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Neuroendocrine Cells During Human Prostate Development: Does Neuroendocrine Cell Density Remain Constant During Fetal as Well as Postnatal Life?

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BACKGROUND. Knowledge concerning differentiation of neuroendocrine (NE) cells during development of the human prostate is rather fragmentary. Using immunohistochemistry combined with a morphometric method, we investigated the distribution and density of NE cells in the developing human prostate, with special emphasis on the topographical relationship of NE cells with the developing gland.

METHODS. Consecutive sections from a total of 42 human prostates taken during autopsy of fetuses (12–38 weeks of gestation), prepubertal males, and young adults were immunostained for chromogranin A and serotonin. Computer-assisted image analysis was used to assess the total number of cells in the different parts of the branching glandular anlage, i.e., budding tips and acini/ducts. Next, the number of NE cells was counted manually. The NE cell density (NE cell index) was then determined.

RESULTS. NE cells could first be detected in the prostate from 13 weeks of gestation. By 21 weeks of gestation, all prostates contained NE cells. NE cells were mainly confined to the acinous/ductal regions, while most of the budding tips lacked NE staining. NE cell indexes of individuals were highly variable, mostly in the youngest age group.

CONCLUSIONS. In the normal prostate, NE cell density probably remains constant in acini/ducts from fetuses to young adulthood. The presence of neuroendocrine cells in well-developed glandular structures at such an early fetal age and their absence in the less differentiated budding tips possibly indicates that differentiation of NE cells is associated with glandular maturation. NE cells occur preferentially in the acinous/ductal region, implying a paracrine function during secretory differentiation of exocrine epithelial cells.

KEY WORDS: prostate development; benign prostatic hyperplasia; neuroendocrine cells

INTRODUCTION

In the epithelial compartment of the human prostate, neuroendocrine (NE) cells are the third type of epithelial cell besides luminal and basal cells [1]. Despite the fact that prostate NE cells were already identified by Pretl in 1944, little is known concerning their functional significance [2]. It has been suggested that NE cells play an important role in prostate growth and differentiation, and in the development of benign prostatic hyperplasia (BPH). Furthermore, large numbers of NE cells seem to be associated with an unfavorable prognosis in prostate carcinoma (PCa) [3].
The distribution of NE cells in the human adult prostate has been the subject of several reports. Studies on adult prostates indicate that NE cells are more frequent in the periurethral ducts than in the peripheral parts of the gland [4,5]. To date, there is little information concerning the distribution of prostate NE cells during ontogenesis, i.e., during fetal development, in early postnatal life, and in the peripubertal adolescent; in particular, data on the distribution of NE cells in the different parts of the branching glandular anlage are lacking. Age-related changes in the number of NE cells were reported in the guinea pig prostate, but this relationship has not been confirmed in humans [6].

Studies on NE cells in the human prostate are mostly descriptive. Techniques such as high-performance liquid chromatography (HPLC) and Northern blotting provide quantitative data but require homogenization of tissues [6]. Therefore, only limited information can be given regarding the number of NE cells and their spatial relationship with other cell types in the epithelial and stromal compartments.

Knowledge on the distribution of NE cells in the prostate glandular tree during development might provide a more profound insight into their functional significance and, in addition, might be helpful in understanding why the prostate is able to maintain its capacity to develop new ducts throughout life, a process that also seems to be implicated in the pathogenesis of BPH [7-9]. In the present study, we attempted to quantify NE cells in relation to the total number of epithelial cells in the prostate by combining a morphometric method utilizing a computer-assisted image analysis system with immunohistochemistry using NE cell-specific markers, i.e., we determined NE cell density within the epithelial compartment during development of fetal, prepubertal, and young adult prostates. Furthermore, NE cell density was compared between age groups. We also examined the regional distribution of NE cells during early development of the human prostate in an effort to establish whether differentiation of NE cells correlates with prostate branching development.

