Comparison of prostatic zones regarding the cell differentiation

PhD thesis

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Introduction

The most common medical problems of elderly men occur in the prostate. Both benign prostatic hyperplasia (BPH) and prostate cancer are significant health problems in western countries. The detailed prostatic anatomy was described by McNeal which superceded previous anatomical models. The prostate consists of four, more or less well defined areas. Three glandular zones (peripheral, transitional and central) and an anterior fibromuscular stroma compose the prostate. The glandular zones cover the two-third of the prostate, while the rest of the prostate made up of the anterior fibromuscular stroma. The peripheral zone comprises approximately 70 percent of the glandular tissue, the central zone 25% and the transition zone 5%. According to widely accepted theories these zones originate from different embryological structures. Similarly to the seminal vesicle the central zone has wolffian origin while the peripheral and transition zones develop from the urogenital sinus. These conclusions were made by using results from comparative studies on the seminal vesicle and the central zone examining histological, immunohistochemical, pathological and biological features. The histological anatomy described by McNeal was supported by immunohistochemical, protein expression, histological, radiological and histopathological studies. During his observations McNeal put emphasis on the clinical features and appearance of prostatic diseases as well. According to his pathological findings the peripheral zone is the most common site of prostate cancer, while the BPH arises in the transitional zone exclusively. Contrary the central zone and the seminal vesicles are rarely affected by these conditions. The background of this different zonal biological behaviour is not understood so far. 70% of the prostate cancers arise in the peripheral zone, 20-25% appears in the transition zone and only 1-5% turns up in the central zone. As previous studies showed the localization of prostate cancer significantly affects the progression of the cancer. Cancer from the transition zone tends to be lower grade cancer (with higher Gleason score) with lower pathological stage at the time of diagnosis than the peripheral zone cancer. Moreover after the radical treatment of peripheral zone cancer the time to biochemical progression is less than the time to progression of transition zone cancer.

The prostate can be divided into epithelium and stroma. The epithelium has three main cell types: secretory luminal cells, basal cells lying on the basal membrane and the neuroendocrine cells (NE). Three times more luminal cells can be seen in the epithelium than basal cells. These three types of cells can be well characterized by immunohistochemical markers. All these cells can be found universally in the prostate. The basal cells may have a

role in the proliferation. It is widely accepted that the basal cells are the precursors of the luminal cells. According to the prostate stem cell theory the multipotent cells localized in the basal compartment can differentiate following induction by certain stimuli. During differentiation intermediate cells and transit amplifying cells develop from stem cells, which are located either in the basal cell compartment or between the luminal and basal cell layer. At the final stage of differentiation the cells transform into secretory luminal cells or neuroendocrine cells. These two terminally differentiated cell types are the final stage of differentiation. The neuroendocrine and luminal cells are not dividing anymore and are terminating by apoptosis. The cell differentiation was studied by numerous cell surface antigens. The most widely used antigens are the citokeratins. Subtypes of citokeratins were used to characterize the above mentioned subpopulation of epithelial cells.

The prostatic stroma consists of smooth muscle, nerves and lymphatic vessels. Smooth muscle cells, fibroblasts, myofibroblasts are the most common cells. The importance of interaction between the stroma and the epithelium was demonstrated earlier both in normal and hyperplasic tissue.

Theoretically the prostate cancer can develop from any cell in the prostate according to the prostate stem cell theory. The prostate cancer cells are luminal phenotype cells predominantly. There are many models describing the prostate cancer development. One of the models hypothesizes that the differentiated luminal cells develop proliferation activity and de-differentiate during cancer progression. Others say that stem cells gives rise to prostate cancer cells, and during differentiation they lose their basal cell characteristics. According to a third model the transit amplifying cells become prostate cancer cells. Investigating the differentiation of normal and cancer cells may be an important step in understanding the cancer development.

