PACAP and Type I PACAP Receptors in Human Prostate Cancer Tissue

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ABSTRACT: We characterized the expression and localization of pituitary adenylate cyclase-activating polypeptide (PACAP) and its specific type I receptor variants in prostatic, hyperplastic, and carcinomatous tissue collected from patients undergoing prostate biopsy and surgery for benign prostatic hyperplasia (BPH) and prostate cancer (PCa). The immunohistochemical studies using an indirect immunoperoxidase technique evidenced positive immunostaining for PACAP in the cytoplasm of epithelial cells of hyperplastic and carcinomatous prostate specimens and in some scattered cells of the stroma. Type I PACAP receptors (PAC1 R) in healthy and BPH tissues were localized in all epithelial cells lining the lumen of the acini and in some stromal cells, while in specimens from PCa the anti-PAC1 R antibody stained the apical portion of a large percentage of cells. Furthermore, our molecular studies provide evidence that several PAC1 R isoforms (*null*, SV1/SV2) are present in normal, hyperplastic, and neoplastic tissue, the *null* variant being the most intensely expressed in PCa. These observations provide additional evidence for a role of PACAP and PAC1 R in the events determining the outcome of PCa.

KEYWORDS: PACAP; PAC1 R; PACAP receptor variants; prostate; prostate cancer; BPH; SV1; SV2; SV3

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a hypothalamic neuropeptide that belongs to the secretin/glucagon/vasoactive intestinal

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polypeptide (VIP)/growth hormone-releasing hormone (GHRH) family of peptide hormones.[1] Two biologically active forms of PACAP, PACAP-38 and the C-terminally truncated PACAP-27, have been characterized, which derive from a 176 amino acid precursor protein by posttranslational cleavage.[2] PACAP exerts a potent stimulatory action on cyclic AMP production in anterior pituitary cells[1] and promotes the release of several pituitary hormones. It also stimulates phosphatidylinositol hydrolysis and increases cytosolic Ca^{2+} in several cell types.[3–6] PACAP is detectable and biologically active in many tissues, including pituitary, brain, adrenal, testis, and nerve fibers of both the gut and the lung,[7,8] where it is considered to act as a neurohormone, neurotransmitter, neuromodulator, and vasoregulator. Recently, the expression and distribution of PACAP in human prostate cancer (PCa) and healthy prostate tissue have been demonstrated by biochemical and morphological procedures.[9] Both PACAP-38 and PACAP-27 act through three classes of membrane G protein–coupled receptors: the specific PAC1 receptor (PAC1 R) and the common PACAP/VIP (VPAC1 and VPAC2) receptors.[10] An alternative splicing of two exons of the PAC1 R gene allows for four major splice variants: the PAC1 R *null*, the PAC1 SV1, the PAC1 SV2, and the PAC1 SV3.[11] PAC1 R and its isoforms are highly expressed in many areas of the central nervous system (CNS)[12,13] and moderately in the neurohypophysis[14] and the pituitary gland,[15] the male reproductive tract,[16] and the adrenal medulla.[17] The presence of functional PAC1, VPAC1, and VPAC2 receptors has been demonstrated in human benign prostatic hyperplasia (BPH),[18,19] and VPAC1 receptors have been detected by autoradiography in human normal prostate and prostate carcinoma.[20] Also, the expression of the mRNAs for the three receptor classes has been demonstrated in the androgen-dependent human LNCaP PCa cell line[21] where they display different abilities to activate adenylyl cyclase (AC) and phospholipase C.[22,23] We have recently demonstrated in LNCaP that the sustained versus transient intracellular cAMP increase induced by the binding of PACAP to its cognate PAC1 receptor is a crucial event determining the outcome of tumor progression.[24] In order to acquire more information about PACAP and PAC1 R variants in normal, hyperplastic, and neoplastic human prostate, we have performed expression studies and immunohystochemical experiments on tissues obtained from patients affected with different degrees of BPH and PCa.

