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Adrenomedullin and Proadrenomedullin N-Terminal 20 Peptide in the Normal Prostate and in Prostate Carcinoma

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ABSTRACT There is increasing evidence for the important role played by regulatory peptides in the physiology of the normal and neoplastic prostate. Adrenomedullin (AM) and pro-adrenomedullin N-terminal 20 peptide (PAMP) are recently discovered regulatory peptides widely expressed in the normal prostate and in prostate carcinoma. AM is produced in secretory, stroma, and endothelial cells and in neurons of the prostate ganglia. PAMP is only produced by neuroendocrine cells. The expression of AM mRNA is regulated by androgens in the rat prostate. The number of neuroendocrine cells expressing PAMP is increased in prostate carcinoma after androgen deprivation, which shows that this peptide could regulate androgen-independent prostate tumor growth. However, the roles of AM and PAMP in the normal prostate and in prostate carcinoma are yet to be elucidated. *Microsc. Res. Tech. 57:98–104, 2002.*

INTRODUCTION

Human prostate is composed of tubuloalveolar glands surrounded by fibromuscular stroma (Stamey et al., 1988). The glands include terminal acini originating from the branching of ducts connected to the prostatic urethra. The epithelium of the glands is composed of three types of cells, called secretory or luminal, basal, and neuroendocrine (NE) cells (reviewed by Luke and Coffey, 1994). Luminal cells are the most abundant type and are responsible for the secretory function of the prostate. Basal cells are considered to contain the stem cell population, which is able to proliferate for regenerating the epithelial compartment (Bonkhoff et al., 1994). NE cells are found scattered among secretory and basal cells and secrete a variety of peptides that control the biology of other prostatic cells (reviewed by Abrahamsson, 1999). The prostate is an androgen-dependent organ which suffers a strong involution after castration. Secretory, basal, and stroma cells express androgen receptor (Iwamura et al., 1994), whereas NE cells do not (Abrahamsson, 1999).

Rodents, such as rats and mice, have frequently been used as models to study the biology and pathology of the human prostate. Rat and mouse prostate is composed of three distinctive types of lobes called ventral, dorsal, and lateral lobes. Ventral lobes are the biggest in size and contain the most developed system of ductal branches. The glands are organized in ducts emerging from the urethra and branching to terminal acini (Jesik et al., 1982). For many years the glandular epithelium was considered to be composed of basal and secretory cells, but not NE cells (Angelsen et al., 1997). However, NE cells have recently been described in the prostate of rodents (Jiménez et al., 1999; Masumori et al., 2001). The prostate of rodents shares some similarities with the human prostate, although no spontaneous prostate tumors arise in mice or rats (Luke and Coffey, 1994). However, prostate tumors can be induced by chemical carcinogens or transgenic expression of oncogenes (Bosland, 1992; Greenberg et al., 1995).

Prostate carcinoma (PCa) is currently the second-leading cause of cancer death in men (Parker et al., 1996). PCa is androgen-dependent in early stages and, therefore, it can be controlled by androgen ablation. However, it frequently becomes androgen-insensitive after androgen withdrawal therapy (Gittes, 1991). This new status is accompanied by a more aggressive behavior of the tumors and poor prognosis for the patients because of the lack of effective treatments. It has been suggested that growth factors secreted by both the secretory and NE cells might play an important role in tumor growth, acting in an autocrine or paracrine manner (Abrahamsson, 1999). In particular, peptides secreted by NE cells could be critical in tumor growth after androgen deprivation, since NE cells are androgen-independent (Abrahamsson, 1999). For all these reasons, there is increasing interest in finding new molecules that play a role in the normal and tumoral physiology of the prostate which can be used as targets for therapy. In this review we present the current information on adrenomedullin (AM) and pro-adrenomedullin N-terminal 20 peptide (PAMP), two peptides originating from the same gene but localized in separate compartments in the prostate. The current data suggest that both peptides could play a key role in normal and neoplastic prostate.

