Expression of Adrenomedullin and Peptide Amidation Activity in Human Prostate Cancer and in Human Prostate Cancer Cell Lines

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ABSTRACT

After therapeutic hormone deprivation, prostate cancer (CaP) cells often develop androgen-independent growth through not-well-defined mechanisms. The presence of neuroendocrine (NE) cells is often greater in prostate carcinoma than in normal prostate, and the frequency of NE cells correlates with tumor malignancy, loss of androgen sensitivity, increase of autocrine-paracrine activity, and poor prognosis. In some CaPs, neuropeptides have been previously implicated as growth factors. Peptidyl-glycine α-amidating monooxygenase (PAM) is the enzyme producing α-amidated bioactive peptides from their inactive glycine-extended precursors. In the present work, we demonstrate that androgen-independent PC-3 and DU145 cell lines, derived from human CaP, express PAM in vitro and in xenografts implanted in athymic nude mice, indicating that they are able to produce α-amidated peptides. Contrarily, barely detectable levels of PAM were found in the androgen-sensitive LNCaP cell line. We also show that whereas PC-3 and DU145 cells produce and secrete adrenomedullin (AM), a multifunctional amidated peptide, no expression was found in LNCaP cells. We further demonstrate that AM acts as a growth factor for DU145 cells, which suggests the existence of an autocrine loop mechanism that could potentially drive neoplastic growth. PAM mRNA levels were found to be 3-fold higher in prostate adenocarcinomas compared with that of human benign prostate hyperplasia (BPH) as demonstrated by real-time quantitative reverse transcription-PCR. The analysis of AM message expression in BPH and CaP (Gleason’s score, 6–9) shows a clear distinction between benign and CaP. The expression was detected only in adenocarcinomas tissues with a marked increase in samples with a high Gleason’s score. Immunochemically, AM was localized in the carcinomatous epithelial compartment. NE phenotype, assessed after the immunocytochemical localization of neuron-specific enolase (NSE), was found in both the epithelial and the stromal compartments of cancers; in BPH, only some sparse basal cells were NSE-labeled. Cancer progression could be accelerated by peptides secreted by a population of cells capable of inducing androgen-independent tumoral growth via autocrine-paracrine mechanisms.

INTRODUCTION

CaP is currently the second leading cause of cancer death in men (1). Because androgens stimulate tumoral growth, hormone depriv-
nografted into nude mice, has been compared with human prostate pathological specimens. To better define the role of PAM in CaP, we have sought α-amidated factors involved in tissue growth. A preliminary screening of amidated peptides present in these types of cell lines have demonstrated that the AM mRNA is by far the predominant message encoding for two α-amidated peptides, namely pro-AM NH₂-terminal 20 peptide (PAMP) and AM. AM, originally identified in the human pheochromocytoma (25) has been shown to mediate a multifunctional response in cell culture and animal systems that includes regulation of cardiovascular tone, bronchodilation, modulation of central brain function, natriuretic and diuretic action, antimicrobial activity, inhibition of hormone release, growth regulation, apoptosis survival, and induction of angiogenesis (reviewed in Refs. 26–29). Both AM mRNA and immunoreactivity are widely distributed in human and rat tissues (30). AM synthesis and secretion were also observed in tumor cell lines of various origins including a prostate cell line DU145 (31).

The present work analyzes the expression of PAM and AM mRNA and the activity of PAM in cultured or xenografted androgen-indepen- dent (PC-3 and DU145) and androgen-dependent (LNCaP) cell lines. The expression of AM and PAM mRNAs was also studied in human specimens of BPH and CaP using a real-time quantitative RT-PCR. In situ hybridization was used to demonstrate the distribution of AM and PAM mRNAs. Finally, the immunocytochemical localization of AM and NSE was studied in BPH and CaP.

MATERIALS AND METHODS

Cell Lines. The human-derived CaP cell lines LNCaP, PC-3, and DU145 were obtained from American Type Culture Collection (Rockville, MD) and routinely maintained in RPMI 1640 (Life Technologies Inc., Paris, France) supplemented with 1 m M sodium pyruvate, 1 m M L-glutamine, 0.5% genta-micin, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 10% fetal bovine serum. The cells were grown at 37°C in a humidified 95% air/5% carbon dioxide atmosphere, passaged weekly, and routinely monitored for mycoplasma contamination by using a detection kit (Roche Molecular Biochemicals, Meylan, France). Before experimentation, cells were plated for 6 days in dextran-coated charcoal steroid-depleted serum (2.5% for LNCaP, 1% for PC3 and DU145) to get density of 30 × 10⁶ for LNCaP, 7 × 10⁶ for PC3, and 4 × 10⁶ for DU145 per 1.9 cm². Cells growing exponentially were harvested by a brief incubation with 0.25% trypsin-EDTA solution.