MATERIALS AND METHODS

Patients and Tissue Procurement

We enrolled in our study 10 normal patients undergoing surgical treatment for pelvic and urogenital primary diseases, 10 patients affected with BPH, and 22 patients 50–70 years of age undergoing radical prostatectomy for previously untreated carcinoma of the prostate. After prostatectomy, a wedge-shaped

specimen of the fresh prostate was cut. Samples were submitted for pathological examination to confirm the prostate origin, the diagnosis, and the absence of other diseases. Hematoxylin-eosin staining was used for the histopathological evaluation, diagnosis, and tumor grading. The TNM score referred to the pathological T stage was used to classify the tumors according to TNM score.[25] The carcinoma tissues were classified according to the Gleason grades and the epithelial/stroma proportion was evaluated. Tissue collected intrasurgically or from biopsies was immediately frozen on dry ice and subsequently kept at –80°C until processing. All patients had given their written informed consent before participating in this study.

Reagents and Antibodies

Maloney-murine leukemia virus (M-MLV) reverse transcriptase was purchased from Life Technologies (San Giuliano Milanese, Italy). Taq polymerase was purchased from Promega Corp. (Milano, Italy). Affinity-purified polyclonal rabbit anti-human PACAP serum was obtained from Peninsula Laboratories, Inc. (San Carlos, CA). Affinity-purified polyclonal rabbit anti-human PAC1 receptor primary antibody was procured by Prof. A. Arimura (Tulane University Hebert Center, New Orleans, LA).

Immunohistochemical Detection of PACAP and PAC1 R

The localization of hPACAP and PAC1 R was performed on 5-μm-thick sections of the fixed healthy prostate, BPH, and PCa tissues, and carried out by the streptavidin-biotin immunoperoxidase method, using a commercial kit (Zymed Laboratories Inc., San Francisco, CA). Sections were incubated overnight at 4°C with the following antisera: 1:50 dilution of the affinity-purified polyclonal rabbit anti-human PACAP, and 1:100 dilution for the affinity-purified polyclonal rabbit anti-human PAC1 R primary antibodies. For controls, the primary antiserum was omitted in some sections. Slides were developed using amino-ethylcarbazole as chromogenic substrate, which peroxidase converts into a red to brownish-red precipitate at the sites of antigen localization in the tissue. The preparations were counterstained with hematoxylin, dehydrated, mounted, and analyzed.

RNA Preparation and RT-PCR

The purity and integrity of the RNA, extracted by the single-step acid guanidinium thiocyanate-phenol-chloroform method,[26] were checked spectroscopically and by gel electrophoresis before carrying out the analytical

procedures. First-strand cDNA synthesis was performed as follows: 1 μg total RNA was reverse transcribed by 200 U of M-MLV reverse transcriptase using 2.5 μM random hexamers in the presence of 250 μM deoxynucleotides triphosphate in a final volume of 20 μL. DNA contamination or PCR carry over controls were performed by omitting M-MLV during RT. The reaction mixture was heat denatured for 5 min at 75°C, and then incubated for 1 h at 42°C. Five microliters of the cDNA obtained was used to amplify hPAC1 or hPACAP. hPAC1 was PCR and nested-amplified as follows: the first round of PCR was carried out using primers designed to amplify a PAC1 cDNA sequence spanning the region 872–1626 [upstream Cat1: 5′-TGTATGCGGAGCAGGACAGC-3′; downstream Cat2: 5′-AGGCCAGACATGCGGATTTGGG-3′, amplified product 754 bp]. The PCR product was nested-amplified using a set of primers flanking the receptor-splicing site [upstream Don1: 5′-TTAACTTTGTGCTTTTTATTGG-3′; downstream Don2: 5′-GAGTCTTTCCCTTTTGCTGAC-3′]; multiple products were amplified relating to the splice variants expressed. cDNAs were amplified using Taq polymerase (2 U per tube) with 15 pmole of both upstream and downstream primers, 1.5 mM magnesium chloride in a final volume of 50 μL. Then, 35 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s, with 5 min final extension for the first round of PCR, and 94°C for 30 s, 48°C for 30 s, 72°C for 30 s, with 5 min final extension for the nested round of PCR) were applied. PACAP PCR was performed using primer designed for the amplification of the fragment 502–1078 spanning exon 5 of the human PACAP gene [upstream hP1: 5′-AAACAAAGGACGACGCCGATAG-3′; downstream hP2: 5′-AGACTCACTGGGAAAGAATGC-3′; amplified product 576 bp].[27] cDNAs were amplified using Taq polymerase (2 U per tube) with 15 pmole of both upstream and downstream primers, 1.5 mM magnesium chloride in a final volume of 50 μL. Then, 30 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s with 10-min final extension) were applied. Finally, a 15-μL aliquot of all the amplified products was analyzed on 2% (wt/vol) agarose gel (NuSieve 3:1, FMC, Rockland, ME) and stained with ethidium bromide. Quantitation of the signals was performed by densitometric analysis using densitometry computer software (Kodak Digital Science 1D Image Analysis software, Eastman Kodak Co., Rochester, NY).