MATERIALS AND METHODS

Specimens

A total of 42 prostate glands was included in this study, comprising formalin-fixed, paraffin-embedded blocks from prostates of 29 fetuses (12-38 weeks of gestation), 7 infants (0-1 year), 3 prepubertal males, 2 young adults, and one 42-year-old male. To avoid overlap with the early stages of BPH, specimens from older patients were not included. Postmortem delay varied between 5-48 hr. In fetuses and infants, organs of the lower abdominal cavity were removed as a block and fixed in 4% buffered formalin for approximately 24 hr. After fixation, a midsagittal section was made through the urethra, and one half of the tissue specimen comprising prostate, prostatic urethra, urinary bladder, and rectum was further processed for paraffin embedding. In adolescents and young adults (n = 6), a midsagittal lamella parallel to the urethra was taken and processed. Gestational age was determined using a foot-length table combined with histologic parameters. In 5 fetuses, abortion was induced because of chromosomal abnormalities (trisomy 21, n = 2; trisomy 18, n = 3). In the other cases, autopsy revealed that intrauterine death was due to infection or placental pathology. Macerated fetuses were excluded from the study population. Based on the clinical data, the findings from gross and microscopic evaluation were classified as “normal” or “without signs of pathologic processes.” Three consecutive 4-μm-thick sections were cut from each paraffin block, mounted on pretreated slides (SuperFrost®/Plus, Menzel-Glaser, Germany), and dried overnight. One slide was for hematoxylin-eosin (H&E) staining; the other two were for immunostaining, using markers for NE cells.

Immunohistochemistry

For NE-cell markers, we used a rabbit polyclonal antibody to serotonin (5-hydroxytryptamine, 5-HT) [10] and a mouse monoclonal antibody (MAb) to chromogranin A (ChrA, clone LK2H10, Boehringer Mannheim Biochemica, Mannheim, Germany).

The procedure used for immunohistochemical staining was published previously [1,11]. Briefly, following dewaxing, rehydration, and preincubation with 10% normal horse or 10% normal goat serum, sections were incubated with primary antibodies, i.e., the mouse monoclonal antibodies to ChrA (1:1,000) or the rabbit polyclonal antiserum to 5-HT (1:250) at 4°C overnight. Sections were then rinsed in phosphate-buffered saline (PBS) and processed according to the avidin-biotin complex (ABC) procedure. For ChrA staining, sections were incubated with rabbit antimouse biotinylated secondary antibodies (1:100). To detect 5-HT, slides were incubated with goat anti-rabbit biotinylated antibodies for 30 min (1:100). Peroxidase activity was detected by incubating with an alkaline phosphatase-conjugated AB complex (1:50, Vector ABC kit, AK5000, Vectastain, Vector Laboratories, Burlingame, CA) and then rinsed in PBS. Immunostaining of NE cells was visualized with a Fast Blue substrate. After a final rinse in demineralized water,
sections were briefly counterstained in hematoxylin and coverslipped.

**Assessment of NE Cell Density**

**Evaluation of immunoreactivity.** Assessment of the number of cells positive for the ChrA and 5-HT antibodies was performed by the two pathologists (F.S., C.S.) by screening the entire specimen, using a Reichert Polyvar microscope (objective 25/0.75, diameter of field of view 0.96 mm). In all prostates, the number of immunostaining cells was determined in the budding tips, and in the acinous/ductal regions if these epithelial structures were present [12]. Any immunostaining in the stromal compartment was also recorded. Staining intensity was not graded, as a clear, strong immunostaining reaction was observed in all cases. For fetal prostates, stained NE cells in the intestine, mostly also present in the blocks, were used as an internal positive control. As a negative control, slides were incubated with 1% mouse serum albumin instead of the primary antiserum.

**Computer-assisted assessment of total number of cells.** Because the reproducibility of visual assessment of the total number of cells in an epithelial compartment is poor, semiautomated image analysis was used. Direct assessment of the number of nuclei, using automated image analysis, was hampered by overlapping nuclei and inhomogeneous staining of nuclei. Therefore, the glandular area was used to obtain a measure for the overall number of cells in outlined compartment.