Animals and Experimental Protocol. Five-to-six-week-old male athymic NMRI (nu/nu) nude mice were obtained from Janvier (Laval Le Genest, France) and watered ad libitum. Institutional guidelines for the proper and humane use of animals in research were followed.

Cell growing exponentially were implanted into six male nude mice by s.c. injection of LNCaP cells (1 × 10⁶), PC3 (3 × 10⁶), and DU145 (5 × 10⁶), in the right flank. When tumors reached a volume of ~600 mm³, mice were killed by direct cervical dislocation at 18 (LNCaP) or 7 (DU145 and PC3) weeks after injection. Tumors were immediately removed and frozen in liquid nitrogen for further experimentation.

RNA Preparation and Analysis. Total RNA was extracted from cell lines, tumor xenografts, and human CaP using the acid guanidinium thiocyanate-phenol-chloroform procedure (32). Northern blot analysis was performed essentially as described previously (33). Briefly, total RNA (20 μg) was resolved on 1% agarose-formaldehyde denaturing gels. The denatured RNAs were transferred to Hybond-N membranes (Amersham Pharmacia Biotech, Orsay, France) by capillary action in 10× SSC [1.5 m M NaCl, 0.15 m M sodium citrate (pH 7.0)], cross-linked by UV irradiation and hybridized to (α-32P)-labeled human 1.1-kb-PAM cDNA (21) and 1.2-kb-AM cDNA (34), respec-tively. Filters were prehybridized, hybridized, and washed as described previ-ously (33). To correct for differences in loading and/or transfer, blots were stripped and hybridized to cDNA probes derived from frog rRNA (33). The autoradiograms were analyzed by measurement of absorbance by scanner-density meter using NIH Image 1.54 Software (NIH, Bethesda, MD). The hybridization signals of PAM and AM mRNAs were normalized to that of 18S rRNA. The results were expressed as the ratio of PAM or AM mRNAs absorbance:18S RNA absorbance.

Preparation of Tissue Extracts for Amidation Assay. Cells were scraped into ice-cold PBS and collected by centrifugation at 2,000 × g for 5 min. The resulting pellet and the tissue from xenografted tumors were homogenized in 20 m M NaTDS [N-Tris (hydroxy-methyl) methyl-2-amino-ethane sulfonic acid; pH 7.4], 10 m M mannitol containing 2 μg/ml leupeptin, 16 μg/ml benzamidine, and 30 μg/ml phenylmethysulfonyl fluoride using a ground glass homogenizer at 4°C. The homogenates were frozen and thawed three times and separated into soluble and particulate fractions, as described previ-ously (33). The crude particulate fractions were resuspended in the same buffer containing 1% Triton X-100. Amidation for 20 min at 37°C with the respective supernatants were used to measure solubilized and membrane-associated PAM activity. All of the samples were stored at −70°C until assay. Protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce Europe, Oud Beijerland, the Netherlands) with BSA as standard.

Amidation assays were performed in duplicate as described previously (35). Reactions were carried out for 2 h at 37°C in a final volume of 40 μl containing 150 m M NaMES [2-(N-morpholino) ethanesulfonic acid (pH 5.5)], 0.5 μM α-N-acetyl-Tyr-Val-Gly, 10 m M CuSO₄, 100 μg/ml catalase, and 10,000–15,000 cpm of [3H]α-N-acetyl-Tyr-Val-Gly, and 1–3 μg of protein. The α-hydroxylated product formed by PHM was converted into α-amidated peptide by the addition of 10 μl of 1 N NaOH. The α-amidated product was separated from the substrate by extraction with ethyl-acetate (35). Reaction velocities were expressed as pmol of product formed per mg protein (specific activity). The variation between duplicate samples was less than 5%. The reaction velocities reported are initial velocities, using a concentration of substrate about 10-fold below the Kₘ of the enzyme for the peptide substrate. In general, no more than 10% of the substrate was converted into product.

Peptide Extraction and RIA. Cell pellets (6 × 10⁶ cells) were boiled in 0.5 m M acetic acid for 20 min (1:10, w/v). After homogenization with a Potter apparatus, cell suspensions were centrifuged at 24,000 × g for 15 min. The pellets were stored at −20°C until assayed for protein content using the bicinchoninic acid protein assay reagent (Pierce Chemical Co.). The supernatant was lyophilized, and the resulting residues were resuspended in RIA buffer (30). The RIA of AM was performed as reported previously (30), using the antisem against human (AM 1–52 amidated) obtained from the Peptide Institute (Osaka, Japan) and was used at a final dilution of 1:30,000. To measure the IR-AM in the culture medium, the medium was extracted by the previously reported method (30) using Sep-Pak C18 cartridges (Waters, Milford, MA). Intra- and interassay coefficients of variation were 6% (n = 10) and 9% (n = 7), respectively.