Aims

In the first part of the examination our aim was to study and compare the morphological, histological, immunohistochemical and cell kinetic features of the normal prostatic zones and seminal vesicles free of diseases. During our laboratory study the distribution and immunohistochemical characteristics of epithelial and mesenchymal cells were examined by using antibodies available from previous studies. These antigens have been proved to be expressed by these cells in earlier works. Cell differentiating immunohistochemical markers were used to identify the subpopulations of cells in the prostatic zones and the seminal vesicle. The zonal distribution of the cells was studied in the zones. Prostates and seminal vesicles form young cadavers may represent the normal prostate in our opinion. Cell kinetic parameters, proliferation activity and apoptosis were compared between the zones and the seminal vesicles. Neuroendocrine cells were examined by serotonin and chromogranin A in the tissues to study the NE cell distribution between the zones. Citokeratins were applied to identify the differentiated cells, while the NE cells were described by widely used NE markers as mentioned before. Our goal was to find differences between the zones and the seminal vesicles which may explain the different zonal susceptibility for diseases. We also used zonal markers described by McNeal's working group to justify that our zonal definition is correct.

In the second part of our investigation various isoforms of CD44 were used to identify the differentiated cells. The CD44 expression study was performed on normal and hyperplasic tissue as well as on cell cultures to find special features. The differences between the distributions of differentiated epithelial cells may explain the biological differences described above.

Materials and methods

Prostates and seminal vesicles were collected from nine organ transplant donors aged between 15 and 36 years old, with a mean of 26. The donors had had traffic accident injuries or cerebrovascular events. Based on the past medical history none of the donors had medical conditions or treatment known to affect the prostate. Nevertheless all the patients had indwelling catheterization during their hospital stay. Prostate samples from transurethral prostate resection were thoroughly selected for the CD44 expression study discarding the tissues damaged by the vaporization of the prostate. Prostate cells were also utilized from

Pre2.8 cell culture and hyperplasic tissue. Prostate and seminal vesicles were sliced at approximately 5 mm intervals from apex to base. The tissue slices were fixed in neutral buffered 4% formaldehyde solution for 24-48 hours. After dehydrating through graded alcohol samples were cleared in Histoclear® solution and embedded in paraffin wax and stored at room temperature until use. Five micrometer thick whole mount sections were cut and mounted on Vectabond® coated slides. One section of each block was stained with haematoxylin and eosin for histological analysis. Histopathological examination confirmed that the prostates were free of diseases, and the zones were also identified on anatomical grounds.

Immortalised cell line, PrE2.8 cell line was used in our study that maintained the characteristics of proliferative prostate cells. This cell line contains immortalised prostate epithelial cells. These proliferating prostate epithelial cells are derived from immortalised BPH tissue by temperature-sensitive SV40 Large T construct. The cells are proliferating at a permissive temperature of 33°C, and differentiating at non-permissive temperature of 39°C. Briefly the following technique was used in our study. Prostate tissue was obtained from patients undergoing TURP for BPH. Burnt areas of the prostate chips and blood clot were removed from the tissue by scalpel. 1 cm^3 pieces were made and washed in 20ml PBS solution. After the PBS was removed with the blood and debris, collagenase was used to digest the acini from the stroma. With the help of trypsin/versene the cells were isolated from the acini. Cells were immortalised by transfection of epithelial cells with SV40 Large T construct. These cells were grown in 75 cm^3 tissue culture flasks on PrEGM medium (Clonetics) at either a temperature of 33°C or 39°C in a humified incubator containing 5% CO_2 enviroment. For immunohistochemical studies the cells were grown on glass cover slips. Before fixing in formaldehyde solution the slips were rinsed in PBS. Immunocytochemical study technique was similar to that of the normal tissue technique.

Tissue chips obtained from TURP technique was also used for immunohistochemistry. These chips were fixed in formaldehyde solution, dehydrated, embedded in paraffin and stored at room temperature until required.

For immunohistochemical staining after short time warming the slides were dipped into Histoclear® solution and rehydrated through graded alcohol. Twenty minute long boiling in Vector Unmasking Solution was used for antigen retrieval to uncover the epitopes covered by conformational changes during formalin fixation. After cooling down the slides were washed in PBS. For horseradish-peroxidase staining technique, endogenous peroxidase was quenched by incubation for ten minutes in 0.3% hydrogen-peroxide solution. 10% bovine

PBS solution was used for blocking. The sections were incubated with the primary antibody for overnight at 4 °C before rinsing the slides in normal saline. Then the sections were exposed to secondary antibodies at a dilution of 1:200 for half an hour. For immunohistochemistry the secondary antibodies were either biotinylated anti-mouse IgG or monoclonal anti-rabbit immunoglobulin. For immunofluorescence staining FITC or TRITC conjugated immunoglobulin subclass specific secondary antibodies were used with Hoechst nucleus counterstaining.

