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## PEPTIDYLGLYCINE $\alpha$ -AMIDATING MONOOXYGENASE- AND PROADRENOMEDULLIN-DERIVED PEPTIDE-ASSOCIATED NEUROENDOCRINE DIFFERENTIATION ARE INDUCED BY ANDROGEN DEPRIVATION IN THE NEOPLASTIC PROSTATE

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**Most PCs show NE differentiation. Several studies have tried to correlate NE expression with disease status, but the reported findings have been contradictory. Prostatic NE cells synthesize peptides with a wide spectrum of potential functions. Some of these active peptides, such as PAMP, are amidated. PAM is the only carboxy-terminal peptide-amidating enzyme identified. We studied expression of PAMP and PAM in normal prostate and prostatic tumors (clinical specimens and human xenograft models) with or without prior androgen-deprivation therapy and found a wide distribution of both molecules in NE subpopulations of all kinds. Although the correlation of either marker to tumor grade, clinical progression or disease prognosis did not reach statistical significance, PAMP- or PAM-immunoreactive cells were induced after androgen-blockade therapy. In the PC-310 and PC-295 androgen-dependent models, PAMP or PAM NE differentiation was induced after castration in different ways, being higher in PC-310, which might explain its long-term survival after androgen deprivation. We show induction of expression of 2 new NE markers in clinical specimens and xenografted PC after endocrine therapy.**

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**Key words:** adrenomedullin; androgen blockade; neuroendocrine; prostate cancer; proadrenomedullin N-terminal 20 peptide; peptidylglycine  $\alpha$ -amidating monooxygenase; peptidylglycine  $\alpha$ -hydroxylating monooxygenase

PC is the most commonly diagnosed cancer in elderly men<sup>1</sup> and is becoming the leading malignant cause of death for men in this age group. The normal prostatic epithelium has a well-developed NE cell population.<sup>2</sup> In PC, the reported incidence of NE cells has increased as detection methods have improved.<sup>3,4</sup> The function of the NE population in the normal and malignant prostate remains unknown. Together with generic NE markers, such as CgA or CgB,<sup>5</sup> prostatic NE cells produce biogenic amines and neuropeptides.<sup>6</sup> The effects of some of these on prostatic cells have been demonstrated *in vitro* in some instances. Serotonin and bombesin/gastrin-releasing peptide act as growth-stimulating factors,<sup>7</sup> while somatostatin is a growth inhibitor.<sup>8</sup> Thus, NE cells could play a role in the regulation of growth, differentiation and local homeostasis of the normal and neoplastic prostate *in vivo*.

PAMP and AM originate from the posttranslational processing of pro-AM.<sup>9,10</sup> PAMP, but not AM, was specifically expressed by some NE cells of the human normal prostate.<sup>11</sup> NE expression in other tissues has also been associated with PAMP production.<sup>12</sup> However, this peptide was first described in the vascular wall and in endocrine organs in normal and pathologic situations.<sup>13</sup> PAMP is a pluripotent peptide initially identified as a vasodilator,<sup>9</sup> but it can additionally act as a neural transmission inhibitor,<sup>14</sup> an endocrine secretion inhibitor<sup>15</sup> or a cell growth suppressor.<sup>16</sup> Some of these roles could contribute to the regulation of prostatic homeostasis or functioning.

Some peptides produced by prostatic NE cells (PAMP, gastrin-releasing peptide and calcitonin) are carboxy-terminally amidated *in vivo*.<sup>13,17</sup> C-terminal  $\alpha$ -amidation is a posttranslational modifi-

cation necessary for many peptides to be completely bioactive.<sup>17</sup> It is sequentially performed by PHM and peptidyl- $\alpha$ -hydroxyglycine  $\alpha$ -amidating lyase,<sup>18</sup> which are included in the PAM precursor. PAM has been detected in endocrine and other tissues in both normal and neoplastic states.<sup>19,20</sup> PAM mRNA has been detected in rat prostate and human prostatic xenografts.<sup>21,22</sup> Although PAM is expressed by many NE cells, this enzyme cannot be considered a general NE marker as it is not present in every cell from the diffuse NE system.<sup>23</sup>

PC is androgen-dependent in early stages but usually relapses to an androgen-insensitive state after androgen-withdrawal therapy,<sup>24</sup> which implies more aggressive tumors and a poor prognosis for the patient. Since prostatic NE cells lack androgen receptors, they have been suggested to be androgen-independent<sup>25</sup> and, therefore, assumed to be involved in the development of refractory tumors. However, several studies on the relationship of NE differentiation (usually represented by universal NE markers) to prognosis in PC have reported contradictory findings.<sup>26</sup> In the present study, we describe PAMP and PAM expression in NE cells of human prostatic specimens obtained from a broad spectrum of histopathologic subgroups. Our aim was to use these 2 new NE markers to gain insight into the relevance of single NE subpopulations producing specific products (with potential effects on tumor biology) on the evolution of prostatic tumors either untreated or pretreated with androgen-deprivation therapy.

