 h, followed by treatment with MTT. Absorbance was plotted against cell number to ensure linearity of the MTT assay across the range of cell numbers used (4,000–40,000 cells). The absorbances obtained at the completion of the proliferation assays were extrapolated to cell number using this standard curve. Statistical analysis was carried out by one-way ANOVA followed by a Tukey post hoc analysis to determine significance between all treatments and respective controls.

ADAM-10 Regulation. The LNCaP cell line was cultured in T75 culture vessels to 70% confluence under the conditions described above. The cells were transferred into serum-free RPMI 1640/0.01% BSA/gentamicin for 24 h. Test medium was then added to the cells after a warm wash (37°C) with PBS. To test for androgen regulation, DHT was added at concentrations of 0 (control), 0.1, 1.0, or 10 nM. For IGF-I regulation, IGF-I was added at concentrations of 0 (control), 10, or 50 ng/ml in the presence or absence of 10 nm DHT in serum-free RPMI 1640/phenol red-free/0.01% BSA/gentamicin. For EGF regulation, EGF was added at concentrations of 0 (control) and 50 ng/ml. The cells were cultured for an additional 48 h under test conditions, with a change of medium to replenish the appropriate growth factors after 24 h. Cells were harvested with ice-cold PBS, pelleted by centrifugation at 175 × g (1000 rpm; CS-6R Centrifuge; Beckman, La Jolla, CA) for 3 min, and stored at −80°C until further manipulation.

Real-Time PCR Analysis

Reverse Transcription-PCR Procedure. Five μg of RNA extracted (TRIZOL reagent; Invitrogen) from control and hormone-treated LNCaP cells, from three independent experiments, were reverse transcribed using Superscript II (Invitrogen) with oligo(dT) priming. All cDNA was found to be free of genomic DNA contamination by screening with β-actin primers that span an intron (12). PCR was carried out on a PE Biosystems 7000 ABI Prism using the following thermocycling protocol (95°C for 10 min, 40 cycles of 95°C for 30 s and 60°C for 1 min), and the fluorescence emitted from the SYBR green dye was read by the instrument after the completion of each thermocycle. The following reaction conditions were used: 1× SYBR Green master mix (Applied Biosystems, Foster City, CA), 50 nM each of ADAM-10 primers (forward, 5'-TCCA-CAGCCCCATTCCGCAA-3'; reverse, 5'-AGGCACTAGGAA-GAACCA-3') or 18S rRNA primers (forward, 5'-TTCGGAA-CTTAGGCATGAT-3'; reverse, 5'-CGAACCTCCGACTTTCC-GTCC-3', housekeeping gene); and 1 μl of cDNA template in a final reaction volume of 20 μl. Each growth factor treatment was run in triplicate. The 18S housekeeping gene (used to normalize ADAM-10) was always quantitated in the same PCR, in triplicate, for each treatment in a 96-well PCR plate format.

Quantitation of ADAM-10 mRNA Transcript. Each primer set was validated for use in quantitation using the “ct-value” direct comparison method as per the manufacturer’s (Applied Biosystems) recommendations. Briefly, cDNA from LNCaP cells was serially diluted and subjected to a PCR under the same conditions described above. The ct value was graphed against cDNA concentration, and an R2 value of >0.97 was obtained, showing that these primer sets could effectively detect relative changes of the target message (ADAM-10 or 18S) in the cDNA to be used for subsequent message level quantitation.

Western Blot Analysis

Whole Cell Protein Extraction. LNCaP cell pellets were resuspended in 500 μl of lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA (pH 8.0), and 1 tablet of Roche Complete EDTA-free protease inhibitor mixture/25 ml]. The cell lysate was frozen on dry ice, thawed in ice-cold water, and then passed twice through a 26-gauge needle. The cell lysate was centrifuged at 12,000 × g for 30 min at 4°C, and the resultant supernatant was collected. Whole cell protein was quantitated in triplicate using the BCA protein assay reagent (Pierce, Rockford, IL) as per the manufacturer’s “microwell” protocol. For confirmation of the specificity of the ADAM-10 antibody in prostate tissue, fresh frozen prostate tissue obtained from cancer patients and sectioned into 8-μm samples was homogenized in lysis buffer.