AM and PAMP are recently discovered regulatory peptides generated from a 185-amino acid precursor through enzymatic cleavage (Kitamura et al., 1993;

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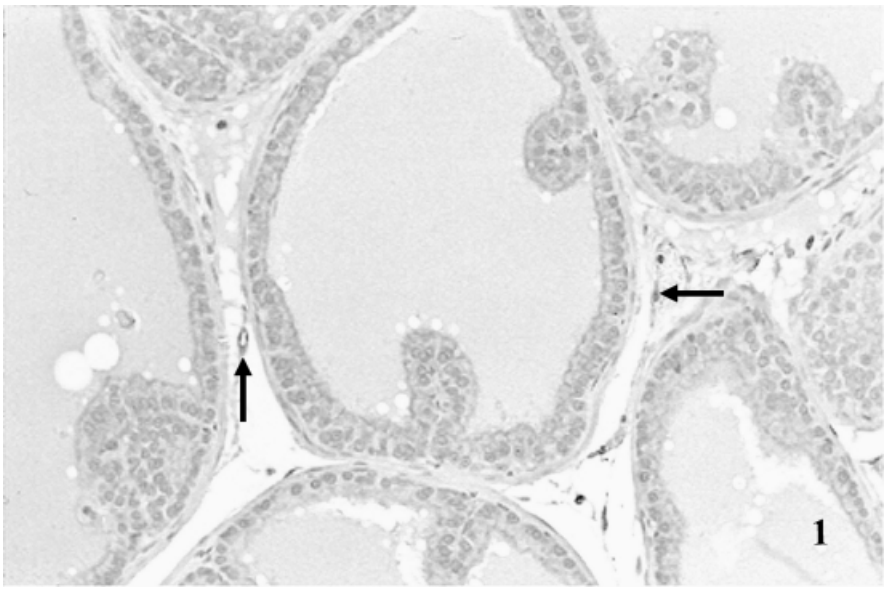


Fig. 1. Immunohistochemical staining for AM in acini of the lateral prostate. Epithelial cells and endothelial cells (arrows) are labeled. ×300. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Hinson et al., 2000, review). These peptides have been detected in a large variety of organs and cell types and exert different roles, depending on the system where they are localized. AM acts as a vasodilator, autocrine growth factor or growth inhibitor, neurotransmitter, and bronchodilator (Hinson et al., 2000). AM also controls hormone secretion and renal homeostasis (Hinson et al., 2000). PAMP is a vasodilator (Kitamura et al., 1994) and inhibits neurotransmission (Shimosawa et al., 1995) and neuroblastoma cell growth (Ando et al., 1997).

Both AM and PAMP are amidated peptides (Hinson et al., 2000). Amidation represents an important step in maturation of many peptides and growth factors in order to become completely bioactive (Eipper et al., 1992). The only amidating enzyme described so far is peptidylglycine alpha-amidating monooxygenase (PAM), which includes two enzymes acting sequentially, peptidylglycine alpha-hydroxylating monooxygenase (PHM), and peptidyl-alpha-hydroxyglycine alpha-amidating lyase (PAL) (Eipper et al., 1992).

AM and PAMP act on the target cells through different receptors. Several AM-receptors have been described, some of them also acting as CGRP-receptors (Hinson et al., 2000). Since AM and PAMP are pluripotent peptides, the presence of a particular type of receptor in a specific cell type will probably determine the function of AM and/or PAMP.

