Prostatic Neuroendocrine Cells Have a Unique Keratin Expression Pattern and Do Not Express Bcl-2

Cell Kinetic Features of Neuroendocrine Cells in the Human Prostate

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We investigated the keratin phenotype and bcl-2 immunoreactivity of neuroendocrine cells in the human prostate to determine whether the postmitotic status of these cells is associated with protection from apoptosis by bcl-2 protein expression and to elucidate the possible cell kinetic relationship between neuroendocrine cells and the other epithelial components of the prostate. Tissue specimens were selected from prostates of 19 patients harboring normal secretory glands (n = 15) and glandular benign prostatic hyperplasia (n = 10). Using a novel sequentially selective destaining immunoenzymatic cytochemical technique we were able to demonstrate the distribution of neuroendocrine cells, keratin markers identifying either basal, luminal, or intermediate cells, and the bcl-2 protein in single sections. Basal cell keratins were expressed in the minority of the neuroendocrine cells. In most of the cells, intermediate and luminal cell keratins were found and bcl-2 was constantly negative. Our findings indicate that neuroendocrine cells and other epithelial cells in the human prostate share a common keratin phenotype and probably originate from a common epithelial precursor. From the absence of bcl-2 we infer that the neuroendocrine cells have no progenitor cell characteristics. (Am J Pathol 1997; 151:1759–1765)

The human prostate contains the largest number of neuroendocrine (NE) cells of any genitourinary organ.1 At birth, NE cells are abundant in the prostate and then disappear from the peripheral zone, only to reappear during puberty, after which their number slowly increases during adult life.2 NE cells are believed to play an important role in prostate growth and differentiation.3 Previous studies have shown that they probably are associated with the development of benign prostatic hyperplasia (BPH) and an unfavorable clinical outcome in prostate carcinoma (PCa).4–6

Although interest in NE cells of the human prostate has increased in the last years, our knowledge concerning the fundamental characteristics of these cells and their relationship with other epithelial cells in the prostate (ie, exocrine cells) is still fragmentary and controversial.7–10 Some authors suggest that they are derived from the neural crest, although others adhere to a unitarian theory stating that they develop locally from a progenitor stem cell common to the other, exocrine, prostatic epithelial cells.11 Another subject to be clarified concerns the status of NE cells with regard to their susceptibility to apoptotic cell death. Studies investigating bcl-2 expression in a variety of tissues indicate that this protein plays an important role in maintaining stem cell status and tissue homeostasis by inhibiting apoptotic cell death.11,12 In the human prostate epithelium, bcl-2 is expressed in basal cells.12,13 In a study devoted to PCa with NE differentiation, in two of six cases a proximate relationship between NE cells and cells expressing bcl-2 was demonstrated. However, this report, based on a limited number of specimens, does not allow decisive conclusions as far as bcl-2 expression by NE cells is concerned.13

In examining cell kinetic features of NE cells in the human prostate, application of immunocytochemical techniques can be instrumental. Particularly, antibodies against the different types of keratin (K) are helpful as they are expressed in specific combinations in various types of epithelia and are related to specific levels of cellular differentiation.14,15 In previous keratin phenotyping studies we were able to distinguish three epithelial
cell types in the prostate, i.e., basal cells expressing K14 and K5, luminal cells expressing K18 and K8, and cells, called intermediate cells, having keratin features of both luminal and basal cells (K5 and K18).\textsuperscript{20-23} These findings seem to support the stem cell model proposed by Isaacs and Coffey indicating that in prostatic epithelium there is a hierarchical differentiation pathway from basally located stem cells to terminal transit cells (columnar luminal cells) via amplifying cells.\textsuperscript{24} The cells we identified as intermediate cells are likely candidates for the amplifying cells. To date, the keratin status of NE cells in the prostate has not been clarified. One study showed that they contain both K14 and K5,\textsuperscript{4} although another report indicates that they are devoid of keratins altogether.\textsuperscript{7}

Recently, we developed a novel immunoenzymatic approach enabling the simultaneous detection of two antibodies in formalin-fixed, paraffin-embedded tissues while maintaining optimal morphology. This technique enabled us to study keratin and bcl-2 expression in single prostate NE cells. The aim of this study was to determine the immunophenotypic characteristics of NE cells to gain insight into the possible cell kinetic relationship between NE cells and other components of the prostatic epithelium and also to examine whether the postmitotic status of the prostate NE cells is associated with protection from apoptosis by bcl-2 protein expression.\textsuperscript{25}