**Abbreviations:** AM, adrenomedullin; APAAP, alkaline phosphatase-anti-alkaline phosphatase; CgA, chromogranin A; GSS, Gleason sum score; NE, neuroendocrine; PAM, peptidylglycine  $\alpha$ -amidating monooxygenase; PAMP, proadrenomedullin N-terminal 20 peptide; PC, prostatic carcinoma; PHM, peptidylglycine  $\alpha$ -hydroxylating monooxygenase; P-TURP, pretreated TURP; RP, radical prostatectomy; TURP, transurethral resection of the prostate; U-TURP, untreated TURP.

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## MATERIAL AND METHODS

*Patients*

Two control prostates were obtained from autopsies of healthy young individuals killed in traffic accidents. Neoplastic tissues were obtained by RP over 10 years at the University Hospital Rotterdam (The Netherlands) or through TURPs mainly performed at the University Hospital Rotterdam and the Reinier de Graaff Hospital (Delft, The Netherlands). RPs were performed on 61 patients with nonmetastasized, clinically localized or locally advanced PC. Patients were followed regularly for a mean period of 82 months (range 13–178). Clinical progression, defined as cytologically or histologically proven local recurrence or the appearance of distant metastasis, occurred in 27 patients. Tumor-specific death, defined as death due to direct tumor effects, metastasis or tumor therapy, occurred in 11/27 patients showing disease progression. TURPs were obtained from 29 patients with extensive tumors. Fourteen of them were pretreated with androgen-blockade therapy, *i.e.*, by antiandrogens, flutamide, androcur or orchiectomy (P-TURPs). The rest ( $n = 15$ ) were untreated before undergoing surgery (U-TURPs). All tissue-procurement protocols were approved by the relevant institutional committees. Prostates were fixed in 10% buffered formalin, dehydrated in alcohol and embedded in paraffin for *in situ* detection. Slides stained with hematoxylin and eosin were used for determining tumor grade according to GSS.

*Human prostate tumor xenograft models*

PC-295 and PC-310 models were established, respectively, from a pelvic lymph node metastasis and from a primary PC at the Department of Experimental Urology, Erasmus University (Rotterdam, The Netherlands).<sup>27</sup> These androgen-dependent models exhibit significantly increased NE differentiation after androgen ablation.<sup>21,22</sup> Small pieces of tumor were heterotransplanted in NMRI athymic nude mice by *s.c.* implantation. Mice (Harlan, Indianapolis, IN) were androgen-supplemented through silicone (Silastic) implants containing testosterone (Sigma, St. Louis, MO). Tumors were grown to a maximal volume of 2,000 mm<sup>3</sup>. For castration experiments, implants were removed and male mice castrated under Hypnorm anesthesia (Janssen, Oxford, UK). Mice were killed at short-term intervals after androgen ablation: days 0, 0.5, 1, 1.5, 2, 4, 7, 14 and 21 for PC-295 and days 0, 2, 5, 7, 14 and 21 for PC-310. A long-term castration experiment was also performed for PC-310, mice being killed 0, 21, 47, 84, 90 and 154 days after androgen withdrawal. Prostatic tumors were then either fixed in 4% buffered formalin and paraffin-embedded for immunohistochemical detection or snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for Western blotting.

We included other models,<sup>27,28</sup> both androgen-dependent and -independent, also established at the Department of Experimental Urology: PC-82, PC-133, PC-135, PC-324, PC-329, PC-339, PC-346, PC-346I and PC-374. All tumors were grown in mice as described above. Mice were killed at 0 and 14 days after androgen withdrawal (though these models did not exhibit NE differentiation after androgen withdrawal previously<sup>29</sup>). Tissues were processed for immunohistochemical analysis.

*Antibodies and peptides*

For PAMP detection, a previously characterized<sup>30</sup> polyclonal antiserum (raised to the C-terminal PAMP<sub>YY13-20AMIDE</sub> peptide) was applied at 1:3,000 on histologic sections and at 1:1,500 for Western blotting. This antibody does not cross-react with other carboxy-terminally amidated peptides, including AM.<sup>30</sup> To detect PHM and PAM, 2 monoclonal antisera (clones G8 and E10, produced by Dr. A.M. Treston, NCI, NIH, Bethesda, MD) were used. They were raised against bovine PHM (bPAM<sub>288-310</sub>; Genbank accession M18683), which shows high homology with the human PAM<sub>288-310</sub> sequence. Both antibodies were applied at 1:2,000 on sections of clinical material and at 1:1,000 on sections of xenograft-derived tissues. A monoclonal antihuman CgA antiserum (Boehringer-Mannheim, Mannheim, Germany) was applied

at 2  $\mu\text{g}/\text{ml}$  to confirm whether PAMP- and PAM-expressing cells had the NE phenotype. Peptides PAMP<sub>YY13-20AMIDE</sub> and bPAM<sub>288-310</sub> were used for the absorption controls.

*Immunohistochemistry*

Sections (4  $\mu\text{m}$  thick) were cut from paraffin-embedded tissues and placed on slides. Some reverse-face sections were mounted to assess colocalization of molecules. To detect PAMP, PAM and CgA single immunostaining, a previously described protocol was used.<sup>21</sup> For absorption controls, solutions containing the specific antiserum preincubated with 10 nmol/ml of its respective peptide overnight at  $4^{\circ}\text{C}$  were applied to slides instead of primary antiserum.