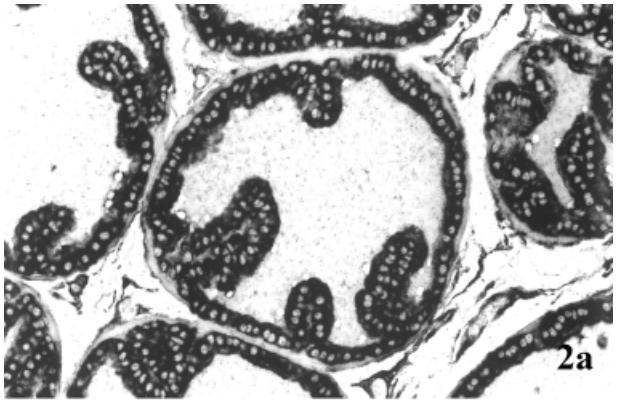
AM AND PAMP IN THE RAT PROSTATE

AM is Expressed in a Large Variety of Cells in the Rat Prostate

AM is abundantly expressed in the rat prostate. In this organ, the expression of AM mRNA is at least 50-fold higher than in the adrenal gland and cardiac atria (Pewitt et al., 1999), tissues where AM expression was reported to be high (Sakata et al., 1994). Both the peptide and the mRNA have been mainly found in the secretory cells of the glandular epithelium (Jiménez et al., 1999; Pewitt et al., 1999). The expression of AM differs, depending on the prostatic lobe and the portion of the gland. The lateral lobes contain the highest amount of peptide and mRNA (Figs. 1, 2) and the ducts in all the lobes contain higher levels than the acini (as demonstrated by immunohistochemistry and in situ hybridization). In the acini of the lateral lobes, AM peptide is localized in the apical cytoplasm and the perinuclear area of the secretory cells (Fig. 1). A similar pattern is observed in the dorsal lobes, whereas in the ventral prostate the staining is restricted to some scattered cells with apical nucleus. In all the lobes, the immunostaining found in the ducts is homogeneously distributed in the cytoplasm of the epithelial cells. A small population of very intensely AM-labeled cells is also found scattered throughout the ducts, in all the prostate lobes, and in the urethra. These cells resemble NE cells (Jiménez et al., 1999).

Western blot analysis also shows lobe-specific differences. A 14-kDa band is found in extracts from the dorsolateral prostate (mostly including tissue from the acini). This band corresponds to the AM precursor and it has been previously described in other organs (Miller et al., 1996; Jahnke et al., 1997). In extracts from the ventral lobes (including tissue from the acini and the ducts), bands of 14-kDa and 6-kDa (fully processed AM) can be detected. Since the 6-kDa band corresponds to the active form of AM, it has been suggested that the ventral lobe could produce more active AM than the dorsolateral lobes, or that the turnover in the dorsolateral prostate could be faster, thus causing a rapid elimination of the 6-kDa product (Jiménez et al., 1999).

Besides the epithelial cells of the glands, both in situ hybridization and immunohistochemistry show that AM is expressed in other prostate-associated tissues (Jiménez et al., 1999). AM is found in the epithelial cells of the ampullary glands, urethra, ureters, and ejaculatory ducts and in the endothelial cells of blood vessels (Fig. 1). Moreover, some stromal cells (mainly



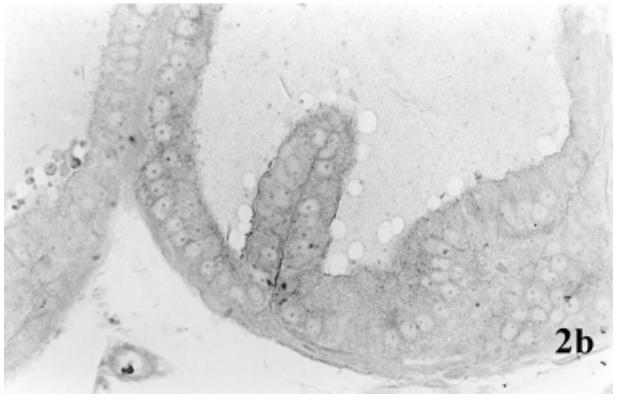


Fig. 2. In situ hybridization of preproAM mRNA in acini of the lateral prostate. **a**: A strong signal in the epithelium is detected with the antisense probe. ×250. **b**: The negative control with the sense probe gives no staining, thus showing the specificity of the labeling. ×500.

muscle cells) and some neurons of the prostate-associated ganglia express AM as well. The widespread distribution of AM in the rat prostate and its localization in such different cellular types points out that this peptide could be involved in a large variety of functions. AM receptor has also been found by RT-PCR in the prostate, although its precise localization is still unknown. The presence of AM and AM-receptor in rat prostate suggests an autocrine-paracrine way of action.

It has been demonstrated that AM causes relaxation of the smooth muscle in the rat prostate, as do also CGRP and amylin (Ventura et al., 2000), two peptides that share homology with AM. Since contraction of the muscular layer surrounding prostate glands facilitates fluid secretion, AM could regulate epithelial fluid secretion by relaxation of subjacent muscle cells. The cellular localization of AM also support this role. In ducts, where there is high expression of AM, the smooth muscle layer surrounding the glandular epithelium is abundant, whereas in acini, where AM is expressed at lower levels, the muscular layer is thinner (Jiménez et al., 1999).