For colour detection of bound primary antibodies avidin-biotin-horseradish peroxidise or avidin-biotin-alkaline-phosphatase were added for 30 minutes. DAB, Novared or Vector Blue chromogenic enzyme substrate were applied for 2-30 minutes. Brief counterstaining with haematoxylin or methylene green was used to visualise the nuclei. After rinsing in PBS the slides were dehydrated through graded alcohol and the sections were mounted in Mowiol 4-88 or DPX before coverslip added. Until microscopical evaluation the slides were stored at dark place. The following primary antibodies were used: citokeratin 5/6, citokeratin 8, citokeratin 14, citokeratin 17, citokeratin 18, citokeratin 19, lactoferrin, andogen receptor, Ki67, PNA, chromogranin-A, serotonin, prostate specific antigen, prostatic acid phosphatase, desmin, smooth muscle α-actin, CD44v3, CD44v4, CD44v5, CD44v7/8. TUNEL technique was used to study apoptotic cells following the manufacturer's instructions for the in situ apoptosis detection kit. The TUNEL technique detects the DNA fragments released by apoptotic cells during cell death cascade.

Immunohistochemical sections were examined by light microscopy. At least three images were captured randomly at 200x magnification from each area. Immunofluorescence stained slides were examined using a Zeiss Axiophot microscope with Photonic Science Coolview 12 camera controlled by Image ProPlus Software. Adobe Photoshop 4.0 was used for picture processing. In every field the positively stained cell nuclei and the unstained nuclei were counted. After scoring the data were recorded and stored by Microsoft Excel. The mean values of the areas were compared with non-parametric Mann-Whitney U-test using SPSS for Windows v11.0 software.

Results

Haematoxylin and eosin staining was used for morphological analysis. The peripheral and the central zone can be distinguished by analyzing the level of the section, position of examined region of prostate and their anatomical features and anatomical appearance. The

acini of peripheral zone are relatively small and round and the stroma contains a meshwork of loosely arranged smooth muscle fibres. Contrary the epithelium of the central zone consists of large, rectangular acini and is separated by closely-packed smooth muscle fibres. The central zone is surrounding the prostatic parts of ejaculatory ducts. In the normal prostate the transition zone is very similar to the peripheral zone and its microstructure is almost the same. The structure and appearance of seminal vesicles are different from that of the prostate. The basal cells are rounded and do not form a continuous layer, making the distinction between luminal and basal cells less clear.

Citokeratin staining was used to examine the basal cells. Basal cells are positive for citokeratin 5. Citokeratin 5 expression was uniform and continuous in the basal cell layer of the prostate. CK5 was not expressed by the luminal cells. Fairly good distinction between the luminal and basal cells can be made by using CK5 staining in all zones of the prostate. In contrast the CK5 staining was not continuous in the epithelium of the seminal vesicles. The cuboidal basal cells sitting on the basal membrane are staining positively and making a patchy appearance of staining. In the prostate the luminal and basal cell ratio was 2.63:1, which means that the 38% and 62% of epithelial cells are basal and luminal or neuroendocrine respectively. No difference was seen between the zones regarding the CK5 staining, though the prostate differed from the seminal vesicles. The citokeratin 14 expression was discontinuous in the prostate zones and absent from the seminal vesicles. In some areas the staining was stronger than in other areas, but no anatomical reason was found behind this anomaly. The CK17 positive cells rate was 20.4%. The CK19 expression was positive only in the basal cell layer as well. The proportion of CK19 cells was 24%. There was no difference between the zones in CK17 and CK19 expression. Basal cells did not show CK8 and CK18 expression.