Human Prostate Specimens. Human prostate specimens from BPH (n = 5) and CaP (n = 15) of different Gleason’s scores were obtained from the Department of Urology (AP-HM, Marseille, France). All of the tissue procure-ment protocols were approved by the relevant institutional committees (University of Aix-Marseille) and were undertaken under informed consent of each patient and all of the participants.

Quantitative RT-PCR. Real-time quantitative PCR method was used to accurately detect the changes of AM, PAM, and ribosomal 18S gene copies. The cycle at which the amplification plot crosses the threshold (CT) is known to accurately reflect relative mRNA values (36, 37). Total RNA (2 μg) DNA-free was reverse transcribed into cDNA using 1 μg of hexamers (Phar-macia Biotech, Orsay, France) and Moloney murine leukemia virus reverse transcriptase as described by the manufacturer (Life Technologies Inc., Paris, France). Human AM and PAM mRNA and 18S RNA were amplified (AM: forward primer, 5′-TGCCGACACCTTATTCGG-3′ and reverse primer, 5′-AAGTTGTTTGCATGTCGTCGGG-3′; PAM: forward primer, 5′-CACTGATT-GGACGCGAGAG-3′ and reverse primer, 5′-CCTACTGAGCTGTC- CACCA-3′; 18S: forward primer, 5′-CTCACCAGATTCAAGAAGACAGA-3′ and reverse primer, 5′-TTTTCTGCTACTCCTCCCG-3′), detected, and quantified in real time using the ABI Prism 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA) as described previously (36, 37).

The Taq Man probes for AM, PAM, and 18S were 5′-ACATGAAGGTT-GCCTCTGGAAGACCC-3′; 5′-TTTGGTGACCTACTGCGTGCA-3′; and 5′-CAGCGCCATATTCCACCTCCCCGAC-3′, respectively. The amplification
mixture contained cDNA derived from 50–150 ng of total RNA, 0.2 μM of primer, and 0.1 μM of Taq Man probe in 50 mM salt and 5 mM MgCl2. A two-step PCR was performed for 35 cycles. Denaturation was done at 94°C for 20 s, and annealing/extension at 60°C for 30 s. The reaction produced a 115-bp PCR product for AM, one of 155 bp for PAM, and one of 70 bp for 18S. To determine the accuracy of the assay, total RNA was reverse transcribed and amplified on 3 separate days. The interassay accuracy of amplification for the 3 days was 8%. For quantitation of the data, AM and PAM mRNA levels were normalized to the 18S rRNA levels in the same reaction. To create standard curves for each gene, RNAs were produced by in vitro transcription from linearized templates corresponding to AM, PAM, and 18S cDNA constructs using T7 or T3 polymerases and reverse transcribed to cDNA.

**Taq Man PCR Assay Conditions for PAM and AM mRNAs.** Using the fluorogenic probes for AM, PAM, and 18S with the experimental conditions defined above, we obtained a linear relationship between the RNA concentration (previously transcribed into cDNA) and the fluorescent signal (ΔRQ) for AM, PAM, and 18S RNA in 1–250 pg DNA target. For each unknown sample, we determined the ΔRQ values for all of the three genes, and the results were expressed as fg of AM or PAM mRNA per ng of 18S RNA.

**Immunocytochemistry and Microscopic Analysis of AM and NSE Proteins.** Resected specimens belonging to three patients presenting with CaP (two grade 5 and one grade 7, according to Gleason’s score), and two Resected specimens belonging to three patients presenting with Proteins. expressed as fg of AM or PAM mRNA per ng of 18S rRNA.

we determined the antibody previously mixed to the AM peptide (10 μg/ml), or with a nonimmunized swine serum instead of the anti-AM antibody, were used as controls. For NSE, only the incubation with a nonimmunized goat serum was used at this stage. The sections were lightly counterstained with hematoxylin. Microscopic analysis was done on a Leitz DMRD microscope (Wetzlar, Germany) equipped with a planachromat 40×/0.70 objective. Microscopic fields were captured with a CoolSNAP CCD camera (RS Photometrics; Roper Scientific Inc., Tucson, AZ) and digitized through a CoolSNAP PCI grabber slotted on a microcomputer. Microscopic magnification was estimated by using a Leitz 2-mm/0.01-mm-interval grid. Image quality was improved using Sharpen Edges command of Photoshop software (Adobe System Inc., Mountain View, CA).