RESULTS

Localization in Normal and Pathological Prostate Tissues

The localization of PACAP and PAC1 R proteins investigated in sections obtained from normal, BPH, and neoplastic prostate glands by an indirect immunoperoxidase technique with anti-PACAP and anti-PAC1 R antiserum demonstrated a specific positive immunostaining for PACAP in the cytoplasm

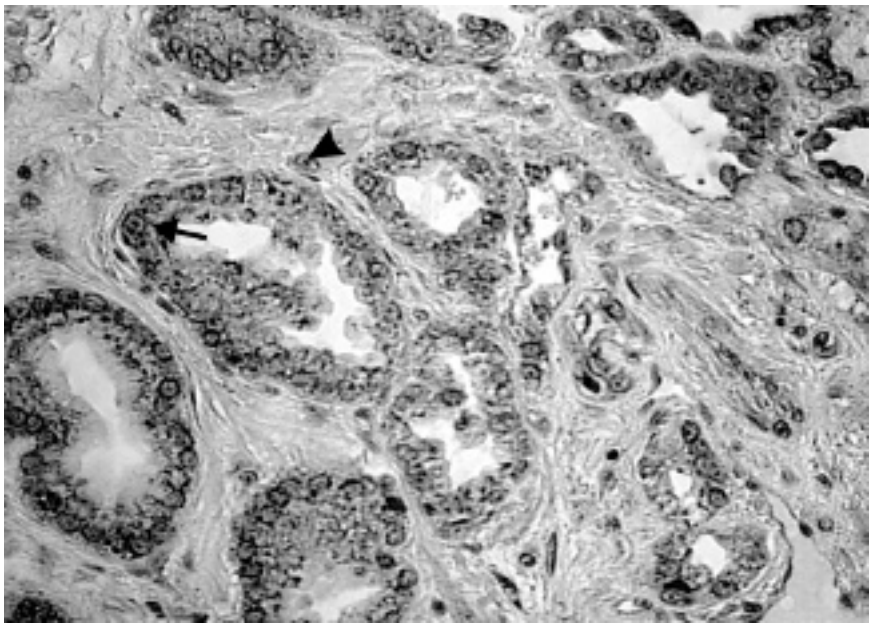

FIGURE 1. Section of human neoplastic prostate tissue showing immunohistochemical staining of PACAP in the PCa epithelium (arrow) and the stromal cells (arrow head).

of the epithelial cells lining the lumen of the acini and in some cells scattered in the stroma (FIG. 1), without significant differences in the distribution pattern between normal, hyperplastic, and tumoral tissues. PAC1 R was found in all epithelial cells and in some stromal cells in healthy and BPH tissues with the immunostaining mostly localized in the apical portion of the cells, while in the specimens from PCa, the anti-PAC1 R antibody stained the apical portion of a large percentage of cells ($75 \pm 10\%$ positive cells).