AM plays a major role in the cardiovascular system. A strong hypotensive effect via NO generation has been described as a consequence of AM action (Hirata et al., 1995). This effect is comparable to that of CGRP (Kitamura et al., 1993). In this regard, the expression of AM

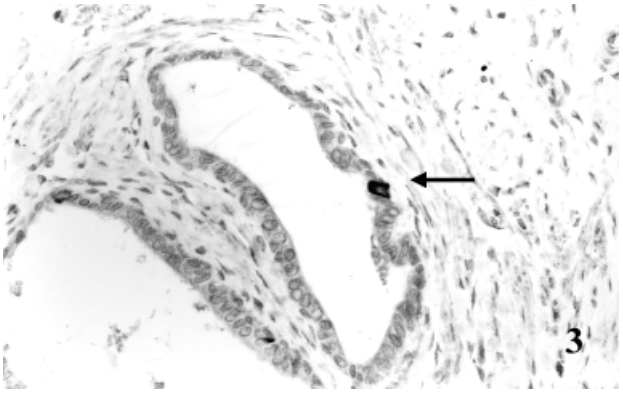


Fig. 3. Immunohistochemical staining for PAMP in a duct of the lateral prostate. The labeling is found in a neuroendocrine cell (arrow). ×500.

in prostate endothelial cells could be related to regulation of prostatic vascular tone.

Expression of PAMP is Restricted to Neuroendocrine-Like Cells

Unlike the human prostate, the glands of the rat prostate contain a very small population of NE cells (Jiménez et al., 1999). The amount and location of rat prostatic NE cells make localization difficult. NE cells are mainly located in the urethra and glandular ducts proximal to the urethra, although scarce numbers can be observed in acini. Another different feature with respect to humans is that in the rat prostate the number of NE cells positive for chromogranin-A (CgA) is lower than the number of NE cells positive for serotonin (Jiménez et al., 1999). Therefore, CgA cannot be considered a pan-marker for NE cells in the rat prostate. Cells expressing PAMP (Fig. 3) constitute a subpopulation of NE cells expressing serotonin in the rat prostate, since all of the PAMP-positive cells colocalize with serotonin-positive cells. However, PAMP-immunoreactive cells do not colocalize with CgA-NE cells and, for that reason, we named them NE-like cells (Jiménez et al., 1999). Four subpopulations of NE cells (or NE-like cells) can be distinguished in the rat prostate, depending on their immunoreactivity for: 1) serotonin, 2) serotonin and PAMP, 3) serotonin and CgA, and 4) AM (Jiménez et al., 1999).

The lack of colocalization of AM and PAMP in the prostate shows that, in spite of the fact that both peptides originate from the same gene and have a common mRNA, different posttranscriptional events will determine which one of these peptides will be expressed by a specific cell type. Other studies have also shown separate expression of AM and PAMP in the same organ (Lopez et al., 1999, in kidney; Montuenga et al., 2000, in the pituitary). The molecular mechanisms responsible for this differential expression have to be elucidated in future studies.

AM Is Regulated by Androgens in the Rat Prostate

A PCR-based cDNA subtraction method isolated AM as an androgen response gene in the rat ventral prostate (Wang et al., 1997). The screening was performed