**In Situ Hybridization and Microscopic Analysis of PAM and AM mRNAs.** In situ hybridization using 35S-labeled riboprobes was performed as described previously (38). Crystall sections (10 μm) of the same fixed specimens used for immunocytochemistry were mounted on gelatin-coated slides, fixed in buffered 4% paraformaldehyde (pH 7.4), and acetylated with 0.5% w/v acetic anhydride in 0.9% w/v NaCl containing 100 mM triethanolamine (pH 8.0). 35S-labeled sense and antisense riboprobes were transcribed from linearized plasmids containing human PAM cDNA (2.2 kb) and human AM cDNA (1.2 kb) using T3 or T7 RNA polymerase (38). The 35S-labeled riboprobe was added to the hybridization buffer containing 50% formamide, 600 μM NaCl, 1× Denhardt’s, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM DTT, 0.2 μg/μl RNA, and 10% dextran to give a final concentration of 1 × 106 cpm/50 μl of buffer. Hybridization was carried out overnight at 56°C in moist-sealed chambers. All of the subsequent steps were performed at room temperature unless otherwise specified. Coverslips were removed under 2× SSC. Slides were incubated in a solution of 2× SSC (30 min, 37°C) containing 10 μg/ml RNase A (Sigma-Aldrich, Saint Quentin, France), and subsequently washed in 1× SSC twice for 10 min, 0.5× SSC for 10 min, 0.1× SSC for 30 min at 60°C, and finally in 0.1× SSC for 10 min. Slides were dehydrated in graded ethanol, air-dried, and exposed to X-ray film (Kodak XAR) for 3 days. For higher resolution analysis, slides were dipped in Ilford K5 photographic emulsion. After exposure for 25 days, slides were developed and lightly counterstained with hematoxylin. Observation was done in a Leitz DMRD (Wetzlar, Germany) light microscope equipped with a planachromat 20×0.50 objective. Representative microscopic fields were captured as indicated for immunocytochemistry, except that each field was captured twice: once under bright field microscopy to assess histopathological characteristics and once under dark-field microscopy for detecting the distribution of the radioautography silver grains. Magnification was assessed with the same Leitz calibration grid.

**Cell Proliferation Assay.** The assay was performed in TIS medium (1640 plus 10 μg/ml transferrin, 10 μg/ml insulin, and 3 × 10−6 M sodium selenite). The effect of the AM at 2 × 10−7 M on cell proliferation was examined by the MTT assay as described previously (39). After 4 and 8 days growth at 37°C in a humidified 95% air-5% carbon dioxide atmosphere, the dye and solubilization solutions were added from the Promega Proliferation Assay (Lyon, France) which was a variation of the MTT assay (40). The Bio-Tek Microplate Manager plate reader and software was used to determine the change in the number of viable cells from dye reduction measured by absorbance at 570 nm.

**Statistical Analysis.** All of the results were expressed as the mean ± SE. Statistical analysis was performed by a one-way ANOVA followed by Fisher’s protected least significant difference (PLSD) test (Statview 512, Brain Power Inc., Calabasas, CA). P ≤ 0.05 was considered significant.

**RESULTS**

**Expression of PAM mRNA in Prostate Carcinoma Cell Lines.** We first sought to assess PAM mRNA levels in hormono-dependent (LNCaP) and in hormono-independent (DU145 and PC-3) cell lines. Total RNA prepared from each of these cell lines was subjected to Northern blot analysis and revealed the presence of an ~4 kb of mRNA hybridizing with the probe for human PAM (Fig. 1A). The PAM cDNA probe was removed from the blots, and the amount of rRNA present in each sample was determined by hybridization to a 50-g aliquot of total RNA (20 μg) was fractionated on a denaturing 1% agarose gel and transferred to Hybond-N membrane. The blot was hybridized with a 1.1-kb human PAM cDNA probe and exposed to X-ray film for 48 h at ~70°C with an intensifying screen. The band was excised from X-ray film and was used to quantify the amount of PAM mRNA to permit correction for the amount of sample actually transferred to Hybond-N membrane. In B, for densitometric analysis of PAM mRNA levels, the amount of PAM mRNA was normalized to the amount of rRNA. Data from three separate experiments were used to calculate the mean of individual values. Data are presented as the mean ± SE.
cDNA probe for 18S rRNA (Fig. 1A). The amount of PAM mRNA in each sample was then normalized to the amount of rRNA (Fig. 1B).

On the basis of the analysis of at least three independent preparations of RNA from each cell line, the amount of PAM mRNA in DU145 cells was 3-fold higher than that found in PC-3 cells (Fig. 1B) and exceeded by far that of LNCaP, in which PAM mRNA could only be detected by RT-PCR (data not shown).