**Determining the linear relationship between epithelial area and cell number.** The relationship between area and number of cells was studied using 15 specimens of different ages. Interactive image analysis procedure was developed using a Vidas+ system (Kontron GMBH, Eching, Germany). Images were recorded with a three-chip CCD RGB camera (DXC-325P, Sony) mounted on a conventional light microscope (Axioskop, Carl Zeiss, Weesp, The Netherlands). The contours of glandular structures were manually delineated by using a mouse operating a cursor on the image monitor. Ductal lumina were excluded from the region of interest, so that the calculated area only reflected the epithelial area expressed in μm². In one case (19 weeks of gestation) in 25 randomly selected fields, all glandular structures were measured (objective 40/0.7). In each field the total number of nuclei was manually counted. These data were used to determine the average size of the glandular cells. The moving average of the area per nucleus (the cell size) showed no significant changes after counting 11 fields of glandular structures. Based on this observation, it was decided to assess only 15 fields of glandular structures in each tissue section for an accurate calculation of the relationship between glandular area and the number of cells. Next, in 12 specimens with ages varying between 19 weeks of gestation to 42 years, 15 fields of glandular structures were assessed in the same way as described above. The data were used to study the possible relationship between cell size and age, using analysis of variance (ANOVA). Linear regression was performed to compute the relationship between the area of glandular structure and the number of cells from this data set. Due to variations in measurement of epithelial cell size and cell number in cases older than 11 years, the numbers of cells in these prostates were individually determined, as described above.

**Obtaining the NE cell index.** In all specimens, every prostate glandular structure was interactively drawn (objective prostate 10/0.30). Using the equations derived from linear regression, the measured areas from each specimen could be transformed to numbers of epithelial cells present in the specimens. The labeling indexes for the ChrA- and 5-HT-staining cells were calculated using the following equation:

\[
\text{NE cell index} = \frac{\text{NE cells number}}{\text{Total cells number}} \times 100,000
\]

(Table I) [13]. This index reflects the NE cell density. Multiplication by 100,000 is used for the sake of clarity.

**Statistics**

Linear regression and analysis of variance were performed, using SPSS 8.0 for Windows software (SPSS, Inc.).

**RESULTS**

**Descriptive Analysis of NE Immunostaining**

For morphologic characteristics of the human fetal prostate, we refer to the literature [12,14–17]. In the epithelial compartment of the prostate, NE cells appeared as early as 13 weeks of gestation. Between 13–21 weeks of gestation, some specimens were negative for NE immunostaining. By 21 weeks, NE cells were identified in all prostates. Usually they were only located in the acinous/ductal region (Fig. 2). In the main collecting ducts, i.e., the proximal part of the glandular structure closer to the urethra, more NE cells were observed compared to the more distal acini. In only 4 cases were limited numbers of cells identi-
fied in the budding tips of the epithelial outgrowth. In all but 2 cases, ChrA antibodies stained more cells than did 5-HT antiserum. The histologic appearance of NE cells has been extensively described elsewhere [4].

**Morphometric Analysis (Table I)**

In the fetal and prepubertal prostates, the ratio between epithelial cell area and cell number was constant. A linear relationship between the total size of the outlined epithelial area and the number of cells was found (see Fig. 3). By linear regression it was possible to determine the number of cells by measuring the area of the glandular compartment.

Evaluation of morphometric data did not show any relationship between NE cell index and fetal age, either in the acinous/ductal compartment (index-A) or

<table>
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<th>No.</th>
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<th>CHrA-BT</th>
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* w, weeks; m, months; y, years; F, fetus; ChrA-A/5HT-A, number of chromogranin A/serotonin-stained cells in acinous/ductal region; 1 ChrA-A/1 5HT, index of chromogranin A/serotonin-stained cells in acinous/ductal region; ChrA-BT/5HT-BT, number of chromogranin A/serotonin-stained cells in budding tips; 1 ChrA-BT/1 5HT-BT, index of chromogranin A/serotonin-stained cells in budding tips; 1 ChrA/1 5HT, general index of chromogranin A/serotonin-stained cells in acinous/ductal region and budding tips; NA, not available.
in budding tips (index-BT). Furthermore, the general NE cell index defined as the sum of NE cells in the acinous/ductal compartment and budding tips, indicated as index-ChrA/index-5-HT, was also not related to gestational age. The NE cell indexes in the fetal prostate were highly variable even within the same age group (e.g., 38 weeks of gestation).

NE cell index as related to the various age groups (Fig. 4) demonstrated that the average index in relation to prostate age is relatively constant, irrespective of whether the prostate was fetal, prepubertal, or adult. However, younger prostates seem to show larger variations in the NE cell density.