For double immunodetection, sections were deparaffinized and hydrated and endogenous peroxidase was inhibited. Next, they were microwave-preheated as previously described.<sup>21</sup> Slides were then incubated for 20 min with normal goat serum (1:20) at room temperature and at  $4^{\circ}\text{C}$  overnight with the specific antiserum mixture (containing 1 monoclonal and 1 polyclonal antiserum). Subsequently, slides were treated with horseradish peroxidase-conjugated goat antirabbit immunoglobulins and with unlabeled goat antimouse immunoglobulins (Dako, Copenhagen, Denmark), diluted 1:50 and 1:100, respectively, for 30 min. Tissues were incubated with monoclonal APAAP (Dako) at 1:50 for 30 min. Goat antimouse antiserum was applied again, followed by APAAP, for 10 min each. Alkaline phosphatase was developed using naphthol AS-TR and new fuchsin as substrate to give a red end product. Peroxidase activity was detected with 3,3'-diaminobenzidine hydrochloride and nickel enhancement, rendering a black precipitate. Slides were embedded in PBS-glycerol.

*Quantification of immunostaining and statistical analysis of data*

For RPs and TURPs, NE cells immunolabeled for PAMP or PAM were evaluated semiquantitatively since this allowed us to consider the distribution pattern (regular or irregular) of positive cells throughout the tissue.<sup>31</sup> Tissue sections adjacent to slides immunostained for PAMP or PAM were stained with hematoxylin and eosin and the neoplastic zones present in every tissue identified and marked. These marks were copied on the corresponding immunolabeled slides. For scoring, we used a microscope equipped with an ocular with an integrated grid of 9 mm along each side. The grid area defined a field. Ten fields of tumor tissue were randomly selected at  $\times 160$ , and cells showing granular cytoplasmic immunostaining were counted. The presence of cell clusters was recorded. The total area scored was 0.03 mm<sup>2</sup>. Quantitative data were transformed to semiquantitative values, which consider not only the observed number but also the distribution of positive cells. The final scoring criteria were as follows: 0, no positive cells; 1, positive cells in 4 or fewer fields; 2, positive cells in at least 5 fields; 3, more than 6 positive cells in at least 5 fields or clusters of 10 or more positive cells in at least 1 field. Therefore, every tumor was assigned 1 semiquantitative score (0–3) for PAMP and another for PAM.

In the xenograft models, quantitative evaluation of PAMP expression was performed to determine how immunoreactive cells evolved after castration. Ten randomly selected fields of neoplastic epithelium were scored at  $\times 400$  by counting the positive cells. The arithmetic mean calculated from the 10 data points was assigned as final value for each tumor. The mean area analyzed was 500  $\mu\text{m}^2$ .

SPSS (Chicago, IL) software was used for statistical analysis. The PAMP or PAM score of each patient was correlated to the respective GSS. The PAMP score was correlated to the PAM score. The means calculated for the xenograft models were correlated with postcastration time. To carry out correlation analysis, the nonparametric Spearman coefficient ( $r$ ) was determined. Scores from patients who underwent RP were also related to clinical progression and survival time (Kaplan-Meier method). The frequency of high PAMP or PAM NE expression (score 3) in U-TURPs and P-TURPs was compared by Fisher's exact test.

### Protein extraction and Western blotting

PC-310 prostatic tissues stored at  $-80^{\circ}\text{C}$  were crushed in a metal cylinder cooled to  $-80^{\circ}\text{C}$ . Tissue homogenates were transferred into a lysis buffer<sup>21</sup> containing a cocktail of protease inhibitors (Complete, Boehringer-Mannheim). After centrifugation of the mixture at  $40,000g$  at  $4^{\circ}\text{C}$  for 10 min, supernatants were recovered and the protein content was measured spectrophotometrically by the Bradford method (protein assay from Bio-Rad, Munich, Germany).