Materials and Methods

Tissue Specimens

Specimens were selected from 40 formalin-fixed and paraffin-embedded blocks of prostate tissue removed from 22 patients with PCa or BPH (average age, 73.6 ± 16.7 years) who had not received any hormonal treatment before surgery. On sections taken from these blocks a standard hematoxylin and eosin staining as well as a serotonin immunostaining (see below) were performed. Only blocks containing normal secretory glands or nodular BPH and at least 10 serotonin-immunoreactive NE cells per field of view (diameter, 0.80 mm) were included. In this way, from 19 patients, 21 tissue blocks were selected. These blocks comprised normal secretory glands in 15 and nodular BPH in 10 areas.

Antisera

As NE cell markers we used a rabbit polyclonal antibody to serotonin (5-hydroxytryptamine, 5-HT)\textsuperscript{26} and a mouse monoclonal antibody (MAb) to chromogranin A (ChrA; Boehringer Mannheim Biochemica, Mannheim, Germany). To examine the keratin expression by the different cell types of prostatic epithelium we used three monoclonal keratin antibodies, i.e., 34/3E12 (Dako, Carpinteria, CA) recognizing K5 and K14 as a marker for basal cells,\textsuperscript{27} RCK103 (MAG, courtesy of Dr. F.C.S. Ramaekers, Maastricht, The Netherlands) recognizing K5 and several other keratins in basal cells and intermediate cells (putative amplifying cell type),\textsuperscript{21-22} and the antibody CK18 (DC-10, Dakopatts, Glostrup, Denmark) recognizing K18 marking the luminal cells in the prostate.\textsuperscript{21,28} A MAb to bcl-2 (124, Dakopatts) was used to demonstrate bcl-2 that is supposed to be expressed selectively in cells protected from apoptotic cell death.\textsuperscript{15,20} Details on the working dilution as well as the staining pattern of these antisera in prostate epithelial cells are reported in these previous studies.

Tissue Preparation and Antigen Retrieval

Consecutive 4-μm-thick sections were cut from the selected blocks, mounted on pretreated slides (Super-Frost\textsuperscript{+}, Menzel-Glaser, Braunschweig, Germany), and dried overnight. After dewaxing and rehydration, an antigen retrieval step was included. Slides to be stained with the antibody 34/3E12 were incubated with 0.1% Pronase XIV solution (P-5147, Sigma Chemical Co., Diessenhofen, Germany) dissolved in 10 mmol/L Tris, 1 mmol/L EDTA, pH 7.5, at 37°C for 10 minutes. For the MAb RCK103, antigen retrieval was performed according to a modified protocol by submerging the slides in a 0.5% periodic acid solution (H₃O₂, Sigma 7875) and microwave heating for two cycles of 5 minutes with power of 180 W.\textsuperscript{24} For CK18 (DC10) and bcl-2,\textsuperscript{25} microwave heating with 0.1 mol/L citrate buffer (pH 6.0) was performed according to the manufacturer's instructions.

Procedure in Detail for Sequential Dual Labeling

Sequential dual labeling was performed for the following antibody combinations: 5-HT/34/3E12, 5-HT/RCK103, 5-HT/CK18, and 5-HT/bcl-2. After preincubation with 10% normal horse serum and 10% normal goat serum in phosphate-buffered saline (PBS), sections were incubated with a mixture of one of the mouse MAb's and the rabbit polyclonal antisera to 5-HT at 4°C overnight, after which they were rinsed in PBS. Sections were then processed with the avidin-biotin complex (ABC) procedure. For detection of the MAb's (34/3E12, CK18, and bcl-2), the sections were first incubated with rabbit anti-mouse biotinylated secondary antibodies (1:100). After rinsing with PBS they were incubated with a peroxidase-conjugated antibody (1:50; Elite Vector ABC kit, PK6100, Vectastain, Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized with diaminobenzidine (DAB) as a chromogen. Slides were then rinsed in tap water followed by PBS. Subsequently, to detect 5-HT, slides were incubated with goat anti-rabbit biotinylated antibodies for 30 minutes (1:100), after which they were again rinsed in PBS. Finally, they were incubated with an alkaline-phosphatase-conjugated ABC (1:50; Vector ABC kit, AK5000, Vectastain) and rinsed in PBS. Immunostaining was visualized with Fast Blue substrate.\textsuperscript{30} After a final rinse in tap water, sections were briefly counterstained in hematoxylin and coverslipped.