Preparation of LNCaP Nuclear and Cytoplasmic Protein Fractions. Cellular compartmentalization of LNCaP cells was achieved by differential lysis and centrifugation using the NePer nuclear/cytoplasmic fractionation kit (Pierce) as per the manufacturer’s recommendations. Each buffer was adapted to contain a final concentration of 0.1 mM phenylmethylsulfonyl fluoride and 10 mM EDTA (pH 8.0), and 1 × Complete EDTA-free protease inhibitor mixture (Roche) was added to inhibit protease activity, as recommended by the manufacturer.
Western Blot Procedure. Five μg of protein from each hormone-treated and corresponding control cell pellet or the nuclear/cytoplasmic fractions were suspended in a reducing loading buffer and boiled for 3 min before loading onto a 10% SDS-PAGE gel, followed by standard Western blotting procedure. The membrane was blocked with 5% Blotto and incubated with either 0.2 μg/ml rabbit anti-ADAM-10 antibody, 0.2 μg/ml mouse anti-β-tubulin monoclonal antibody, or 0.2 μg/ml mouse anti-proliferating cell nuclear antigen monoclonal antibody in 0.8% Blotto in Tris-buffered saline/0.05% Tween 20 at 4°C overnight. The membrane was incubated for 2 h at room temperature in either antirabbit horseradish peroxidase (Dako) or antimouse peroxidase (Chemicon) in 1% Blotto/Tris-buffered saline/0.05%/Tween 20. For detection, the membrane was incubated in 2 ml of Femto (Pierce) for 5 min at room temperature and exposed to X-ray film. The size of the protein was determined by comparison with Bio-Rad (Hercules, CA) prestained protein markers.

Quantitation of Signal Intensity. For the housekeeping gene β-tubulin, all autoradiographic detections were subjected to over- and underexposures to determine that the blots used for subsequent densitometric analysis were in the linear range of detection. Densitometric analysis was conducted using a GS-690 image densitometer (Bio-Rad). Signal intensity obtained for ADAM-10 expression was normalized for the signal obtained for β-tubulin for the same sample on the same Western blot. All Western blots were performed on protein samples collected from three separate, treated-cell preparations, giving an experimental value for each set of treatments of n = 3. Western blots for each set of treatments were performed at least in duplicate, giving possible n values of at least 6, and subsequent densitometric analysis was performed for each control and test concentration. Absolute ratios of intensity between control and test concentrations were calculated, and the entire data set was subjected to a one-way ANOVA followed by a Tukey post hoc analysis to determine significance between treatments and their respective control.

RESULTS

In Situ Hybridization and Immunohistochemistry. To determine the cellular localization of ADAM-10 in prostate tissue and to assess if benign, non-neoplastic and cancerous prostate tissues differ in their expression of ADAM-10, archived prostate tissue samples from 23 men with differing pathological conditions were studied.

ADAM-10 mRNA Expression in Benign, Nonneoplastic, and Cancerous Prostate Tissue. The hybridization signal from the ADAM-10 antisense riboprobe was predominantly detected in the cytoplasm of glandular epithelial cells and varied in intensity between different patients and different glands. Benign glands revealed strong ADAM-10 mRNA expression in basal cells but mostly weak staining in secretory cells (Fig. 1A, inset, arrow). Similarly, glands exhibiting characteristics of benign prostate hyperplasia demonstrated strong expression in basal cells and heterogeneous staining in secretory cells, which varied from very weak to strong staining (data not shown). Most glands exhibiting characteristics of prostatic intraepithelial neoplasia, a putative precursor of PCa (13), showed a high level of ADAM-10 mRNA expression (Fig. 1B). Staining intensity varied from weak (Fig. 1C), to strong (Fig. 1D) in tumor cells in different samples. High-grade PCa exhibited very strong staining intensity in the disrupted epithelial cells as well as in scattered cells in the stroma (Fig. 1E). The negative control sense riboprobe gave no positive signal (Fig. 1F).