CK8 and CK18 expressed only in luminal cells uniformly in the whole prostate with no difference between the zones. The seminal vesicles also expressed these citokeratins. Using the CK8, CK18 and CK5 luminal and basal cells can be distinguished both in the prostate and seminal vesicles.

Neuroendocrine cell distribution was examined by chromogranin-A and serotonin. Our results showed that both markers stained the same number of cells. The seminal vesicles and the ejaculatory ducts were absent from neuroendocrine cells. In contrast large numbers of cells were detected in the prostatic utricle and in the prostatic ducts opening into the urethra. In these ducts the proportion of neuroendocrine cells was 8%, while this number was

approximately 1-2% in the prostate. The frequency of neuroendocrine cells in the central zone was 50% lower than in the peripheral and transitional zones.

Functional markers (PSA, PAP) expressed by the luminal secretory cells. Both the prostate specific antigen and the prostatic acid phosphatase showed strong expression in the cytoplasm of the luminal cells. Expression was especially strong in the apical apart of the luminal cells, where the secretory granules can be found. None of these markers was seen in the seminal vesicles or in the ejaculatory ducts. Androgen receptor was strongly and uniformly expressed in the nuclei of the luminal cells of the prostate and the seminal vesicles. No differences were observed between the zones.

We have used two of the zonal markers previously applied by McNeal's workgroup to make difference between the zones defined by McNeal. Some of the basal and luminal epithelial cells were PNA positive in all three prostate zones and in the SV. The intensity of the PNA staining was stronger in the basal cells of the peripheral zone, whereas in the central zones the luminal cells expressed the PNA stronger. Staining in the SV was similar to that of the central zone. The transitional zone stained similarly to the peripheral zone. The PNA staining only appeared in the epithelium, while the stroma was negative for PNA. Lactoferrin was the second zonal marker previously shown to be expressed differently in the prostate. Lactoferrin is expressed most strongly in the central zone and the seminal vesicle epithelium, though the central zone showed weaker expression. The Lactoferrin expression was also stronger in the apical part of the luminal cells. Over 70% of the epithelial cells in the SV stained for Lactoferrin, compared to 31.2% in the central zone and 2.1% in the peripheral zone. This difference was statistically significant. No lactoferrin expression was seen in the stroma.

Androgen receptor expression was assessed in the stroma. The AR is expressed in the nuclei of the cells. We have not seen difference between the zones and the SV. 50% (43%-53%) of the cells of the stroma were positive for AR. AR expression was more frequent in the SV stroma, but no statistical difference was experienced. In the prostate, smooth muscle α-actin (SMA) and desmin are expressed by smooth muscle cells, but rarely by fibroblasts. Myofibroblasts are negative for desmin and positive for actin. Myofibroblasts were not identified at all in our study. There were statistically more smooth muscle cells in the central zone than in the transitional and peripheral zones. From this aspect the SV was similar to peripheral zone. Desmin expression was most frequent in the seminal vesicle, 71.6% of stroma cells were desmin expressing cells. In the prostate 61.4% of stroma cells expressed the desmin. No statistical difference was seen between the zones.

The proliferation and apoptosis were observed by Ki67 expression and TUNEL test. The proliferation index measured by Ki67 was almost identical in the peripheral and transitional zones at 0.8%. In the central zone and SV the Ki67 positive cell proportion was lower at 0.5%. The difference between the peripheral zone and SV was significant. The proliferation activity in the peripheral zone stroma was approximately 50% higher than in the SV. The apoptosis observed by TUNEL test appeared in the luminal cells of the epithelium. The apoptosis index was around 2.2% in the peripheral and transitional zones, whereas 0.8% in the central zone. This was significantly different. The TUNEL test was not assessable in the seminal vesicle. The proliferation of cells was studied further in the peripheral zone epithelium. 89% of the proliferation activity was observed in the basal cell compartment. The proliferation rate of CK14 expressing cells in the peripheral zone is around 2.1%, while this was 0.9% in the central zone. The central zone contained the most CK19 and Ki67 co-expressing cells.