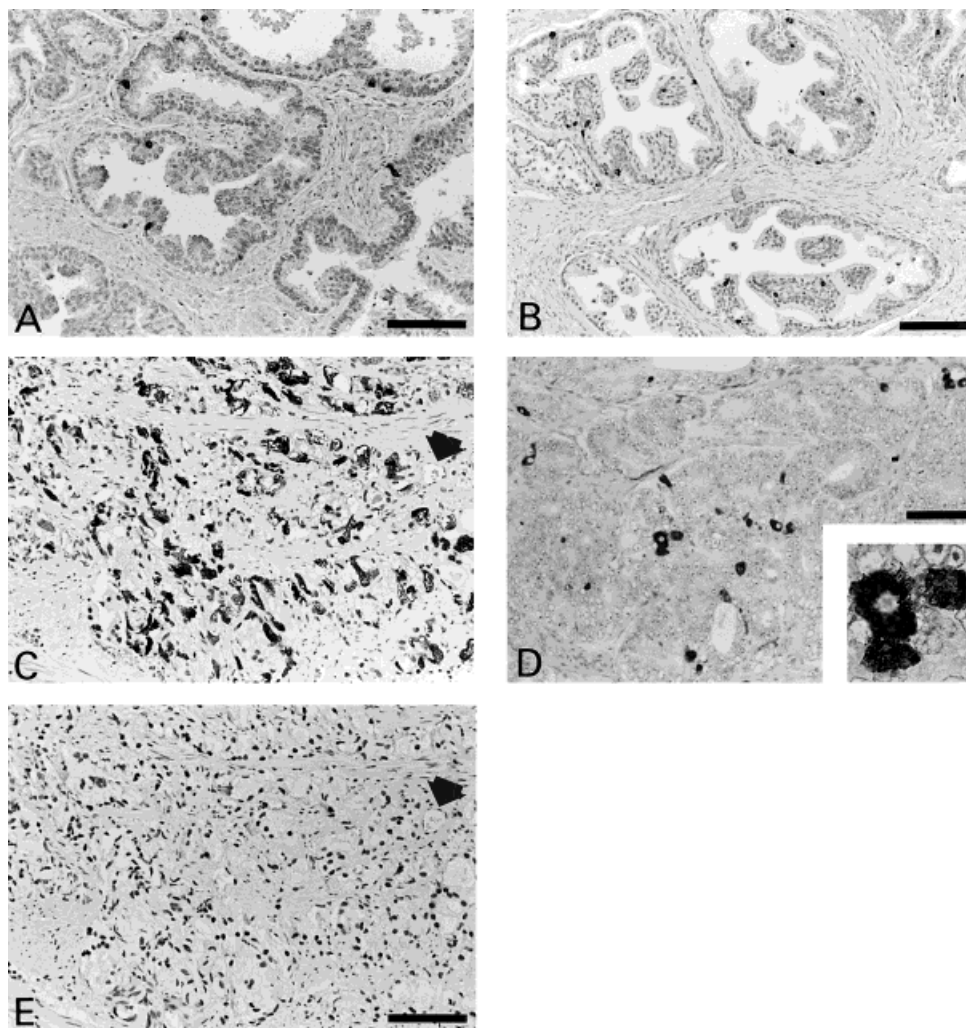
For Western blotting detection of PAMP, each sample ( $35\ \mu\text{g}$ ) was transferred to 10–20% tricine SDS-PAGE gels (Novex, San Diego, CA). Synthetic PAMP (5 ng) was loaded in separate wells as a positive control. Unstained Mark12 Wide Range Protein Standard (Novex) was loaded to determine relative m.w. Proteins were electrophoretically separated under reducing conditions. Gels were blotted to PVDF membranes (Immobilon; Millipore, Bedford, MA). The immunoblot was blocked overnight at  $4^{\circ}\text{C}$  with PBS (pH 7.7) containing 5% dry milk. Anti-PAMP antiserum diluted in PBS/milk was added subsequently, and the membranes were incubated for 1 hr on an orbital shaker at room temperature. After rinsing membranes with PBS containing 0.1% Tween-20 (Fluka, Buchs, Switzerland), incubation with horseradish peroxidase-conjugated antirabbit secondary antibody (Amersham, Amersbury, UK) diluted 1:5,000 in PBS/milk was performed for 1 hr

at room temperature. Membranes were rinsed with PBS/Tween, and Western blotting luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA) was added for 1 min. Blots were then exposed to high-performance chemiluminescence film (Hyperfilm ECL, Amersham). Finally, blots were stained with Serva Blue R (Serva Electrophoresis, Heidelberg, Germany) following the manufacturer's instructions, to visualize protein standards.

## RESULTS

### Immunodetection and quantification of PAMP and PAM in clinical specimens

PAMP-like and PAM-like immunostaining, granular and cytoplasmic, was detected in normal and neoplastic prostates (Fig. 1*a–d*). PAMP- or PAM-immunoreactive cells were mainly isolated in the epithelium of ducts and acini in the normal glands (Fig. 1*a,b*) and other prostate-associated structures, such as the utriculus and the urethra. In the neoplastic glands, positive cells were distributed as either isolated or clustered cells (Fig. 1*c,d*). Double immunostaining and serial reverse-face staining demonstrated that both PAMP<sup>+</sup> and PAM<sup>+</sup> cells showed the NE phenotype as they were subpopulations of CgA<sup>+</sup> cells (data not shown). Absorption controls confirmed the specificity of staining (Fig. 1*e*).



**FIGURE 1** – NE PAM-like (*a,c*) and PAMP-like (*b,d*) expression and PAM absorption control (*e*). (*a,b*) Normal prostate. (*c,e*) P-TURP, GSS 10, serial sections. (*d*) RP, GSS 8. Inset, PAMP cytoplasmatic and granular expression. (*c,e*) Arrows point to the same blood vessel. Nuclei appear stained by the counterstaining (Mayer's hematoxylin). Scale bar = 100  $\mu\text{m}$ ; inset  $\times 500$ .