**Tissue Localization of ADAM-10 Protein.** ADAM-10 immunostaining was observed across the range of clinical samples tested, although distinct and differential staining patterns were apparent between glands of differing pathology, even within a single section (e.g., Fig. 1K). Only minor differences in staining intensity were observed between benign glands within benign prostatic hyperplasia (1+ to 3+) or cancer (1+ to 4+) sections. Staining in benign tissue revealed cytoplasmic immunoreactivity in nearly all of the basal cells, with weak membranous staining in the secretory cells of some glands (Fig. 1G), whereas the majority of benign glands showed strong membranous staining patterns in secretory cells (Fig. 1, H and I). Interestingly, some perinuclear/nuclear staining, along with weaker membrane staining, was evident in some benign glands (Fig. 1J, arrow). No reactivity was observed in stroma. In stark contrast, PCa tissues demonstrated heterogeneous nuclear and perinuclear intensities, and staining varied from weak to strong (1+ to 3+), but across the whole cohort (n = 17) no apparent correlation with cancer grade was evident (Fig. 1, K and L). Benign glands within neoplastic tissue revealed some perinuclear clear as well as membrane-bound labeling (Fig. 1K, arrow). The localization of ADAM-10 immunostaining to the nucleus was strikingly obvious in all cancer glands studied, in all patients. To assess the binding specificity, ADAM-10 primary antibody was preabsorbed with purified ADAM-10 peptide. No positive staining was observed with the preabsorbed antibody (Fig. 1M). Western blotting was performed to assess whether the presence of ADAM-10 found in the nuclei of prostate tissue via immunohistochemistry could be confirmed in a PCa cell line (Fig. 1N). LNCaP cells were subjected to subcellular protein fractionation, and the relative purity of each fraction was evaluated using nuclear (proliferating cell nuclear antigen) and cytoplasmic (β-tubulin) markers. Fig. 1N shows that the two subcellular compartments were successfully isolated with only minor contamination of the cytoplasmic fraction with the nuclear marker, proliferating cell nuclear antigen. Immunoreactive bands representing the unprocessed proform of ADAM-10 (~100 kDa) and the mature (active) form of ADAM-10 (~60 kDa) were clearly present in the nucleus as well as cytoplasmic protein fractions. An additional band (~80 kDa) representing a partially processed form was also apparent in this fractionation study. A similar intermediate band is sometimes seen in whole cell lysates (e.g., Fig. 4).

**LNCaP Cell Proliferation.** To test for biological responsiveness of the LNCaP PCa cell model to each of the hormones/growth factors used, a MITT cell proliferation assay was performed. As expected, and as shown in Fig. 2A, LNCaP cell proliferation was significantly increased after the addition of DHT (19% increase), with a further increase in proliferation with the addition of IGF-1 (an additional 24% increase). For EGF-treated cells (Fig. 2B), a 4-fold increase in cell proliferation was observed. These findings parallel the same proliferative response found by Iwamura et al. (14) and Siu et al. (15) and
demonstrate the expected functional activity of DHT, IGF-I, and EGF in the LNCaP PCa cell model. Because it has been shown that PSA mRNA is stimulated on DHT treatment (16), the action of DHT in our system was further demonstrated by measuring the up-regulation of PSA mRNA in LNCaP cells by quantitative PCR. This result also validated our quantitative PCR method.