To determine the effect of differentiating and mitogenic factors present in the local cell environment on PAM expression, PAM mRNA levels were analyzed in prostate cell lines xenografted in nude mice. Northern blot analysis of the xenografted tumoral tissue demonstrated that only PC-3 and DU145 expressed PAM mRNA and activity in hormono-independent cell lines (PC-3 and DU145), either maintained in vitro or xenografted, demonstrates that these malignant cells are able to produce α-amidated peptides.

Identification of PAM Activity in Prostate Carcinoma Cell Lines. Cultured cells were homogenized and separated into soluble and particulate fractions. All of the extracts were assayed for amidation activity and contained detectable levels of PAM activity (Fig. 3). Compared with LNCaP, PC-3 and DU145 consistently contained higher levels of soluble and membrane PAM activity. The soluble PAM activity in PC-3 (33.78 ± 9.33 pmol/mg/h) and DU145 (62.26 ± 20.90 pmol/mg/h) was higher than LNCaP (0.21 ± 0.048 pmol/mg/h; Fig. 3). The membrane PAM activity was 38- to 46-fold higher in PC-3 (17.29 ± 2.79 pmol/mg/h) and DU145 (20.72 ± 2.08 pmol/mg/h) than LNCaP (0.45 ± 0.068 pmol/mg/h; Fig. 3). Soluble: particulate PAM activity ratio was cell-type specific.

The next experiments were carried out to quantitate the levels of both soluble and membrane-associated PAM activity in xenografted prostate cell lines. Fig. 4 shows PAM activity measured from xenograft tumoral tissue. Interestingly, levels of both soluble and membrane PAM activity in PC-3 xenografts (41.77 ± 6.49 pmol/mg/h) were significantly (3- to 4-fold) higher than those found in DU145 xenografts (11.83 ± 0.98 pmol/mg/h) and 92- to 93-fold higher than in LNCaP xenografts (0.45 ± 0.1 pmol/mg/h). No effect of the local environment can be observed on the soluble PAM activity, whereas the membrane-associated PAM activity showed a 2.4-fold increment in PC-3 xenografts compared with extracts from PC-3 cells in vitro (Figs. 3 and 4). Surprisingly, compared with extracts prepared from DU145 cells maintained in vitro, the extracts from DU145 xenografts demonstrated a 5- to 6-fold decrease in soluble, and a 2-fold decrease in membrane, PAM activity (Figs. 3 and 4). These data suggest that the expression of PAM might be affected by the local cell environment.

The presence of PAM mRNA and activity in hormono-independent cell lines (PC-3 and DU145), either maintained in vitro or xenografted, demonstrates that these malignant cells are able to produce α-amidated peptides.

Human AM-mRNA Expression in Prostate Carcinoma Cell Lines. Total RNA prepared from the three cell lines maintained in culture or xenografted was subjected to Northern blot analysis, and AM mRNA was visualized using a human-AM-radiolabeled cDNA. The size of this messenger transcript was ~1.6 kb (Fig. 5, A and B), corresponding to what has been found in other AM-producing tissues, such as adrenal gland, lung, kidney, heart, spleen, duodenum, and salivary gland in the rat (41). The amount of AM mRNA in each sample was then normalized to the amount of rRNA (Fig. 5C). The Northern blot analysis demonstrated that only PC-3 and DU145...
expressed AM, whereas no expression was found in LNCaP, even after prolonged exposure. The absence of AM expression in LNCaP cells was also corroborated by RT-PCR (data not shown). Interestingly, in both PC-3 and DU145 cell lines, a significant 4- to 6-fold increase in the mRNA levels of AM was found in tumor xenografts as compared with the expression in vitro (Fig. 5C).

Production and Secretion of AM in Prostate Carcinoma Cell Lines. IR-AM was detected in both the cell extracts and the culture medium. PC-3 and DU145 cells produced and secreted IR-AM. Dilution curves of the extracts were parallel with the AM standard curve (Fig. 6). Concentrations of IR-AM were 71 ± 9 fmol per mg protein in PC-3 cells, and 78 ± 10 fmol per mg protein in DU145 cells. When expressed as the amount of IR-AM secreted into the medium reported to the cell number (as measured by the cell lysate protein level) both PC-3 and DU145 cells secreted 3.3 ± 0.5 fmol per mg protein per hour. No IR-AM was detected in the medium and in the cell extracts of LNCaP.