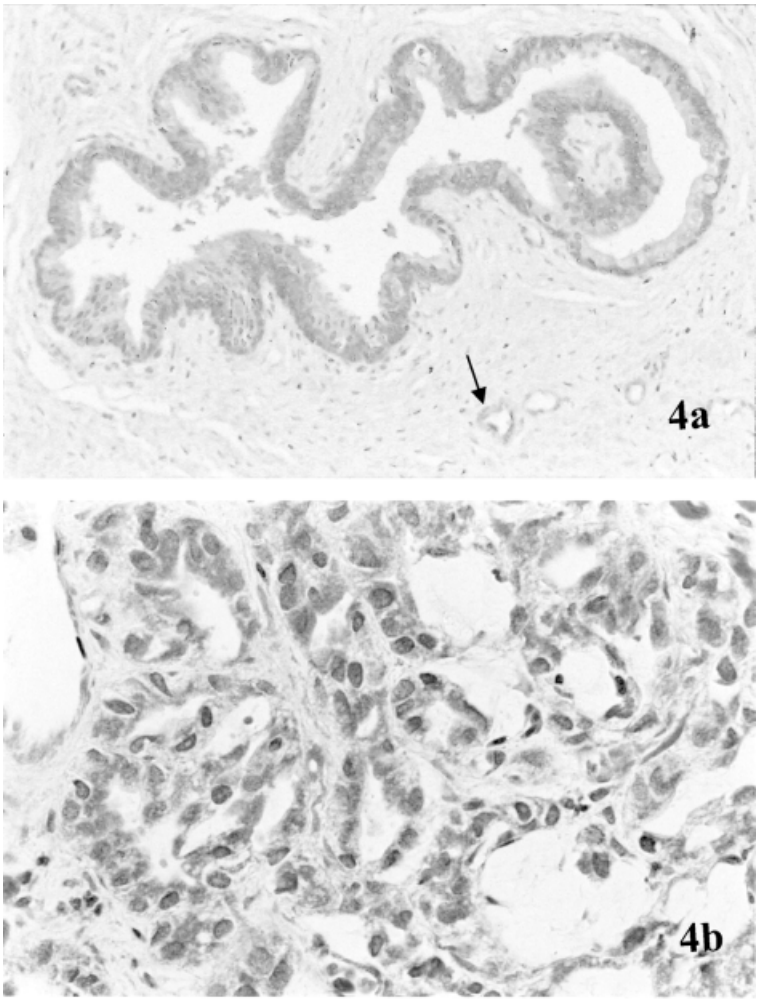


Fig. 4. Immunoreactivity for AM in human prostate. **a**: In normal prostate, the basal compartment of the epithelium is strongly stained. AM is also found in blood vessels (arrow). ×300. **b**: In prostate carcinoma, most tumor cells are labeled with anti-AM antibody. ×600. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

using mRNA from ventral prostates of 7-day castrated young male rats and 7-day castrated rats after 14 and 48 hours of androgen replacement. AM was identified among the upregulated genes after 14 hours of androgen replacement following castration and was therefore classified as an early androgen response gene (Wang et al., 1997).

Further studies by Northern blot analysis demonstrated a 25-fold decrease in AM mRNA levels in the prostate of castrated rats with respect to controls, the decrease occurring mostly within 1 day (Pewitt et al., 1999). Androgen replacement elevated AM expression within only 14 hours. The upregulation of AM after androgen replacement was maintained as long as androgens were present, which confirms that the regulation does not occur transiently. Interestingly, 5-alpha-dihydrotestosterone (DHT) was able to super-induce AM expression in primary prostate organ cultures even in the presence of cycloheximide, a protein synthesis inhibitor, suggesting that androgens directly stimulate AM expression (Pewitt et al., 1999). In the same study, the presence of cycloheximide alone partially increased the AM signal, probably by AM mRNA stabilization, as suggested by other authors (Minamino et al., 1995).

Previous studies in vascular endothelial and smooth muscle cells showed that androgens did not affect AM expression (Imai et al., 1995; Minamino et al., 1995). Indeed, studies by Pewitt et al. (1999) showed no AM androgen regulation in organs other than the prostate (including androgen-dependent organs such as the seminal vesicles). Moreover, a genetic analysis of the 5′ upstream region of the AM gene performed by Minamino et al. (1995) failed to find any androgen response element (ARE). Taken together, these results suggest the existence of a prostate-specific transcriptional mechanism that activates AM gene under androgen control. However, further studies should be done in order to clarify how this process occurs.

AM AND PAMP IN HUMAN PROSTATE: IMPLICATIONS OF THEIR EXPRESSION IN PROSTATE CARCINOMA

AM in Normal and Neoplastic Prostate

Similar to the rat prostate, AM is widely expressed in the normal human prostate (Jiménez et al., 1999). In situ hybridization and immunocytochemical studies show AM expression in the glandular epithelium of the ducts and acini (mainly in the basal compartment) (Fig. 4) and in the epithelium of the urethra, ejaculatory ducts, and squamous glands. Moreover, some stromal cells, endothelial cells, and nerves are also stained

for AM. Another similarity with the rat prostate is that PAMP is only localized in NE cells (Jiménez et al., 1999).