Regulation of ADAM-10 mRNA Levels. Real-time PCR analysis was performed on mRNA extracted from LNCaP cells treated with DHT, IGF-I, IGF-I plus DHT, or EGF alone. The changes in ADAM-10 mRNA levels were normalized for levels of mRNA initially reverse transcribed by quantitating 18S rRNA in the same cDNA in the same PCR. Fig. 3A shows that both DHT (10 nM) and IGF-I alone (50 ng/ml) significantly decreased levels of ADAM-10 mRNA, whereas DHT plus IGF-I significantly elevated (1.8-fold) ADAM-10 mRNA levels. For EGF (50 ng/ml), a significant elevation (1.7-fold) of ADAM-10 mRNA levels was observed (Fig. 3B).

3 D. R. McCulloch, A. C. Herington, and D. M. Odorico, unpublished data.

Fig. 1 In situ hybridization (A–F) and immunohistochemical staining (G–M) of the disintegrin metalloprotease ADAM-10 mRNA and protein in prostate tissue sections. In benign epithelium, basal cells stained for ADAM-10 mRNA (A, inset, arrow). A very strong signal was observed in secretory cells of prostatic intraepithelial neoplasia (B, arrow), whereas secretory cells in cancerous epithelium varied in staining intensity from weak to strong (C and D, arrows), to very strong staining in high-grade cancer (E). ADAM-10 sense cRNA probe (negative control) shows no positivity (F). The pattern of immunostaining for ADAM-10 protein in benign epithelium varies from a strong signal in basal cells and weaker staining on the cell membrane of secretory cells (G, arrow) to predominantly strong cell membrane staining of secretory cells (H and I, arrow; I represents a magnification of the boxed area in H) and a mix of uniform basal cell staining, secretory cell membrane staining, and some perinuclear/nuclear (arrow) staining in secretory cells (J). In contrast, a perinuclear/nuclear staining pattern was seen in all cancer epithelia (K, arrow) with high-grade cancer cells demonstrating strong nuclear staining (L, arrow). The preabsorbed antibody (negative control) shows no immunoreactivity (M). Scale bar, 30 μm.

Western blot (N) of ADAM-10, β-tubulin (cytoplasmic marker), and proliferating cell nuclear antigen (nuclear marker) on nuclear and cytoplasmic fractions from LNCaP cells, demonstrating both the purity and presence of ADAM-10 in both subcellular fractions.
image analysis, using treated with DHT, IGF-I, IGF-I plus DHT, or EGF. Quantitative position of the active 60-kDa form. Western blots were performed on 100-kDa proform and a doublet at the expected prostate tissue sections closely resembles the pattern found in tissue lysates. As depicted in Fig. 4, the banding pattern in the Western blot analysis of LNCaP cells alongside two prostate LNCaP cells as found in prostate tissue was confirmed by of the ADAM-10 antibody to detect the same protein forms in human prostate cancer cell lines.

Purified ADAM-10 (or its orthologs in species from Drosophila 319 -H9251 [-dihydrotestosterone and growth factors (in- insulin-like growth factor I (o r B epidermal growth factor (B)) for an additional 96 h. The 3-(4,5-dimeth-ylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye method was used to determine change in cell number. Data shown are derived from a single experiment with each condition replicated in 16 wells/96-well plate. Similar results were observed in two subsequent cell proliferation assays. *A, b,b,c P < 0.05; *a,c,d,e P < 0.01. B, *a,b,c P < 0.01.