Expression of PAM and AM mRNAs in Human CaP. Total RNA was prepared to assess steady-state levels of PAM and AM mRNA transcripts. The expression of both of the genes in human prostate adenocarcinomas for which Gleason’s scores ranged from 6 to 9 as well as in BPH was analyzed by real-time quantitative RT-PCR. Quantification of PAM mRNA transcripts revealed higher PAM mRNA levels in prostate adenocarcinomas compared with BPH samples (Fig. 7A). The mean level of PAM mRNA expression was 27 ± 9.9 fg/ng 18S rRNA in prostate adenocarcinomas, whereas it was 24 ± 0.6 fg/ng in benign samples (P ≤ 0.017). Quantification of AM mRNA transcripts shows a clear distinction between benign and adenocarcinoma samples. Fig. 7B shows that AM-mRNA was expressed in all of the samples prepared from prostate adenocarcinomas. The mean level of AM mRNA expression was 0.95 ± 0.05 fg/ng 18S rRNA for samples with Gleason’s score 6 (ADK6), 1.44 ± 0.17 fg/ng for ADK7, 1.23 ± 0.029 fg/ng for ADK8, and 2.95 ± 0.284 fg/ng for ADK9. Very low-to-null expression was detected in samples

Fig. 6. AM RIA. A standard curve (•) of human AM and dilution curves of the cell extracts (1/4, 1/2, 1/1) of PC-3 (○) and DU145 (●). 1/1, the measurements in cell extracts equivalent to 280 μg of protein of DU145 and PC-3 cell extracts. LNCaP cell extracts are not shown because the cell line lacks detectable IR-AM. R/B0, the ratio of measured bound peptide to basal bound labeled peptide.

Fig. 7. Real-time quantitative RT-PCR analysis of PAM and AM mRNA levels in human prostate tissues. Total RNA DNA free from BPH and CaP (Gleason’s scores, 6–9) were transcribed to cDNA and subjected to real-time quantitative RT-PCR for the estimation of relative PAM mRNA (A) and AM mRNA (B) to 18S rRNA ratios as described under “Materials and Methods.” The PCR products were fractionated on agarose gels, prepared for Southern blot analysis, and hybridized with the corresponding probe to confirm the identity of the amplified products (not shown).
from benign prostate pathologies (0.019 ± 0.011 fg/ng; Fig. 7B). Omission of the reverse transcriptase eliminated the signal, which indicated that it was not attributable to contaminating genomic DNA (not shown).

**Immunocytochemistry of AM and NSE Proteins.** Comparing BPH and CaP, microscopic examination showed different localizations for the reaction products of AM and NSE. As shown in Fig. 8A, which is a representative field of the two cases of BPH examined, AM labeling was absent from the majority of the epithelial compartment of the glands and was only slightly detectable in the stroma; however, a mild staining was observed over restricted segments of the glandular epithelium. Cancer specimens (Fig. 8B) displayed overt AM labeling of both epithelial and stromal cells. It is most likely that the intensity of the labeling is related to the degree of malignancy. In the case of the two grade 5 cancers, irregularly shaped adenocarcinomatous formations displayed heavily labeled segments of monostratified epithelium.
lium as well as of multiple pluristratified sprouts. Horseradish peroxidase reaction product was detected in the cytoplasm, but many cell nuclei were also labeled. Dispersed among the stromal collagen septa, numerous clusters of labeled cells were consistently found. The degree of epithelial labeling was intensified in the grade 7 adenocarcinoma (Fig. 8, C and D), in which multiple solid carcinomatous masses were all deeply stained, particularly at the cell nuclear level. In this highly malignant cancer stage, some tissue areas presented with labeled stromal cells (Fig. 8C), but the majority of examined territories were constituted by intensely stained carcinomatous infiltrations surrounded by practically unstained stroma (Fig. 8D). Sections proceeding from BPH or CaP and previously neutralized with the AM peptide displayed both the epithelial and the stromal compartments completely unlabeled (Fig. 8, E and F).

NSE labeling was restricted to the stroma in BPH; in these cases, the majority of acinar epithelial cells was unlabeled, although some rare isolated cells located in the basal epithelial stratum contained a distinct deposit of reaction product (Fig. 9A). On the contrary, in the three adenocarcinomas analyzed, the epithelial compartment was consistently labeled for NSE. In the two grade 5 adenocarcinomas, heavily labeled transitional-like epithelial formation protruding into the acinar lumen alternated with epithelial segments composed of completely unlabeled cells; here, some cells located in the basal epithelial stratum were also heavily labeled (Fig. 9B). Intensely NSE-labeled solid carcinomatous masses were characteristic of the grade 7 adenocarcinoma (Fig. 9C). The sections in which the specific antibody was replaced by a nonimmune goat serum were unlabeled (Fig. 9D).