Expression Studies

The expression of PACAP mRNA in normal, hyperplastic, and neoplastic human prostate examined in order to determine if these tissues synthesize PACAP and PAC1 R variants, demonstrated in all tissues the expression of PACAP and PAC1 R mRNAs. The nested RT-PCR of cDNA, in addition to the semiquantitative amplification from these tissues, identified the isoform without SV1/SV2 cassettes (PAC1 R *null*) as the predominant product (FIG. 2). The expected products corresponding to PAC1 R containing SV1or SV2 cassettes were all present in the normal and hyperplastic tissues while in the PCa, the PAC1 R *null* isoform was the only one clearly expressed. The PAC1 SV3

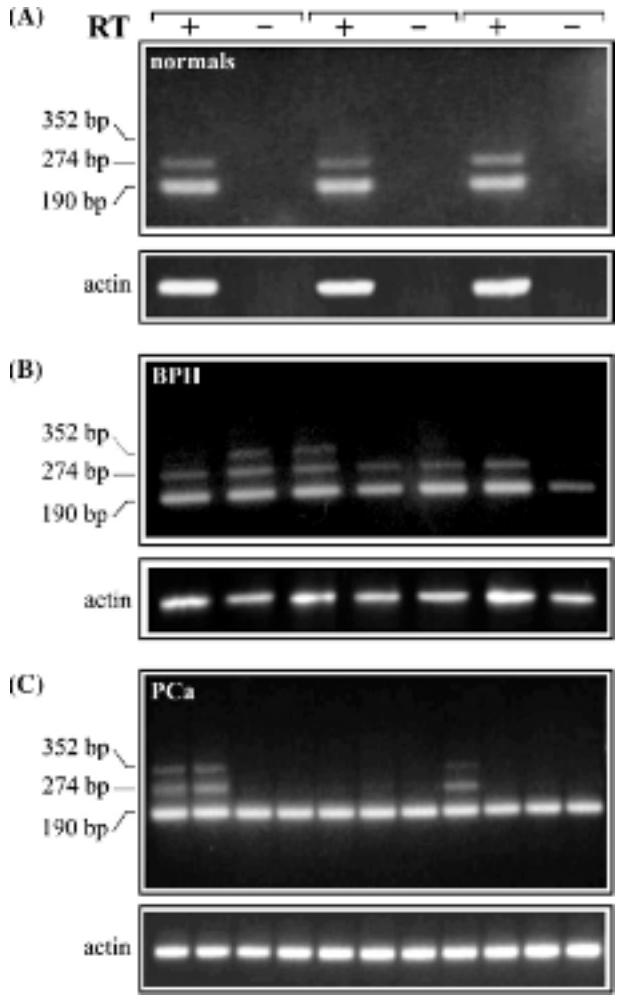

FIGURE 2. Analysis of type I PACAP R splice variants in normal (**A**), hyperplastic (BPH) (**B**), and neoplastic (PCa) (**C**). Reverse transcriptase (RT) was omitted in the control experiments (-RT). cDNAs were synthesized by RT-PCR using total RNA from tissues with cat1 and cat2 oligonucleotide as forward and reverse primers, respectively, followed by a second round of PCR products with don1 and don2 nested primers. PCR products were electrophoresed and stained with ethidium bromide. The product of expected size (190 bp) is related to the *null* variant. The product of expected size (274 bp) is related to the SV1/SV2 variant. The product of the expected size (352 bp) is related to the SV_3 variant. The β-actin transcript was analyzed as control.

isoform, a splice variant of the PAC1 receptor containing both the SV1 and SV2 boxes, was occasionally measurable with our method independently from the pathological state of the prostate tissue examined.