**Western Blot Analysis and Quantification.** The ability of the ADAM-10 antibody to detect the same protein forms in LNCaP cells as found in prostate tissue was confirmed by Western blot analysis of LNCaP cells alongside two prostate tissue lysates. As depicted in Fig. 4, the banding pattern in the prostate tissue sections closely resembles the pattern found in LNCaP cells, a 100-kDa proform and a doublet at the expected position of the active 60-kDa form. Western blots were performed on whole cell protein extracted from LNCaP cells treated with DHT, IGF-I, IGF-I plus DHT, or EGF. Quantitative image analysis, using β-tubulin as an internal housekeeping marker, was used to correct for protein loading between the control and test lanes in each experiment. Quantitating the volume and density of each signal band allowed us to report ADAM-10 expression as an absolute ratio between the control and test treatments. DHT or IGF-I alone did not significantly alter ADAM-10 protein levels (Fig. 5), despite the reduction in ADAM-10 mRNA levels (Fig. 3). However, IGF-I (10 or 50 ng/ml) in the presence of 10 nM DHT up-regulated both the unprocessed 100-kDa proform (~1.8-fold) and the processed active 60-kDa form (~3–4-fold) of ADAM-10 (Fig. 5), paralleling the change in ADAM-10 mRNA (Fig. 3). EGF (50 ng/ml) also significantly stimulated the expression of unprocessed 100-kDa proform (~2-fold) and stimulated the processed 60-kDa form (almost 3-fold) of ADAM-10 (Fig. 5), again in complete agreement with observed changes in mRNA (Fig. 3).

**DISCUSSION**

This study describes, for the first time, the expression and cellular localization of ADAM-10 mRNA and protein in prostate tissues and provides novel data regarding the hormonal response of ADAM-10 mRNA and protein in the LNCaP cell model to three key PCa cell growth promoters: DHT, IGF-I, and EGF. The expression of ADAM-10 in the epithelial cells of prostate tumors may provide these cells with the capacity to assist in ECM remodeling and cell mobility, key characteristics of tumor cell invasion. Additionally, our data demonstrating a primarily nuclear localization of ADAM-10 in PCA epithelial cells raises the novel hypothesis that ADAM-10 may have further and distinct actions in this disease.

ADAM-10 (protein and/or mRNA) is expressed in all prostate glands, where a variation of staining intensity was observed for ADAM-10 protein in specimens representative of the same histological grade. This may well be a result of the inherent heterogeneity of glands within any given biopsy as being characteristic of normal, benign, prostatic intraepithelial neoplasia, or low- to high-grade cancer. Although no obvious trend toward an up-regulation of ADAM-10 in high-grade PCa compared with lower grade cancer or benign glands was observed, a most striking and unexpected finding was the differences in the localization of ADAM-10 staining between benign and cancer glands. In benign tissue, epithelial cell staining was largely concentrated on the cell surface with some nuclear/perinuclear localization, whereas all tumor cells demonstrated marked nuclear/perinuclear immunoreactivity with membrane staining essentially absent. The absence of membrane staining in high-grade cancer suggests differing roles for ADAM-10 as compared with the initial hypothesis that ADAM-10 may be involved in cell migration and cell invasion. Benign glands within nonneoplastic and neoplastic tissue all show consistent membrane localization in secretory epithelial cells. The strong membrane association is consistent with the documented ability of several ADAMs (in particular, ADAM-9, ADAM-10, and ADAM-17) to act as cell surface "sheddases," cleaving the extracellular (ecto-) domains of membrane-bound proteins from the cell surface (1, 17). This process can lead to the activation of growth factors or the solubilization of extracellularly active molecules. The proteolysis by ADAMs can also change the active states of remnant surface molecular complexes and hence affect signaling pathways inside cells (18). Purified ADAM-10 (or its orthologs in species from Drosophila 319 -H9252
to human) is highly active as a “sheddase,” being able to proteolytically cleave/ degrade, for example, the developmental regulator Notch receptor ligand Delta (19), precursor tumor necrosis factor α (4), amyloid precursor protein (20), and ephrin-A2 (a well-characterized axon repellent; Ref. 21).

Cell membrane-localized ADAM-10 in epithelial cells of benign glands suggests that ADAM-10 may be important in cell-cell and cell-matrix/cell-basement membrane interactions. Although ADAM-10 is not known to interact directly with any integrins (22), it has been shown to indirectly influence integrin-related adhesion activity by cleaving the L1 membrane adhesion molecule, a type I membrane glycoprotein implicated in the cell migration of neural and tumor cells (23). L1 is overexpressed in several carcinomas and is capable of acting as a substrate for integrin-associated cell adhesion (24). L1 has also been shown to trigger cell migration of Chinese hamster ovary cells (24).