**In Situ Hybridization for PAM and AM mRNAs.** Sections hybridized with antisense and sense riboprobe for AM and PAM mRNAs were observed after 25 days of autoradiographic exposure. Both AM and PAM mRNA antisense-riboprobes, tested on sections of Gleason 7 prostate CaP, consistently displayed deposits of silver grains over the adenocarcinomatous formations, on the stroma as well as on the epithelium; however, there was a clear predominance of the labeling to be localized over the epithelial compartment (Fig. 10, A...
and B). The sections hybridized with the sense riboprobes for AM and PAM messengers displayed all over the structures a much fainter concentration of grains (Fig. 10C).

**Effect of AM on the Proliferation of Prostate Cell Lines in Vitro.** AM had been shown to elevate cAMP, a signal transduction pathway known to modulate cellular growth (42). To investigate this suspect effect, we used MTT assay techniques to examine the effects of AM on the growth of prostate cell lines. DU145, PC-3, and LNCaP cells cultured in vitro were exposed to $2 \times 10^{-7}$ M of AM, and the effect on the proliferation was followed by the MTT assay. As shown in Fig. 11, AM at $2 \times 10^{-7}$ M stimulated the proliferation of DU145 by 20% ($P < 0.05$) and 25% ($P < 0.02$) after 4 and 8 days of treatment, respectively. Both PC-3 and LNCaP cells showed no proliferative responses. Because PC-3 cells produce AM peptide, we assumed that this inability to stimulate growth with extrinsic ligand could possibly mean that the cells had already achieved maximal proliferative effects using the endogenous factor. The absence of expression of AM receptor, or of a functional AM receptor in the LNCaP cell line, may be the reason why AM had no effect on its growth.

**DISCUSSION**

On the basis of the detection of NSE (5, 6) and chromogranin A (12), the occurrence of NE phenotype in human prostate is well documented but not well understood. Much of the uncertainty about the pathogenetic significance of NE phenotype derives from the lack of knowledge concerning prostate NE cell biology. NE cells are rare in the normal human prostate epithelium (43, 44). With growing recognition of cancer as a multifactorial process, the identification and characterization of specific cellular phenotypes contributing to the malignant process may open the possibility of targeting the responsible cells to improve therapy. Tumor cell populations have been...
reported to become enriched for NE cells after long-term antiandrogen therapy (18). The presence in CaP of NE cells points them as secretors of paracrine proliferation stimuli for the surrounding carcinomatous cells. It is, therefore, probable that the expression of the NE phenotype be associated in human pathology, as well as in experimental models, to the shift of CaP toward the androgen-independent state (45, 46). Identifying factors produced in the tumor and engaged in NE phenotype regulation, assessing the potential paracrine activity of NE cells, and elucidating the role of NE cells in the development of androgen independence can provide clues for improving therapy. Greater benefit may be gained by identifying control points in the pathways of the synthesis of growth factors. In this report, we have investigated a major pathway for peptide processing. The NE phenotype implies the presence of peptides that are most probably produced and secreted in situ. A majority of peptides are active only after amidation through PAM activity. In the present study, we showed the presence of PAM mRNA in two androgen-independent cell lines (PC-3 and DU145), in CaP and BPH tissues as demonstrated by Northern blot analysis, a real-time quantitative RT-PCR, and in situ hybridization, which suggests the synthesis of amidated peptide(s) in these systems. As shown by the enzyme assays, PAM is active in both cell lines, which indicates the capacity to α-amidate products if the appropriate prepropeptides, endo- and exoproteases as well as reducing equivalents, are available. In addition, barely detectable levels of PAM activity were found in the androgen-dependent cell line LNCaP. The low-to-null PAM expression seen in some well-differentiated tumors can account for the concomitant low-to-null content of amidated peptides. For example, no amidated peptides have been detected in NE breast carcinomas (47–49). On the basis of our data, it seems that PAM content is related to the degree of NE expression of the carcinomatous cell. The increase in PAM biosynthesis is associated to the production of α-amidated peptides (38), some of which could act as autocrine-paracrine factors and influence the response of neighboring neoplastic cells.

As found in previous studies, using PAM as a marker for the presence of α-amidated peptides in NE tumors could be a potential avenue for the discovery of novel peptides or for gaining a better insight into known peptides the distribution of which has not yet been detailed (50). The demonstration that circulating levels of pancreastatin are strongly influenced by enterochromaffin-like cell number and activity (51) is consistent with the reported relation between plasma PAM activity and gastrin levels (52); gastrin is a well-known growth factor for enterochromaffin-like cells (53). A preliminary survey of amidated peptides present in CaP-derived cell lines has demonstrated that AM is predominantly represented. Both AM mRNA and peptide are present specifically in androgen-independent cell lines PC-3 and DU145 as well as in CaP. Although our data must be confirmed on a large series of CaP samples, it seems that AM mRNA levels are higher in samples with high Gleason’s scores. However, expression of AM was practically absent in the androgen-dependent cell line LNCaP and in tissues derived from benign pathologies. Recently, Jiménez et al. (54) reported that in human normal prostate, AM was expressed in the prostate epithelium. Additional studies are needed to elucidate this issue.