The CD44 v5 expressing cells were seen in all the hyperplasic samples. Numerous cells expressed both CD44 and CK14. We successfully identified a new cell population located between the basal and luminal cell layer. This cell population may have represented the transit amplifying cell population. We have observed a higher expression rate in the hyperplasic tissue compared to normal tissue. In every sample the CD44 v5 positive cells were covered by luminal cells. All CD44 isoforms (v3, v4, v7/8) showed a similar expression pattern to CD44 v5. According to these observations we can say that the CK14 negative, differentiated cells are expressing all isoforms of CD44.

Conclusions

The zones of the prostate described by McNeal can be reproducibly distinguished by haematoxylin and eosin staining if the level of the prostate section is known. Recognizing of the transition zone in the normal prostate is the most challenging according to our experience. Histological appearance of the transition zone resembles to that of the peripheral zone. The transition zone is becoming more recognizable and distinct by progressing of age. In our study we could identify the transition zone in six samples out of nine. The seminal vesicle can be distinguished from the prostate easily and its morphological features make the SV similar to the central zone. So far the identification of prostatic zones is important mainly for scientific investigations and studies. Distinction between the zones has no impact on the clinical treatment modality to date.

In our study we examined expression of numerous citokeratins in the prostate epithelium. Citokeratins are widely used to study the level of cell differentiation. We could not find difference between the zones in distributions and frequency of luminal, basal or intermediate cells. The luminal and basal cell ratio was significantly different between the SV and the prostate. The different biological behaviour of prostatic zones cannot be explained by the distribution of differently differentiated epithelial cells. The lower number of basal and proliferating cells in the seminal vesicles may be explained by lower turnover of the epithelium that may contribute to low frequency of proliferating diseases in the SV.

Lactoferrin and PNA are good and useful zonal markers of markers described by McNeal's study group. In our opinion it is only possible if at least two zones are present in the examined sample and they can be compared. If small fragment of tissue sample must be analyzed the usefulness of these markers are limited.

The functional markers (PSA and PAP) are expressed strongly in the apical part of luminal cells uniformly in the prostate, but no expression was seen in the seminal vesicles. From this functional point of view all three zones of the prostate differed from the seminal vesicle. Regardless of speculation on similar embryological origin of the central zone and the seminal vesicles the function of these two anatomical structures is very different.

The high number of neuroendocrine cells in the prostatic utricle and in the glandular ducts opening in the urethra led us to two conclusions. Neuroendocrine cells may have a role in sustaining the motility of spermiums. The prostatic utricle is more likely to origin from the remnant of the Mullerian duct than from the urogenital sinus or the Wolffian duct.

No difference was seen between the zones using mesenchymal markers, which means that no explanation for differential disease susceptibility can be concluded.

Confirming the results of previous studies no neuroendocrine cells were detected in the seminal vesicles. Higher number of neuroendocrine cells in the peripheral and transitional zones comparing to central zone may have a role in the development of diseases in these two zones. Regarding the neuroendocrine cell numbers the central zone is very similar to the seminal vesicle.

In our CD44 expression study we could identify an intermediate cell population both in the normal and hyperplasic tissue, though no difference in the distribution of these cells was seen between the zones.

The higher proliferation activity and cell turnover of the peripheral and transitional zones may contribute to higher frequency of prostate cancer in the peripheral zone and hyperplasia in the transitional zone.

In summary we can conclude the followings:

- the prostate anatomy can be determined reproducibly by H&E staining,
- the transition zone is very similar to peripheral zone,
- the central zone resembles both to the peripheral zone and the seminal vesicle,
- the lower incidence of diseases in the seminal vesicle can be explained by lower turnover of its epithelium,
- lactoferrin and PNA may help to identify the prostatic zones,
- regarding the neuroendocrine staining the prostatic utricle may be Müllerian remnant, and the NE cells may have a role in the motility of spermiums,
- zonal differences cannot be explained by stromal differences according to our limited study,
- similarity between the central zone and the seminal vesicle may explain the low disease susceptibility to proliferative diseases of these two structures,
- intermediate cells can be identified, although no difference between the zones was noticed,
- the higher proliferation activity of the peripheral and transition zones may explain the susceptibility to proliferative diseases of these two zones.

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