**Stromal Immunostained Cells**

A few dispersed clusters or individually ChrA-immunostained cells were identified in the stromal compartment of 24 prostates ranging from 16 weeks of gestation to 16 months after birth. 5-HT-immunostained cells were found in 7 cases; coexpression of ChrA/5-HT-immunostained stromal cells was also observed in several cases by localizing identical cells in two consecutive sections, one stained for ChrA, and the other for 5-HT. Stromal immunostained cells were large, with a well-developed cytoplasm containing intensely stained granules. These cells were mostly located in the peripheral parts of the prostate, sometimes in the vicinity of budding tips. Around these cells, vascular structures were usually present.

**DISCUSSION**

In this study we examined the number and spatial distribution of NE cells in prenatal, infantile, prepubertal, and young adult human prostates. From these prostate specimens were taken according to a standardized protocol within 48 hr after death. By combining immunohistochemical and morphometric techniques, we determined the number of NE cells in the...
prostate in relation to the total number of epithelial cells, i.e., the prostate NE cell density during pre- as well as postnatal ontogenesis.

NE cells first appear in the prostate at 13 weeks of gestation. By 21 weeks, all prostates studied contained NE cells. These findings are in line with the observations of Jen and Dixon [18], who also observed these cells as early as 13 weeks, and Aumüller et al., who found these cells for the first time in the 16-week-old prostate [14]. Information regarding the anatomic distribution of these cells cannot be inferred from their data. In some studies, the glandular segments of the developing gland are classified according to their topographic association with the prostate capsule, the urethra, and the verumontanum [4]. However, we observed that in these regions glandular architecture might be different. For example, in the subcapsular region, solid budding tips as well as canalizing structures are present. In the more central parts of the prostate, solid epithelial structures can also be identified which are undoubtedly budding tips of ramifications. Previously, we showed that in the human fetal as well as adult prostate, budding tips, acini, and collecting ducts can be identified by histology and by differentiation-related keratin expression, and by a marker for stromal-epithelial interactions (tenascin-C) as well [7,12]. In a recent study, we showed that in these areas, characteristic differences in proliferative activity occur (unpublished data). Therefore, we prefer the subdivision of the glandular component of the prostate into segments of budding tips, acini, and collecting ducts rather than using the anatomical classification based on the topographic relationship between glandular structure and urethra or capsule. As our object was to focus on the possible role of NE cells in growth and differentiation of epithelial cells, we evaluated the gland as budding tips and acini/ductal regions only, without further differentiating acini from main collecting ducts.

Most surprising was our observation that in the developing fetal prostate, NE cell density, expressed as NE index, does not correlate with gestational age. Furthermore, comparison of the NE index in fetal prostates with that in prepubertal and young adult prostates reveals no age-associated correlation. Although in prenatals NE cell number is highly variable, it seems as if at an early age, NE cell density in the prostate already reaches a set point, remaining constant till adult life.

Constant NE cell density in young prostates was also reported in the guinea pig [6]. Using HPLC and immunostaining combined with semiquantitative morphometry, di Sant’Agnese et al. [6] showed that in the guinea pig prostate, the serotonin content increased with age from an undetectable level in weanlings to an intermediate level in prebreeding animals. In the retired animals this level was even higher. From their data we are able to infer that NE cell density in weanlings and young mature guinea pigs remains constant and increases only in old animals, implying that the functional differentiation of these cells is an age-related process. Cohen et al. studied NE cell distribution semiquantitatively in the human prostate in various age groups [4]. From their data it is also deducible that irrespective of the location in the prostate, NE cell density is actually constant in specimens from age groups between 1 month and 13 years old. These authors, however, reported that NE cells disappeared in the peripheral part of the prostate gland shortly after birth, implying that in this part of the prostate, NE cell differentiation is androgen-dependent. We failed to confirm this observation, although the zonal classification these authors used is not entirely comparable with ours.

