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Immunohistochemical determination of age related proliferation rates in normal and benign hyperplastic human prostates

Siegfried Claus, Markus Wrenger, Theodor Senge, Harald Schulze

Urologische Klinik der Ruhr-Universität Bochum im Marienhospital II, Widumer Strasse 8, D-44627 Herne, Germany

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Summary. To study whether benign prostatic growth in aging men correlates with an increase in proliferation, proliferation rates were determined immunohistochemically using the antibody Ki-67 in 20 benign hyperplastic prostates (BPH) and in four normal prostates (NPR). There was no significant correlation between age and proliferation rate in epithelium or stroma in BPH. In addition, no significant correlation between prostate weight and proliferation rate could be demonstrated in either compartment. In NPR the proliferation rate in epithelium and stroma was 9 times and 37 times lower, respectively, than in BPH. Obviously the induction of BPH from NPR may be associated with a distinct increase in proliferation. The further increase in BPH volume, however, is not correlated with a further increase in proliferation rate.

Key words: BPH – Proliferation – Age – Normal prostate – Ki-67

Although benign prostatic hyperplasia (BPH) is the most common benign neoplasm in men [13] the aetiology of the disorder remains obscure. The development of BPH is both under endocrine control and associated with aging [2, 4, 17]. On the basis of autopsy studies there is an age-dependent increase in volume of the prostate throughout the entire life of a man [10]. During this growth phase of the prostate, the ratio between cell proliferation and cell death rate must be greater than 1. Therefore cell proliferation is a necessary factor for the increase in prostate volume with age.

Proliferating cells can be identified in tissue sections by labelling with Ki-67 antibody. This antibody recognizes a proliferation-associated nuclear antigen that is expressed in the G1, S, G2 and Mphases of all cycling human cells but is lacking in G0 and early G1 cells [6, 7].

Correspondence to: Harald Schulze, M.D.; FAX: 49(2323) 499-388

To study the question of whether the age-related growth of the prostate may be based on an increase in proliferation, in patients of different ages with BPH proliferating cells in epithelium and stroma were localized immunohistochemically and proliferation rates were determined and correlated with the patient's age and prostate weight. In addition these results were compared with data obtained in normal prostates (NPR) to show possible differences.

Materials and methods

Reagents

The monoclonal antibody Ki-67, a mouse immunoglobulin (IgG) (Dako Diagnostika, Hamburg, Germany), was used to localize proliferating cells. Biotinylated horse anti-mouse IgG (which was used as the secondary antibody), normal horse serum (which was used to reduce nonspecific binding) and an avidin-biotinylated-peroxidase complex (ABC) kit were purchased from Camon Labor-Service (Wiesbaden, Germany).

Prostate tissue

Normal prostates (NPR) were obtained from four organ donors 15–52 years old; mean age 30 years). The mean tissue weight was 19.5 g (range 9.5–33.5 g). These had been clearly identified by a pathologist as normal prostate tissues. Prostates with BPH removed by suprapubic prostatectomy were obtained from 20 men (43–94 years old; mean age 70 years). The mean tissue weight was 85 g (range 30–200 g).

Immediately after the prostates had been removed they were opened anteriorly and longitudinal and sagittal sections through the verumontanum were obtained. The frozen samples were mounted in Tissue-Tek OCT Compound (Miles, Ill.) on precooled cryostat chucks. Frozen sections (5 µm) were cut in an SLEE Model HR Mark II cryostat at −20 °C and thaw mounted onto subbed slides (0.5% gelatin, 0.05% chrome alum) [12].

Ki-67 antibody labelling

Indirect immunohistochemical antibody staining was performed using the monoclonal antibody Ki-67 and the standard ABC method according to Hsu et al. [8, 9]. All procedures were carried out at room temperature as follows:

1. After air drying for 5–10 min, cryostat sections were fixed in acetone for 10 min.

2. Sections were washed twice for 1 min in phosphate-buffered saline (PBS), pH 7.4.

3. Sections were placed in a moist chamber and flooded with 1% normal horse serum in PBS for 20 min.

4. Primary antibody Ki-67 was applied to the sections at 1:16 dilution in PBS containing 1% horse serum for 120 min. Control sections were treated with PBS instead of primary antibody.

5. Sections were washed twice in PBS for 3 min each time.

6. Secondary antibody, biotinylated horse anti-mouse IgG, was applied to sections at a concentration of 7.5 µg/ml PBS with 1% horse serum for 45 min.

7. Sections were washed twice in PBS for 3 min each time.

8. The avidin-biotinylated-peroxidase complex (ABC reagent), prepared according to the instructions supplied by the manufacturer was applied to the sections for 45 min.