AM is expressed in PCa as well (Fig. 4). Rocchi et al. (2001) recently described the presence of AM in PCa and suggested that AM expression is increased in correlation with tumor progression, although few cases of PCa were studied. In our hands, immunohistochemical analysis did not show any correlation between AM expression and tumor progression (Jiménez et al., in prep.).

Expression of AM mRNA has also been reported in androgen-independent PCa cell lines PC-3 and DU-145, but not in the androgen-dependent cell LNCaP (Miller et al., 1996; Rocchi et al., 2001). However, we have found preproAM mRNA in LNCaP (Jiménez et al., in prep.). The effect of AM on PCa cell proliferation was analyzed by Rocchi et al. (2001). The addition of 2×10^{-7} M AM in the culture media had no effect on LNCaP or PC-3 and produced a slight increase in DU-145 growth after 8 days in culture. We have stably transfected PC-3 cells with an expression vector carrying the AM gene. Clones with different levels of AM expression were analyzed to compare in vitro and in vivo proliferation. Our results show that the ectopic expression of AM inhibits proliferation of PC-3 cells, both in vitro (60–80% inhibition) and in vivo (50–70% inhibition, 5 weeks after injection in nude mice). Moreover, a G0/G1 cell cycle arrest and a reduction of anchorage-independent growth in soft agar were observed in the PC-3-AM transfectants (Abasolo et al., in prep.). New studies in this direction are needed to further clarify the role of AM in growth control, in prostate cancer cell lines and prostate tumors.

PAMP in Normal Human Prostate and in Prostate Carcinoma

PAMP is only expressed in NE cells in normal and neoplastic human prostate (Fig. 5) (Jiménez et al., 1999; 2001). In the normal prostate, NE cells expressing PAMP are more abundant in the utriculus and urethra than in the glands. Moreover, the portion of the glands located near the urethra contains more PAMP-positive cells than the periphery. Unlike the rat prostate, all the PAMP-stained cells colocalize with CgA- and serotonin-NE cells in the human prostate. However, not all the cells labeled for serotonin or CgA were positive for PAMP, which shows that PAMP-NE cells represent a subgroup of the NE population.

We have extensively examined the distribution of PAMP staining in a variety of prostate tumors and PCa xenograft models and quantified the number of NE cells positive for PAMP in these samples (Jiménez et al., 2001). Clinical material included neoplastic tissues obtained through radical prostatectomy (RPs) and transurethral resection of the prostate (TURPs) with different Gleason sum scores (GSS). Some of the patients undergoing TURP had been pretreated with androgen-blockage therapy. PAMP was immunolocalized in 80% of the prostate tumors from the clinical samples. The NE phenotype of PAMP-positive cells was confirmed by colocalization with CgA. As previously described for CgA (Noordzij et al., 1995), no significant correlation was found between the number of PAMP-positive cells and tumor grade, progression, or prognosis. However, we found that TURPs from patients pretreated with antiandrogens showed a significantly higher number of PAMP-positive cells than TURPs from untreated patients. This result shows that the subpopulation of NE cells expressing PAMP increases after androgen deprivation in PCa and points out that this peptide might regulate androgen-independent prostate tumor growth.

Previous studies on some human prostate tumors xenografted into nude mice (PC-295 and PC-310 models) showed an extensive NE differentiation after castration (Jongsma et al., 1999, 2000). Both models (especially PC-310) exhibited a significant increase in PAMP-positive cells after castration, which is in accordance with our data on clinical TURPs (Jiménez et al., 2001). Unlike in PC-295, PAMP-positive NE cells differentiated sharply in PC-310, in parallel with the CgA population. Interestingly, PC-310 xenograft survives much longer than PC-295 after androgen removal. We hypothesized that the differences in survival time could be due to the different PAMP expression program (Jiménez et al., 2001).