Recent studies show that in the prostate, NE cells are likely involved in neoplastic transformation [5]. Furthermore, they could also play a role during the process of branching of the glandular anlage before birth. The constant NE cell density in the prostate from early fetal life till adulthood may reflect the functional significance of these cells. Perhaps NE cell density reached at an early age is sufficient to maintain some kind of steady state, e.g., prostate cell turnover under normal conditions. In line with this assumption is the observation that the paracrine functions of these cells include secretion of hormones involved in growth regulation and even propagation [19]. Additional proof for this hypothesis may be gleaned from the study of Cockett et al. [5], which shows that in early forms of BPH, the number of NE cells is higher than in normal prostate tissue, while in mature nodules the number of NE cells is lower. Thus, NE cells
may play a fundamental role in the homeostasis of the glandular structure, and if this is the case, perhaps disturbance in NE cell density may be associated with the pathogenesis of neoplastic diseases.

Accumulating evidence supports the opinion that NE cells share a common origin with exocrine epithelial cells, probably developing from an intermediate cell type which can commit itself to either an endocrine or a secretory differentiation [20]. It could well be that by paracrine secretion, NE cells are involved in this differentiation process, maintaining a balance between their numbers and the number of differentiating secretory cells. This last speculation is not entirely hypothetical because, as is shown in the present study, the time by which NE cells seem to be fully developed, i.e., by 20 weeks of gestation, coincides with the first appearance of cells with a secretory function [12,21]. Another argument supporting the hypothesis that in the prostate NE cells are involved in the development and maintenance of secretory functions might also be inferred from our study, i.e., in the majority of specimens, NE cells were only found in the acinous/ductal compartment and were absent in the active growth foci (budding tips). Furthermore, in cases where NE cells were present in the budding tips, their numbers were quite low. These findings might indicate that NE cells probably exert little, if any, effect on early growth. This supposition is contradictory to reports suggesting that NE cell products have mitogenic effects. For example, in prostate carcinoma Bonkhoff et al. found ChrA-positive NE cells preferentially situated in the proximity of proliferating tumor cells labeled by Ki-67 [22]. But the results of others failed to support this observation [23]. To explain these contradictory data, it is important to note that NE cells present elsewhere in the human body are known to secrete a variety of peptides with different functions, under different circumstances. So far, more than 200 NE peptides have been identified; at least 15 of these peptides have been reported in the prostate [19,24]. It is therefore likely that detection of NE cells using antisera to only a few NE cell markers will give only limited information concerning their function. Only a few NE cells were found in the budding tips. However, this may not reflect the true situation, as the markers we used to detect these cells are only capable of identifying terminally differentiated NE cells. Therefore, we cannot rule out that in the budding tips, a premature type of NE cells is present with a differentiation status parallel to that of the surrounding exocrine cells. As far as NE cell products are concerned, these cells may be in an immature stage, as suggested in a recent in vitro study [25].

It is generally accepted that NE cells are confined to the epithelial cell compartment in the prostate [26]. However, in 24 of our cases we noted strong immunostaining for the NE cell markers in dispersed single as well as small groups of stromal cells. We also observed these cells during our previous study [1]. In the course of this study, dual immunostaining of 5-HT/34E812 (keratins 1, 5, 10, and 14), 5-HT/CK18 (keratin 18), and 5-HT/bcl-2 was performed in some cases, revealing that stromal NE cells were negative for high/low molecular weight keratins and bcl-2. Although these data do not allow a final conclusion, probably these cells constitute part of the paraganglionic system [27]. As opposed to a previous report [4], we regularly found cells positive for NE markers in the stroma of young prostates, thus supporting observations by Feyrter [28]. The function of prostate paraganglionic cells remains uncertain; in the fetal bladder, it has been suggested that they may be involved in innervation [29]. In our prenatal specimens, portions of the small intestine were included. The NE cells present in the intestinal epithelium were used as an internal control. It is our impression that the number of staining NE cells in the intestine remained more or less constant, irrespective of postmortem delay, suggesting that at least in the above-mentioned duration of postmortem delay (5–48 hr), damage to the NE epitope is limited, as was also shown by Bird [30].

In conclusion, in the human prostate, NE cells appear early during fetal development and by midterm gestation are found in all prostates. NE cell density in the normal prostate remains constant, from fetuses to young adulthood. Prostate NE cells are preferentially distributed in the acinous/ductal region, implying a paracrine function during secretory differentiation of exocrine epithelial cells.

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