NE PAMP-like expression was detected in 80% of RPs, 80% of U-TURPs and 79% of P-TURPs. PAMP scores in relation to GSS are shown in Tables I and II. There was no correlation between PAMP score and GSS for the RPs ( $r = -0.012, p > 0.05$ ) or the U-TURPs ( $r = -0.078, p > 0.05$ ). However, P-TURPs showed a positive correlation between scores ( $r = 0.489$ ), though it did not reach statistical significance ( $p = 0.076$ ). To score PAM-expressing NE cells, the anti-PHM antiserum clone G8 (which is more sensitive than antibody clone E10) was used. NE-associated PAM expression was present in 46% of RPs, 27% of U-TURPs and 36% of P-TURPs (Tables I, II). Spearman's coefficient indicated no correlation between GSS and PAM score for the RPs ( $r = 0.070, p > 0.05$ ). The correlation coefficient was negative for U-TURPs ( $r = -0.302, p > 0.05$ ) and positive for P-TURPs ( $r = 0.347, p > 0.05$ ). There was a noticeable increase in the percentage of patients expressing high PAMP (score 3) from untreated (1/15, 7%) to pretreated (5/14, 36%) TURPs ( $p = 0.08, n = 29$ ); 27% of P-TURPs and 7% of U-TURPs exhibited high PAM expression, but these percentages were not significantly different ( $p = 0.169$ ). Kaplan-Meier curves did not show a prognostic value of PAMP or PAM expression in relation to clinical progression or tumor-specific survival of the RPs (data not shown).

Colocalization of PAM and PAMP immunoreactivity revealed 3 types of NE cell: cells positive only for PAM, cells immunolabeled only for PAMP and cells immunostained for both PAMP and PAM. To assess whether the relationship between PAM and PAMP expression exists, the correlation between both scores was calculated. For the RPs, there was a positive and significant correlation ( $r = 0.494, p < 0.001$ ). For the TURPs, the correlation was smaller and not significant ( $r = 0.208$  for U-TURPs and 0.307 for P-TURPs).

*Immunodetection and quantification of PAMP and PAM in xenograft models*

PAMP-like immunoreactive cells showed a marked increase in the PC-310 model with postcastration time (Table III). At 7 days postcastration (T-7), positive cells increased mainly in the peripheral area of the tumor; in time, they became numerous in the tumor central zone after androgen deprivation (Fig. 2a). In the short-term castration experiment done with the PC-295 model, PAMP-associated NE differentiation was lower. The first PAMP cells were detected at T-7, and the maximum number of positive cells (mean  $0.9 \pm$  SEM) was observed at T-14. There was a positive and significant correlation between postcastration time and the number of PAMP-immunostained cells for PC-310 ( $r = 0.976, p < 0.001$ ) and PC-295 ( $r = 0.822, p = 0.007$ ). Both models showed induction of CgA-expressing cells after androgen deprivation. CgA<sup>+</sup> cells were found at low levels at T-0 but increased in tumor areas from the peripheral to the central zone after longer periods of androgen deprivation (Fig. 2b). PAMP<sup>+</sup> cells were a subpopula-

**TABLE II – RELATIONSHIP BETWEEN PAMP OR PAM SCORE AND GSS IN THE TURPS (N = 29)**

| Tumor score | Pretreated patients |   |   |    |       | Untreated patients |   |   |    |       |
|-------------|---------------------|---|---|----|-------|--------------------|---|---|----|-------|
|             | GSS                 |   |   |    | Total | GSS                |   |   |    | Total |
|             | 7                   | 8 | 9 | 10 |       | 7                  | 8 | 9 | 10 |       |
| 0           |                     |   |   |    |       |                    |   |   |    |       |
| PAMP        | 1                   | 2 | 0 | 0  | 3     | 0                  | 0 | 2 | 1  | 3     |
| PAM         | 2                   | 4 | 3 | 0  | 9     | 1                  | 1 | 3 | 6  | 11    |
| 1           |                     |   |   |    |       |                    |   |   |    |       |
| PAMP        | 1                   | 2 | 3 | 0  | 6     | 1                  | 1 | 4 | 5  | 11    |
| PAM         | 0                   | 0 | 1 | 0  | 1     | 0                  | 0 | 3 | 0  | 3     |
| 2           |                     |   |   |    |       |                    |   |   |    |       |
| PAMP        | 0                   | 0 | 0 | 0  | 0     | 0                  | 0 | 0 | 0  | 0     |
| PAM         | 0                   | 0 | 0 | 0  | 0     | 0                  | 0 | 0 | 0  | 0     |
| 3           |                     |   |   |    |       |                    |   |   |    |       |
| PAMP        | 0                   | 2 | 2 | 1  | 5     | 0                  | 0 | 1 | 0  | 1     |
| PAM         | 0                   | 2 | 1 | 1  | 4     | 0                  | 0 | 1 | 0  | 1     |
| Total       |                     |   |   |    |       |                    |   |   |    |       |
| PAMP        | 2                   | 6 | 5 | 1  | 14    | 1                  | 1 | 7 | 6  | 15    |
| PAM         | 2                   | 6 | 5 | 1  | 14    | 1                  | 1 | 7 | 6  | 15    |

**TABLE III – TIME COURSE OF THE PRESENCE OF PAMP-LIKE POSITIVE CELLS IN ANDROGEN-DEPRIVATION EXPERIMENTS USING THE PC-310 PROSTATE XENOGRAFT MODEL**

| Postcastration time (days) | PAMP cells (mean value) | SEM |
|----------------------------|-------------------------|-----|
| 0                          | 0.0                     | 0.0 |
| 2                          | 0.2                     | 0.1 |
| 5                          | 0.6                     | 0.3 |
| 7                          | 5.8                     | 1.2 |
| 14                         | 12.5                    | 2.9 |
| 21                         | 10.3                    | 2.6 |
| 47                         | 24.4                    | 3.8 |
| 84                         | 19.7                    | 2.7 |
| 90                         | 35.0                    | 5.8 |
| 154                        | 42.7                    | 5.9 |