Coexpression of AM and PAMP With Amidating Enzymes in the Human Normal Prostate and in PCa

As mentioned above, AM and PAMP are amidated peptides. Amidation, which is required for full activity of many peptides and hormones, is performed by PAM. The presence of PAM was initially described in the prostate by Samos and Gkonos (1996) in rats. Rocchi et al. (2001) recently reported the expression of PAM mRNA in human prostate. They found a correlation in the expression of PAM mRNA and AM mRNA in benign hyperplastic prostates, prostate tumors, and PCa cell lines. This result suggests that PAM enzyme could modulate AM function through amidation (and, therefore, maturation) in human prostate. Studies in other types of tumors have shown that increase in amidation is frequently associated with increase in tumor growth (Iwai et al., 1999).

Using a battery of antibodies against the PHM region of PAM enzyme in human prostate, we have immunolocalized PHM in NE cells of human normal and neoplastic prostate (Jiménez et al., 2001). All the NE cells positive for the amidating enzyme were also stained for CgA and many of them for PAMP. However, there was not a complete colocalization between PAMP- and PAM-positive cells. In fact, there were more cells stained for PAMP than for PAM. Several explanations can be given for these results. 1) The presence of the PAM enzyme in the cells could be transient. 2) Although our antibodies against PAMP recognize its amidated form, they could partially label the nonamidated peptide. 3) The antibodies used for PAM might not recognize all the enzyme isoforms. Despite lacking a perfect match at the protein level, PAM enzyme and PAMP seem to be expressed in a coordinated way in normal prostate and in PCa (Jiménez et al., 2001).

CONCLUSIONS AND FUTURE DIRECTIONS

Recent studies have shown that AM and PAMP are largely expressed in rat and human (normal and neoplastic) prostate, although in different cellular com-

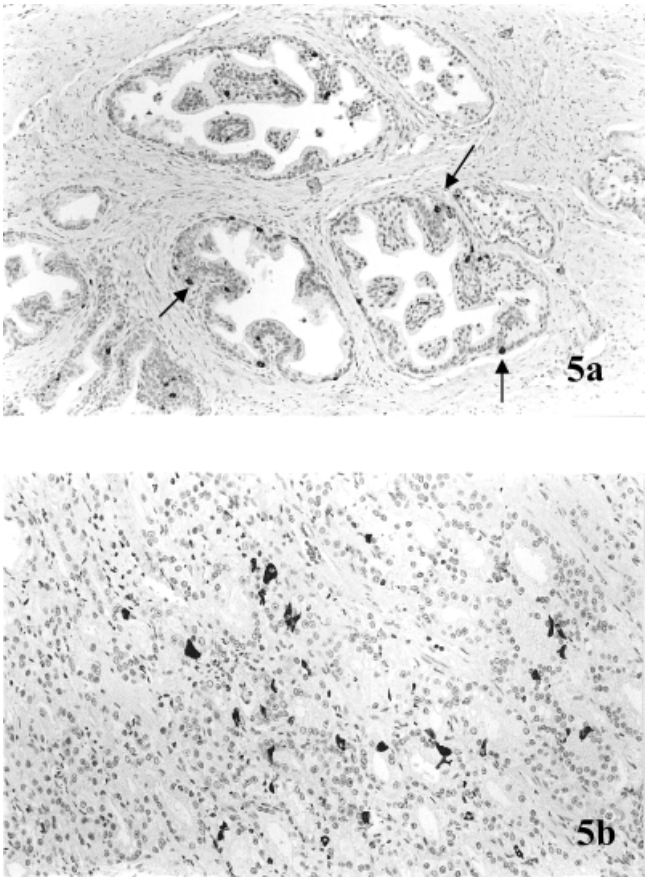


Fig. 5. PAMP immunostaining in human prostate. **a**: In normal prostate, PAMP is localized in neuroendocrine cells (arrows). ×150. **b**: In prostate carcinoma, PAMP is also exclusively found in neuroendocrine cells. ×150.

partments. These results suggest that both peptides might have a significant role in the biology of normal and tumoral prostate. However, many unresolved questions arise from these studies. In particular, some important issues should be addressed in the future: 1) the mechanisms responsible for the differential expression of AM and PAMP in the prostate; 2) the concrete physiological role of these peptides in both normal and neoplastic prostate; 3) the characterization of the receptors that mediate AM/PAMP functions; and 4) the intracellular pathways activated by AM/PAMP.

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