Human ADAM-10, through the shedding of transmembrane collagen XVII (BP180) from the cell surface of keratinocytes, leads to the release of a soluble basement membrane collagen and an increased cell motility in vitro (25). Collagen XVII (one component of hemidesmosomes) provides stability for the interactions between the basement membrane and basal cells. Immunohistochemical studies of collagen XVII in human prostate have shown that in normal prostate and prostatic intraepithelial neoplasia, it is expressed along the basal aspect of the basal cells, together with a range of other hemidesmosomal-related proteins. PCs, on the other hand, were found to completely lack immunoreactive collagen XVII (26). Whether a direct causal link can be established between expression of ADAM-10 and the loss of collagen XVII in PCs remains to be investigated. The loss of both collagen XVII and ADAM-10 from the membrane of PCs may provide a mechanism for loss of cell-cell or cell-basement membrane contact and allow for the regulation of the mobility of such cells. ADAM-10 is known to cleave one of the major constituents of the basement membrane, collagen IV (7), and its activity is regulated by the tissue inhibitors of metalloproteinases (27). Tissue inhibitors of metalloproteinases serve as a key physiological regulatory mechanism for MMP-mediated ECM-degrading activity, providing additional support for functional similarities between the

![Fig. 4](image-url) Western blot analysis of the disintegrin metalloprotease ADAM-10 in the prostate cancer cell line LNCaP (Lane 1) and in two different prostate cancer tissue section extracts (Lanes 2 and 3). Both LNCaP and tissue extracts depict a similar banding pattern of a 100-kDa ADAM-10 band (proform) and a doublet at ~60 kDa (processed active forms).

**Fig. 3** The regulation of the disintegrin metalloprotease ADAM-10 mRNA extracted from LNCaP cells treated with 5α-dihydrotestosterone ± insulin-like growth factor I or epidermal growth factor alone. ADAM-10 mRNA levels were significantly elevated by a synergistic response to treatment with 5α-dihydrotestosterone ± insulin-like growth factor I (A) and with epidermal growth factor alone (B). Each histogram represents three independent experiments analyzed in triplicate in two separate PCR reactions. The changes in ADAM-10 mRNA levels were normalized to the levels of the housekeeping gene 18S rRNA in the same cDNA sample measured in the same PCR. A, *, P < 0.05; b,c,d, P < 0.01. B, *, P < 0.01.
ADAMs and MMPs (28, 29). The above observations are consistent with a putative role for membrane-bound ADAM-10 in the early stages of ECM degradation and facilitation of subsequent invasion of metastatic cells.

The primary nuclear localization of ADAM-10 in PCa tissue sections and the presence of ADAM-10 in the nuclear protein fractions of LNCaP cells, however, suggest an additional but quite different role, which has yet to be defined. We hypothesize that nuclear ADAM-10 functions by interacting with nuclear protein or DNA to directly influence nuclear processes. Evidence for nuclear localization and function of extracellular/membrane-bound proteins, previously thought to have no role in the nucleus, is becoming more common. Recent examples include growth hormone/growth hormone-binding protein (30), parathyroid hormone-related peptide (31), IGF-binding protein-3 and -5 (32), EGF receptor (33), and heparin-binding EGF (34). Evidence suggests that some of these have direct effects on transcriptional activity of target genes (33). The mechanism by which ADAM-10 translocates to the nucleus is not known. However, analysis of the ADAM-10 protein sequence reveals several consensus "nuclear localization signals" (35), including two Pattern 4 and two Pattern 7 motifs and a COOH-terminal bipartite nuclear localization signal (aa721-RRRPPQPIQQPQRQRPR-). Small proteins [up to 45 kDa (36)] that are not constitutively present in the nucleus can diffuse freely through the nuclear pore complex. However, many larger proteins, such as ADAM-10, require the presence of the largely highly basic nuclear localization signal motif and are taken up through one of several regulated, active-transport mechanisms (35, 37). Additional studies are required to determine which nuclear localization signal-mediated transport system is used by ADAM-10.