For dual staining with the MAb RCK103 and the antibody to 5-HT, a modified procedure was followed, because periodic acid appeared to interfere with the antigen-antibody binding of 5-HT. After removal of the paraffin and rehydration, tissues were first stained with...
5-HT-positive cells in section I, also present and immunoreactive to ChrA as well. Three consecutive 4-μm tissue sections were then incubated with the MAb RCK103 after which they were incubated with linker antibodies and the ABC (peroxidase labeled) followed by staining with DAB.

**Evaluation of Immunoreactivity**

From each of the 21 paraffin blocks, sets of four consecutive sections of 4-μm were examined for the co-expression of 5-HT (NE cells) and one of the keratins or bcl-2 by removal of the Fast Blue deposit (labeling 5-HT) using saturated potassium chloride (KClO₃, see next paragraph). When no DAB staining was observed we decided that no co-expression existed, and intense DAB staining was classified as a positive dual staining. The staining results were qualified as partial expression when there was a dual staining with a weakly and/or focally expressed DAB label in the cell. Immunoreactivity evaluation was performed in a stepwise procedure for the four antibody combinations, ie, 5-HT/34E12, 5-HT/RCK103, 5-HT/CK18, and 5-HT/bcl-2 (for details, see Table 1).

**Validation of the Selective Substrate Deposit Removal Procedure**

In the literature, several immunoenzymatic cytochemical procedures are described to detect more than one substance in a single cell. These procedures are based on the use of antibodies labeled with different enzymes employing different chromogens. However, when applied to the NE cells of the human prostate the different deposits seem to mask each other. Therefore we developed a new procedure, depicted in Figure 1, based on the selective destaining of one of the deposits.

The validity of this procedure was tested making use of the fact that NE cells can be stained by antibodies to 5-HT and ChrA as well. Three consecutive 4-μm tissue sections were cut from paraffin blocks and numbered I to III. Section I was stained with the antibody to 5-HT using Fast Blue as a chromogen. Section III was stained with a MAb to ChrA using DAB as a substrate. The position of 5-HT-positive cells in section I, also present and immunoreactive to ChrA in section III, were marked with a diamond pen on the underside of the slides. Section II was dual labeled with a mixture of both antisera (5HT/ChrA) using the procedure we applied to examine the co-expression of 5-HT and keratins or bcl-2 as described in a previous paragraph. Cells marked in section I as well as section III were then relocated in section II. After removal of the coverslip, section II was incubated for 1 hour at room temperature with saturated potassium chlorate solution (2 g of KClO₃ (Merck 4944) in 700 μl of 37% HCl and 180 ml of 96% ethanol), rinsed with water, dehydrated, and again coverslipped.

Identical NE cells could be retrieved in all three numbered sections, ie, stained blue (5HT-Fast Blue) in section I, dark brown (ChrA-DAB) in section III, and black blue in section II (indicating co-expression for both antibodies). After incubation in the saturated KClO₃ solution, these cells showed only a brown cytoplasmic staining, proving that KClO₃ was able to remove the Fast Blue staining deposit leaving the DAB staining unaffected.

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**Figure 1.** Schematic presentation to illustrate the development of a positive control for selective substrate deposit removal. Consecutive 5-μm sections containing prostate NE cells are numbered I to III. Section I was immunostained with antibody to 5-HT and Fast Blue as a substrate. Section III was immunostained with antibody to ChrA and DAB as a substrate. Section II was dual immunostained with a mixture of antisera to 5-HT/ChrA. Validation of the selective substrate deposit removal procedure was based on the comparison of section II after KClO₃ treatment with the staining results obtained in sections I and III.
Results

NE cells were mostly confined to the basal and suprabasal compartments of the epithelium (closed type, Figure 2A), and some possessed long dendritic processes (open type, Figure 2C). Consistent with previous results, basal cells were stained positive by 34βE12, RCK103, and the bcl-2 antisera (Figure 2G), whereas K18 was observed only in the luminal cells. In dual-labelled slides it was difficult to discern the immunoreactivity of the second marker when the KClO₄ substrate deposit removal was not applied.