DISCUSSION

As in other human neoplasms, in the prostate gland, the process of forming a malignant tumor depends on a series of intermediate changes existing between the normal prostate epithelium and the newly formed prostate tumor. These changes involve a modification of the physiological reciprocal cellular interactions between stroma and epithelium that have been shown to accelerate local tumor growth[28,29] and distant metastases,[30] increase the genetic prostate cell instability,[31] and its subsequent androgen independent progression. The intricate intercellular communication between stromal and epithelial cells alters the physiology of the prostate epithelium in the malignant transformation of which two distinct circuits are involved: one operating in the nucleus and one in the cytoplasm, both of which are required for cell transformation.[32] Sex steroids are very important in the control of such mechanisms in that they alter within the prostate the main cell growth and survival as well as the action of several growth factors. The central characteristic of fatal PCa is androgen independence, a condition in which a perturbation of the androgen receptor signaling enables the androgen receptor to be activated by nonandrogenic steroid hormones and growth factors.[33] The androgen independence is markedly related to neuroendocrine (NE) cell activity and NE cells may represent an independent indicator of poor prognosis in patients with prostate carcinoma.[34] NE cells are dispersed throughout the prostate tumor and their number increases after long-term antiandrogen therapy.[35] They exhibit a fusiform morphology with neuritic processes and their ultrastructural characterization shows the presence of neurosecretory granules. Although they appear to be nonmitotic, proliferating carcinoma cells have been found in close proximity to them,[36] suggesting that NE cells provide paracrine stimuli for proliferation of the surrounding carcinoma cells. Among the factors that regulate NE differentiation from epithelially derived prostatic tumor cells, we investigated the localization and action of the PACAP and of the different variants of its specific PAC1 receptors. Our immunohistochemical studies show that, in the normal prostate, PACAP and PAC1 R are mainly present in the epithelial cells lining the lumen of the acini, while in the neoplastic tissue the anti-PAC1 R antibody stained the apical portion of a large percentage of cells, suggesting a different localization of the protein among tissues without variations in staining intensity. Furthermore, our expression studies show that the PAC1 R *null* isoform, which is related to the AC activation, is the most important receptor present in the neoplastic prostate. Considering the importance of the cAMP milieu in the mechanism of NE differentiation, the potential role of PACAP, as a natural cAMP inducer, may be reevaluated in the prostate neoplastic progression. We have recently demonstrated *in vitro* that PACAP induces the development of a NE morphology and NE differentiation in LNCaP cells[24]: LNCaP cells treated with different concentrations of PACAP-27 added at the beginning of the culture, showed a rapid but transient development of neuritic processes both in the

presence and in the absence of 5% FBS; the acquisition of an NE morphology was evident after 15 min of treatment but had almost completely reverted after 24 h. This effect was partially prevented by pretreatment with PACAP6–27, indicating that PAC1 R is involved in this phenomenon. Chronic PACAP-27 treatment or co-treatment with PACAP-27 and IBMX, conditions that maintain a sustained cAMP accumulation, exerted the same effect as Fsk, and the NE morphology of LNCaP cells was still observable after 3 days. These data suggest that the inhibitory effect of the peptide on cell growth correlates with the maintenance of NE differentiation. Besides PACAP, several peptides produced by epithelial and prostate NE cells have been shown to possess the ability to elevate cAMP and to induce NE cell differentiation, capable of sensitizing the response of LNCaP PCa cells to growth factors.[24] A relevant consideration in the physiology of PCa is that cells with NE phenotype increase in number as cancer progresses to the androgen refractory condition. NE cells are particularly concentrated in the vicinity of proliferating cancer cells, on which they act in a paracrine fashion by secreting mitogenic factors.[30,36,37]It has been shown that the PACAP receptor antagonist PACAP6–27 suppresses the growth of a human PCa cell line, suggesting that this peptide could be an important prostate local regulator of cell growth and differentiation.[38] Thus, we propose that PACAP, through its specific receptors, may modulate the tissue availability of cAMP in the prostate. The evidence that the PAC1 R *null* variant, which is the predominant isoform expressed in human neoplastic tissue, is able to induce a cAMP rise may explain why in the neoplastic microenvironment it might be a determinant factor with the ability to change the fate of the prostate tumor. A large and sustained PAC1 R *null*–induced production of cAMP might be involved in the loss of androgen sensitivity. Further studies need to be performed in this direction.

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