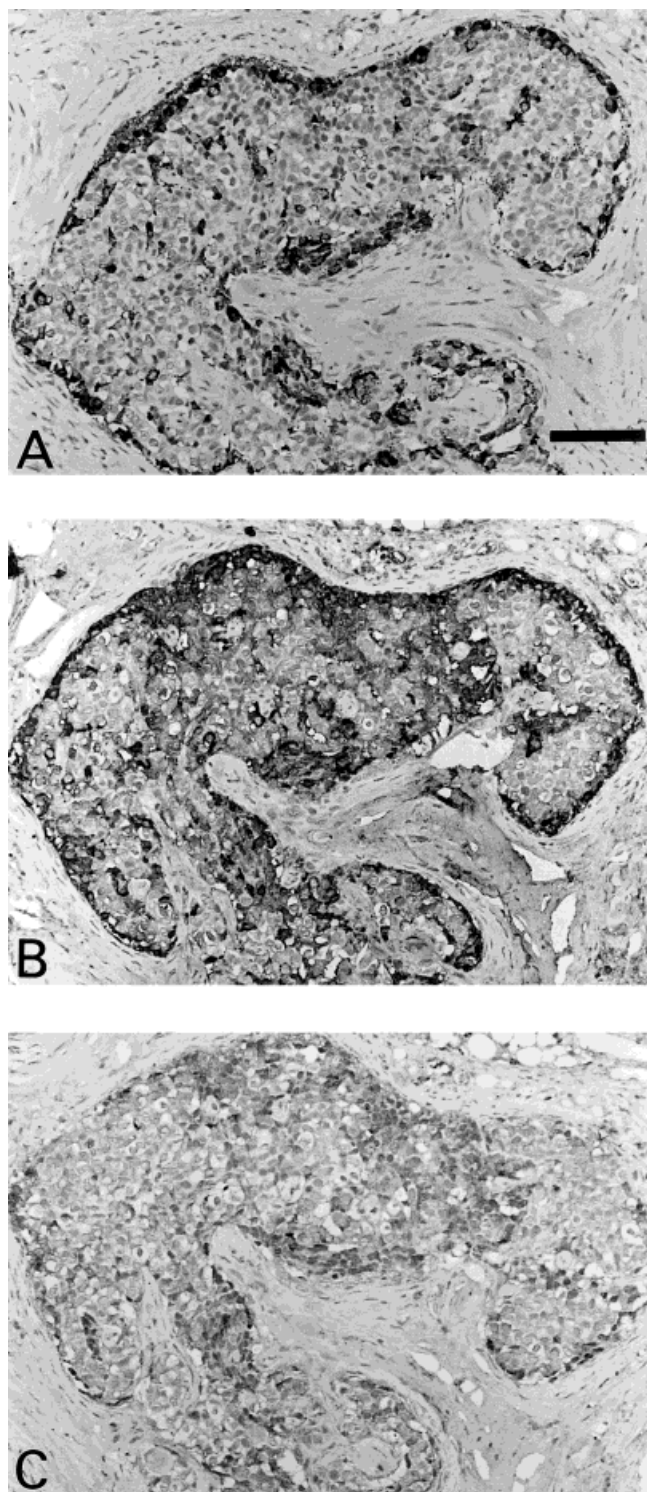
**TABLE I – RELATIONSHIP BETWEEN PAMP OR PAM SCORE AND GSS IN RP (N = 61)**

| Tumor score | GSS |   |   |    |   |    |    | Total |
|-------------|-----|---|---|----|---|----|----|-------|
|             | 4   | 5 | 6 | 7  | 8 | 9  | 10 |       |
| 0           |     |   |   |    |   |    |    |       |
| PAMP        | 0   | 1 | 1 | 4  | 3 | 3  | 0  | 12    |
| PAM         | 1   | 2 | 5 | 12 | 6 | 5  | 2  | 33    |
| 1           |     |   |   |    |   |    |    |       |
| PAMP        | 1   | 1 | 7 | 13 | 3 | 7  | 2  | 34    |
| PAM         | 1   | 1 | 3 | 9  | 2 | 6  | 0  | 22    |
| 2           |     |   |   |    |   |    |    |       |
| PAMP        | 1   | 0 | 0 | 3  | 0 | 0  | 0  | 4     |
| PAM         | 0   | 0 | 0 | 1  | 0 | 1  | 0  | 2     |
| 3           |     |   |   |    |   |    |    |       |
| PAMP        | 0   | 1 | 0 | 4  | 3 | 3  | 0  | 11    |
| PAM         | 0   | 0 | 0 | 2  | 1 | 1  | 0  | 4     |
| Total       |     |   |   |    |   |    |    |       |
| PAMP        | 2   | 3 | 8 | 24 | 9 | 13 | 2  | 61    |
| PAM         | 2   | 3 | 8 | 24 | 9 | 13 | 2  | 61    |

tion of prostatic CgA-expressing cells (Fig. 2a,b). In both models, CgA cells showed a trend to be the main tumor cell type at later stages in the postcastration period. This was also observed for the PAMP<sup>+</sup> subpopulation in PC-310 but not in PC-295.