34βE12 Expression Pattern in NE Cells

After Fast Blue deposit removal in slides stained for 5-HT and 34βE12, only 20% of the NE cells showed a weak and focal expression with the basal cell marker. Eighty percent of cells were negative, indicating that the majority of 5-HT-positive NE cells did not express the basal cell keratin marker (compare Figure 2A with 2B).

MAb RCK103 Expression Pattern in NE Cells

Substrate deposit removal of Fast Blue from slides dual stained for 5-HT and the MAb RCK103 revealed positivity in 75% of the NE cells, indicating that these cells expressed the intermediate cell marker as well. Focal expression of the MAb RCK103 was seen in 16% of NE cells whereas 7% of the NE cells did not show the intermediate cell keratin phenotype (compare Figure 2C with 2D).

CK18 (DC 10) Expression Pattern in NE Cells

After removal of the Fast Blue deposit from the dual-stained sections, the luminal cell marker CK18 was observed in 63% of the NE cells. Weak focal positivity also, indicating co-expression of 5-HT and the luminal keratin marker, was present in 28% of the NE cells, and 9% of NE cells apparently contained only 5-HT (compare Figure 2E with 2F).

Bcl-2 (124) Expression Pattern in NE Cells

All cells that were double stained for 5-HT and bcl-2 were negative after deposit removal of Fast Blue, indicating that bcl-2 (124) was not expressed in 5-HT-positive NE cells in the human prostate (compare Figure 2G with 2H).

The staining results of NE cells are summarized in Table 2.

Discussion

In this report we present a number of cell biological characteristics of the human prostate NE cells achieved by the use of a novel sequentially selective destaining immunoenzymatic staining technique. We have applied this method to investigate the keratin and bcl-2 expression by NE cells.

Bcl-2 protein expression has been described in the basal cell compartments of various epithelia. The expression of bcl-2 by these cells is considered to be indicative for pluripotent stem cells. However, knowledge concerning the bcl-2 status of NE cells is fragmentary. In some organs, such as thyroid, skin, gastrointestinal tract, and lung, these cells express the bcl-2 protein whereas NE cells in the adrenal gland and pancreas are negative.30,31 In our study, NE cells of the human prostate did not show bcl-2 immunoreactivity. The absence of bcl-2 staining in those cells might be due to the low sensitivity of the detection technique. However, this is not likely as adjacent basal cells were intensely labeled by the same antibody using the same procedure. Therefore, we assume that in the human prostate NE cells actually are devoid of bcl-2, indicating that they are terminally differentiated cells. Several studies suggest that cells containing the bcl-2 protein are protected from apoptosis. However, bcl-2 is part of a multigene family of proteins involved in protecting cells from apoptosis.12,13,15 For example, bcl-X is also found to inhibit apoptosis, but mainly in postmitotic cells with extended survival, such as neurons in the brain.34,35 In the human prostate, MCL1, another member of bcl-2 gene family, is found only in luminal cells, which implies a unique role for this molecule in regulation of the survival of differentiated cells.32,33 Therefore, the absence of bcl-2 in prostate NE cells does not exclude the possibility that other member(s) of the bcl-2 gene family are protecting prostate NE cells from apoptosis.

In cell biological studies on epithelial cells, keratin markers are helpful in determining the origin and differential status of specific epithelial cells in normal tissues as well as their malignant counterparts. Using antibodies to these keratins, we have previously suggested that various types of (exocrine) epithelial cells in the secretory acini of the prostate possess a characteristic keratin expression pattern and seems to be related to cellular differentiation and functional status. This pattern is observed in human early prostate development, normal adult prostate, BPH, PCA, and rat prostate during involution-regeneration processes.20-22 Implicit in these observations is the assumption that there is a lineage relationship between basal and luminal cells via intermediate cells. This relationship has been called the hierarchical differential pathway in the stem cell model.24

To date, investigations in human prostate are mainly focused on evaluating the role of NE differentiation in the behavior and prognosis of PCA. The cell kinetic features of NE cells and their interrelationships with other epithelial cells in the normal human prostate have not been extensively pursued. In a recent report on the peptidergic innervation of the human fetal prostate, NE cells were observed as early as 13 weeks gestation.97 However, no information concerning their relationships with other exocrine cells in terms of glandular morphogenesis and cellular origin were given as epithelial markers were not included in this study. Bonkhoff et al reported that some NE cells focally express K5 and K14.8 In another study on the human prostate tumor cell line LNCaP, modulation of a conditioned medium resulted in an increased expression of luminal keratins in many cells accompanied by the appearance of cells with dendritic processes that were
frequently positive for NE cell markers. This observation implies that at an early stage of development prostatic epithelial cells are pluripotent and capable of differentiation into NE cells. In addition, there is some evidence that NE cells have an exocrine function as well.