AM has been shown to have a remarkable range of actions, from regulating cellular growth and differentiation, through modulating hormone secretion, to antimicrobial effects (26). Herein, we demonstrate that the addition of external AM to DU145 cells significantly stimulates cell growth, which opens the possibility of AM being an autocrine/paracrine growth factor in CaP. DU145 cells have been previously shown to express both AM and AM-receptor (L1) message as reported by Miller et al. (31). These authors demonstrate a potential autocrine growth effect of AM with a variety of human tumor cell lines. Taken together, these findings implicate AM as having a possible regulatory role in human tumor promotion.

Our data showed a marked increase in AM mRNA levels during xenograft growth for both PC-3 and DU145 cell lines. The observed elevated response in AM transcript expression could be a result of xenograph hypoxia, a common feature of solid tumors. Our hypothesis is supported by the work reported by Garayoa et al. (55). Recently, these authors demonstrated that the expression of AM mRNA in a variety of human tumor cell lines, including DU145 cells, is highly induced by reduced oxygen tension (1% O2) or exposure to hypoxia mimetics such as desferrioxamine mesylate (DFX) or Co(OAc)2 through a hypoxia-inducible factor-1 dependent mechanism (55). This group of investigators, have shown the intranuclear accumulation of fluorescent immunoreactivity after the induction of cell hypoxia; in the present work, we have found great numbers of horseradish peroxidase-labeled cell nuclei in CaP. The correlations and significance of this finding deserve to be further investigated.

Prior studies have demonstrated the ability of reduced oxygen tension to mediate elevations in AM message/protein expression in several animal and cell systems. Nakayama and colleagues (56) have demonstrated that hypoxia can elevate AM mRNA and protein expression in a single human colorectal carcinoma cell line, DLD-1, and a similar relationship has been reported for rat ventricular cardiac myocytes mediated through a hypoxia-inducible factor-1-dependent mechanism (57). The production and secretion of AM at the hypoxic areas present in tumors (58) could establish an autocrine/paracrine-mediated proliferation that lead to tumor growth. In addition, because AM has angiogenic and vasodilator capabilities (25, 29), the secreted AM could induce neovascularization and facilitate nutritional supplementation to the tumor cells.

Thus, the localized production of AM suggests that it could stimulate some growing processes within tumor. Bonkhoff et al. (15) reported that NE cells in human do not contain detectable levels of androgen receptor. A number of markers indicate that NE phenotype is better represented in androgen-independent human CaP. These observations suggest that human NE cells do not require androgens for their growth and survival; their number increases in CaP in response to the selective pressure of the androgen-ablation therapy (18). Alternatively, if the number of NE cells does not change in response to androgen ablation, tumors with clear-cut NE phenotype may exhibit androgen-independent growth because NE cells are able to elaborate factors that influence the androgen responsiveness of the non-NE subpopulation of neoplastic cells. AM is a very good candidate for autocrine-paracrine interactions in prostate malignancies. In addition, AM may have an adaptive value for tumors by increasing the intratumoral blood flow through its well-known vasodilative function (25, 42).

In both normal and malignant prostate tissue, growth may be regulated by prostate NE cells by means of peptide secretion. Prostate NE cells express a variety of potentially mitogenic hormones, including parathyroid hormone-related protein (8), neurotensin (59), calcitonin (9), bombesin-like factor (7), and thyroid-stimulating hormone-like peptide (60). In the present article, we demonstrate the expression of AM. The ability to produce these factors should be responsible for the observed increased growth rate in carcinoma cells juxtaposed to the foci of NE cells in some tumors (61). If NE cells are able to contribute to the proliferative capacity of the surrounding tumor cells, then tumors containing them would gain a strong selective advantage by getting androgen independence (45, 46). The deployment of NE phenotype can occur gradually in response to other phenotypic potentialities of the cell as well as to environmental influences. The shift from androgen-dependent to androgen-independent modes of regulation may be gradual, but the rate of the process can be accelerated by
peptides originated from a different population of cells capable of inducing tumoral growth through autocrine-paracrine mechanisms, independently of androgens. Knowledge of NE cells and their products could have important implications in the treatment of hormone-resistant CaP.

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REFERENCES


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Expression of Adrenomedullin and Peptide Amidation Activity in Human Prostate Cancer and in Human Prostate Cancer Cell Lines

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