Labeling for PAM, usually strong and granular, was localized in some epithelial cells later assessed as NE in the PC-310 and PC-295 models. Faint, diffuse PAM staining was also found in the non-NE neoplastic epithelium. NE-associated PAM expression colocalized not only with CgA- but also with PAMP-expressing NE cells (Fig. 2a-c). In the PC-310 model, PAMP<sup>+</sup> NE cells appeared relatively late postcastration (*i.e.*, T-21). The increase in PAM NE differentiation appeared later after androgen deprivation than PAMP differentiation as the positive cells became numerous from T-84 on. The PC-295 model showed a slight increase in PAM-expressing NE cells following androgen ablation. PAM NE cells were not counted because the parallel increased staining of non-NE tumor cells made the scoring unreliable.

Most of the other xenograft models studied did not exhibit PAMP immunolabeling. Some sporadic immunostained cells were observed at T-14 in the androgen-dependent PC-82 and PC-329 models and in the androgen-independent PC-374 model. Tumor epithelia in the androgen-dependent PC-82, PC-329 and PC-346 models showed weak epithelial PAM expression, which was stronger in the androgen-independent PC-133, PC-135, PC-324, PC-339 and PC-374 xenografts. The androgen-independent subline PC-346I, derived from the parental androgen-dependent PC-346 xenograft, exhibited areas with strong epithelial staining surrounded by areas with low PAM expression. In the parental line as well as the subline of this model, overall PAM expression increased after 14 days of androgen deprivation. In every case, PAM-stained cells were assessed as non-NE, *i.e.*, CgA<sup>-</sup>, tumor cells.



**FIGURE 2**—Immunoreactivity in a tumor epithelial node of the PC-310 model at T-90 for PAMP (a), CgA (b) and PAM (c). More stained PAM cells are situated in coincident zones with the cells immunoreactive for CgA and PAMP. Counterstaining by Mayer's hematoxylin. Scale bar = 100  $\mu$ m.

#### PAMP Western blotting

The PC-310 model was studied with proteins from short- and long-term experiments (Fig. 3). The 3 kDa band, corresponding to fully processed PAMP, was not detectable in any of the

extracts. However, some larger bands (14 to 22 kDa) were observed from T-21. The intensity of the signal increased with androgen deprivation time, especially for species corresponding to 15 and 14 kDa bands, which became stronger from T-21 to T-84, though they were weaker at T-154 (Fig. 3b). In the absorption controls, no signal was detected (data not shown). PHM/PAM quantification by Western blot analysis was not possible in PC-310 due to the strong background derived from the mouse immunoglobulins present in the extracts, which were recognized by our detection system.

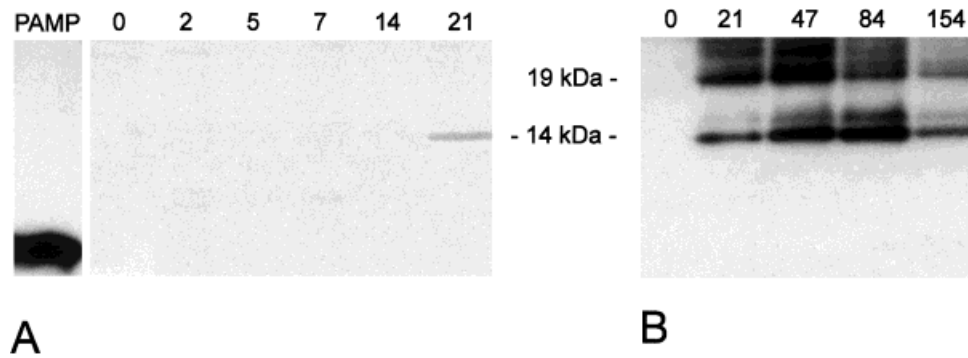
#### DISCUSSION

The generalized presence of NE cells in PC and the androgen-independent behavior of this cell type<sup>25</sup> have suggested that the prostatic NE population may be involved in the progression of cancer and in the development of refractory tumors after endocrine therapy. The contradictory conclusions on the relevance of NE cells in PC reported by several groups<sup>26</sup> encourage studies of the relation between the expression of newly discovered prostatic NE products and PC progression. We have studied the expression of 2 new NE markers with potential roles in tumor biology (PAMP and PAM) in normal prostate and prostatic tumors (both clinical and experimental) with and without prior androgen-deprivation treatment.