Our study revealed that a minority of 5-HT-positive NE cells contain K5 and K14, keratins expressed by basal cells. Many NE cells were stained by the antiserum CK18 (DC 10) marking luminal epithelial cells as well as the antiserum RCK103 predominantly staining the basal cell compartment. Our results seem to indicate that in the human prostate NE cells represent a heterogeneous population of cells as far as the cytoskeleton pattern is concerned, sharing phenotype characteristics common to exocrine cells. Therefore, it is most likely that NE cells are derived from the same precursor cells as the exocrine prostate cells but not from neural crest cells.

The complexity of the keratin phenotype in NE cells may also indicate that their keratin expression pattern does not always parallel their functional status. For example, the existence of NE cells with low levels of basal cell keratins could indicate that these cells have already reached terminal differentiation in terms of functional status (eg, positive for 5-HT) whereas phenotypically their keratin phenotype still carries basal cell characteristics. A comparable phenomenon has been observed in the cervical epithelium of the uterus.

So far there are only a few reports dealing with the proliferative status of NE cells in the human prostate. By immunocytochemical methods, Bubendorf et al did not find any association between the presence of NE cells and Ki67-labeled tumor cells in the prostate carcinoma samples studied. Using dual immunoenzymatic labeling with antibodies to ChrA and MIB-1, Bonkhoff and coauthors showed that proliferative activities are restricted to only exocrine epithelial cells; ie, NE cells lack MIB-1 immunoreactivity. NE cells in general may, however, undergo some degree of proliferation, as reported in rodent models using the bromodeoxyuridine (BrdU) labeling method.

So far, there is no information concerning the NE cell proliferative status in human prostate tissue using incorporation of radioactive thymidine or the thymidine analogue BrdU to identify dividing cells.

Cell proliferation and differentiation are complex biological events. Proliferating cells are usually less differentiated, and after turnover, these cells gradually acquire differentiation-related functions although not entering the cell cycle again. It is therefore possible that prostate NE cells are derived from a functionally less mature cell type and gain their NE-related functions afterwards, as proposed in our putative stem cell model (Figure 3). As the conventional NE markers, such as ChrA and serotonin, are associated with secretory functions of NE cells, those with NE potential, but not yet fully differentiated, proliferating cells might escape identification by these markers. This might explain why Noordzij et al could not identify the incorporation of BrdU counting 1000 ChrA-positive cells in a human prostate tumor model in mice, although there is an increase in the number of NE cells by castration. It is suggested by the authors that NE differentiation takes place only in cells that are in the G0 phase. Interestingly, in a recent study by the same group, using the same tumor models, a time course experiment was performed showing that cells double labeled for ChrA and BrdU were indeed observed early after castration (2 to 4 days) albeit in low numbers. Taken together, these data indicate that in the human prostate epithelium some proliferating cells might exist having NE differentiation potential. If this is the case, the amplifying cells described in our model are most likely the candidates for these not yet fully differentiated NE-potent cells. It is possible that by androgen deprivation a subpopulation of these amplifying cells is induced to enter the cell cycle followed by differentiation into NE cells. Therefore, NE cells in the prostate most likely undergo a similar differential pathway parallel to the exocrine cells. By the same token, the observations reported by Noordzij et al may also explain the high percentage of recurrence in PCa with NE differentiation after androgen ablation. Finally, as luminal cells, which are terminally differentiated, regress apoptotically after androgen withdrawal, it is highly unlikely that NE cells originate from them as has been suggested elsewhere.

In conclusion, using a novel sequential destaining immunoenzymatic technique we were able to demonstrate that the keratin phenotype of NE cells in the human prostate overlaps that of exocrine epithelial cells. These findings indicate that NE cells and other prostate epithelial cells in the human prostate share a common keratin phenotype and probably originate from a common epi-
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the precursors. From the absence of bcl-2 we infer that the NE cells have no progenitor cell characteristics.

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