With respect to studies on the regulation of ADAM-10, the effects of DHT and IGF-I are particularly relevant in the transition from early- to late-stage PCa, when both IGF-I and DHT may be promoting growth and PCa development synergistically, whereas later stage PCa is known to have elevated levels of EGF (9). Previously, we reported the regulation of ADAM-10 mRNA levels by DHT in the LNCaP cell line (12). The current study has extended these observations to analyze changes in both ADAM-10 mRNA and protein levels in response to DHT and the growth factors IGF-I and EGF. Alone, IGF-I and DHT decreased the levels of ADAM-10 mRNA, but together, they significantly increased ADAM-10 mRNA. Our previous observations showed ADAM-10 mRNA levels significantly elevated at low DHT concentrations (0.1 and 1.0 nM), whereas at 10 nM DHT, the dose used here, levels of ADAM-10 mRNA declined, resulting in a bell-shaped dose-response curve (12). Because this current study focused on a possible synergistic response to DHT/IGF-I, we used the higher 10 nM concentration of DHT, so that any changes detected could be attributed to the addition of IGF-I. The use of the higher dose of DHT in these experiments is a likely explanation for the lower response to DHT observed in this study, compared with our previous report (12). EGF alone also elevated levels of ADAM-10 mRNA significantly, which is not surprising, given the ability of EGF to regulate gene expression of other genes (e.g., syndecan-1, collagens, and MMPs) involved in ECM maintenance (38).

With respect to ADAM-10 protein expression, two distinct bands were observed on Western blots. The larger band (100 kDa) likely represents the unprocessed proform of ADAM-10, whereas the ~60-kDa band corresponds to that found in plasma membrane preparations from bone MG-63 cells (39) and is
believed to represent the mature, active form of ADAM-10. An additional band (~80 kDa), which may represent a partly processed intermediate form of ADAM-10, was inconsistently observed.

Neither 10 nm DHT nor 50 ng/ml IGF-I alone resulted in a significant change in either ADAM-10 protein band. However, in response to IGF-I in the presence of DHT, a significant increase in protein expression was seen for the ADAM-10 proform (1.8-fold) and the mature, active form (3–4-fold). The addition of EGF also gave rise to a significant up-regulation of ADAM-10 protein (proform, ~2-fold; mature, active form, ~3-fold), in the absence of DHT. The apparent differences in regulation of the proform versus the mature form may reflect a specific effect on transcription (for the proform) and/or an effect on the furin-mediated processing mechanism or stability of the mature ADAM proteins (40).

The synergistic effect of DHT and IGF-I is consistent with known positive interactions between the IGF signaling pathway and androgen receptor activation (41). The subsequent loss of androgen dependence in late-stage PCa might be expected to cause a decrease in ADAM-10 because IGF-I alone was unable to increase ADAM-10 in vitro. However, because ADAM-10 is maintained at equivalent or greater levels in cancer glands in vivo (as demonstrated by immunohistochemistry), it would appear that additional regulatory factors, such as EGF [which has also been found to specifically up-regulate the metalloprotease matrilysin (MMP-7) in LNCaP cells (11)], are involved in regulating ADAM-10 expression.

The behavior of normal and cancer cells with respect to cellular contacts, cell migration, and invasion of surrounding tissues may well be determined, at least in part, by hormone and growth-factor regulation of ADAMs, such as ADAM-10, expressed and coordinately regulated within those cells. It is clear from the present study that the particular roles of ADAM-10 in PCs require further investigation but that ADAM-10 is very specifically expressed in epithelial cells of the prostate and appears to demonstrate differential regulation and function (nuclear versus membrane) in vivo, as PCA progresses from a benign to a malignant state. Because the ADAMs have a multidomain structure and are therefore potentially multifunctional, the ADAMs may have multiple roles depending on their cellular localization.

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