Immunohistochemical analysis showed PAMP- and PAM-immunoreactive cells distributed with patterns similar to other NE markers in the normal<sup>32</sup> and malignant<sup>6</sup> prostate. Both types of immunoreactive cell are subpopulations of NE CgA-expressing cells. Therefore, our findings in prostate and the data obtained from other systems<sup>23</sup> demonstrate that neither PAMP nor PAM is a general NE marker. The incidence of NE differentiation in PC is variable depending on the NE marker considered.<sup>3,33</sup> The existence of NE subpopulations producing specific substances is interesting since the secreted products could potentially have different effects on neighboring non-NE tumor cells. In the clinical material, we observed PAMP-like expression in 80% of tumors while PAM was present to a lesser extent. PAM- and PAMP-like signals colocalized in several NE cells, pointing to PAM, the only amidating enzyme identified, as the candidate for PAMP amidation. The absence of PAM in some PAMP-expressing cells might reflect the accumulation of PAMP in cells requiring late coexpression of PAM.

In accordance with observations made for other markers,<sup>31</sup> we did not find a correlation between PAM or PAMP expression and GSS for either group of RPs or the TURPs. PAMP- or PAM-associated NE differentiation does not appear to be directly associated with tumor grade and, furthermore, does not have clinical value for estimating prognosis. However, we found high PAMP and PAM scores more frequently in palliative TURPs (PAMP 36%, PAM 27%) than in untreated TURPs (7% for both markers), PAMP incidences being almost significantly different. This points out that at least PAMP-immunoreactive NE cell differentiation and/or selection is induced in hormone-refractory tumors after antiandrogen therapy.

The xenograft models studied were heterogeneous with respect to the presence and evolution of NE subpopulations after castration. This can be related to the variability found in the clinical specimens as well as each of the models originated from a different patient. During androgen-deprivation experiments, the PC-295 and PC-310 models exhibited tumor regression<sup>21,22</sup> but PC-310 tumors survived as dormant residues for at least 5 months. PC-295 tumor epithelium regressed completely in a short period after androgen ablation. In both models, CgA<sup>+</sup> cells become the main epithelial cell type after prolonged androgen withdrawal. In the present study, PAM- or PAMP-associated NE differentiation was induced in 2 different ways: in PC-310, PAMP cells increased sharply in parallel to the increase of CgA<sup>+</sup> cells and high PAM NE differentiation was induced (relatively late); in PC-295, the number of PAMP<sup>+</sup> or PAM<sup>+</sup> cells induced was limited. The differences in survival time after hormone deprivation in both models could be related to the different patterns of induction of



**FIGURE 3** – PAMP Western blotting in short-term (*a*) and long-term (*b*) PC-310 postcastration experiments. Products of  $M_r$  14, 15, 19, 20 and 22 kDa were found, but only bands corresponding to 14 and 19 kDa are indicated. Synthetic PAMP was loaded in a separate well (*a*).

NE subpopulations. For the PC-310 model, NE products could act as alternative growth factors, promote survival of dormant tumors or exert a vasodilatory effect on mouse stromal vessels in the xenograft after androgen depletion.

Immunohistochemic studies in organs such as normal prostate and pituitary have concluded that PAMP and AM do not always colocalize in the same cells,<sup>11,34</sup> though they are processed from the common precursor pro-AM. In Western blot analysis for PC-310-derived material, we did not detect fully processed PAMP. This may indicate that the PAMP-immunoreactive molecule produced by the PC-310 model is not fully active or that the mature peptide is present below the detection threshold of the technique. The latter possibility could be due to regulation of the final steps of protein processing, resulting in a slow rate of PAMP production or fast secretion and/or degradation of mature PAMP. The immunoreactive bands we found (14–22 kDa) may correspond to the previously reported precursor form and processing-derived peptides or even to species related to the pro-AM alternatively spliced mRNA.<sup>35</sup> In any case, the intensity of the bands corresponding to PAMP-related molecules (especially the 14 kDa peptide) increased as postcastration time advanced. The diminished intensity observed at T-154 is very likely due to the increase of mouse stromal protein relative volume present at this time point.

Expression of PAMP and PAM was related to androgen-insensitive human NE cells. The postcastration increase of immunoreactivity for PAMP observed by Western blotting and immunohistochemistry in the PC-310 model and the detection of PAMP-like expressing cells in clinical specimens after androgen-ablation therapy are in apparent contrast to another report of downregulation of pro-AM gene expression after castration.<sup>36</sup> In that report, the mRNA encoding the tradi-

tional pro-AM in the ventral lobe of the rat prostate was analyzed. Rat prostate is a very complex organ composed of ventral, lateral and dorsal lobes, which differ from each other. The dorsal, but not the ventral, rat prostate is embryologically close to the human organ. In the rat, the epithelial cells of every lobe are androgen-dependent but some genes, such as the one encoding for C-CAM epithelial cell-adhesion molecule, are distinctly regulated in the dorsal or lateral lobes with respect to the ventral ones after castration.<sup>37</sup> Thus, regulation by androgens of pro-AM gene expression or its alternative splicing may be different in the rat prostatic ventral lobe compared to the human prostate.

In summary, NE differentiation of PC cells producing PAMP-related peptides, and PAM is induced by androgen deprivation in 2 xenograft models and in tumors from patients pretreated with androgen blockade, showing the similarity between the experimental and the clinical settings. Our results implicate specific NE subpopulations in the progression of the disease after antiandrogen therapy. Despite the variability between individuals, further experiments to determine the putative effect of NE products on the evolution of PC are necessary to determine if the concept should be considered when new treatment strategies for hormone-refractory prostatic tumors are developed.

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