9. Sections were washed twice in PBS for 3 min each time.

10. Sections were flooded with diaminobenzidine (0.5 mg/ml PBS) plus H_2O_2 (0.01%) and stored in the dark for 5 min. After washing in deionized water for 3 min the sections were counterstained with haematoxylin (for a few seconds) and then dehydrated with alcohol and cleared in xylene. Coverslips were mounted with DePex (Serva, Heidelberg, Germany).

Specific immunohistochemical staining of proliferating cells was defined as staining that was observed when the monoclonal antibody Ki-67 was used but that was absent when PBS was substituted for Ki-67.

Calculation of proliferation rates

Immunohistochemically stained proliferating cells were counted in an area of approximately 5 mm^2 (approximately 8000 nuclei) at a magnification of ×200 in each specimen for epithelium and stroma separately using a computer-assisted image analysis system (Quantimet 520, Cambridge Instruments, Cambridge, UK). For calculation of proliferation rates, in sequential sections stained with H&E, the total number of epithelial and stromal cells in an area of approximately 0.5 mm^2 at a magnification of ×400 was determined and the proliferation rates expressed as a proportion of the total cells.

Statistical analysis

Statistical significance of differences between the means was determined using Student's *t*-test, assuming a normal distribution of the values. Regression lines were calculated by the method of least squares. To study any correlations between data the Spearman rank correlation coefficient (R) within the bounds 2α was calculated. P and $2\alpha < 0.05$ were considered significant.

Results

Immunohistochemical localization of proliferating cells

Immunoreactive specific staining was observed only in sections treated with the primary antibody Ki-67 and not in the PBS-treated control sections. Background staining was completely absent. Immunostaining was exclusively in the nuclei of the cells and was localized predominantly in the periurethral and central zone of the prostates. Epithelial cells of the gland (Fig. 1) and the duct, as well as a few basal cells, were detectable using immunohistochemical methods. Stained stromal cells could be observed in inter- and periglandular locations.

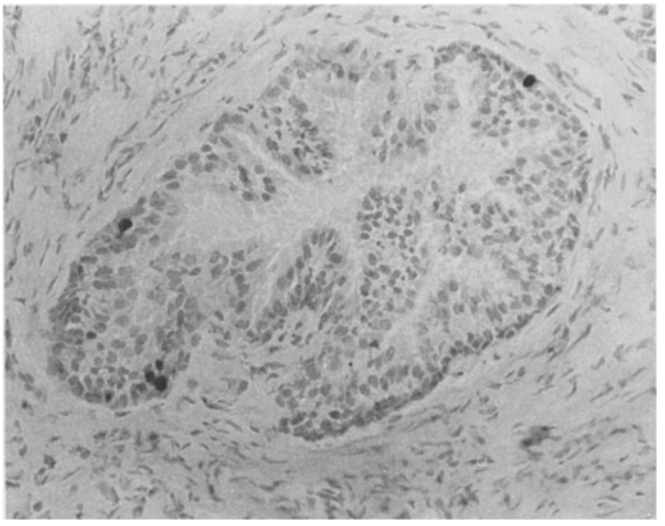

Fig. 1. Ki-67 immunostaining of epithelial nuclei in a prostate with benign prostatic hyperplasia (BPH). The darkly stained nuclei of the counterstained section are Ki-67 positive and the light nuclei are Ki-67 negative. ×200

Proliferation rate in benign hyperplastic prostates

The mean proliferation rate ± standard deviation (SD) in epithelium (0.132 ± 0.087%) was slightly higher than that in stroma (0.121 ± 0.083). The difference was not statistically significant.

To investigate age-related changes in growth the proliferation rates of epithelium and stroma in BPH were plotted against the patient's age. Using the Spearman rank correlation test it could be demonstrated that there was no significant correlation between proliferation rate and age in either epithelium ($R = -0.082$; $2\alpha = 0.731$) (Fig. 2a) or stroma ($R = -0.068$; $2\alpha = 0.774$) (Fig. 2b). The regression lines obtained give the impression of a nearly constant growth in prostate tissue throughout the life of patients with BPH.

In addition, no significant correlation between prostate weight and proliferation rate in epithelium ($R = 0.289$; $2\alpha = 0.217$) (Fig. 3a) or stroma ($R = 0.061$; $2\alpha = 0.800$) (Fig. 3b) in BPH could be demonstrated. However, the regression lines for epithelium (Fig. 3a) could indicate a possible weak correlation.

Proliferation rate in normal prostates

In NPR the mean proliferation rate ± SD in epithelium (0.015 ± 0.006%) and in stroma (0.003 ± 0.007%) was 9 times and 37 times lower, respectively thamin BPH (Table 1; Fig. 4).

Table 1. Mean proliferation rate (%) ± standard deviation in epithelium and stroma in NPR and BPH

Compartment	NPR ($n = 4$)	BPH ($n = 20$)
Epithelium	0.015 ± 0.006	0.132 ± 0.087
Stroma	0.003 ± 0.007	0.121 ± 0.083

NPR, normal prostate; *BPH*, benign prostatic hyperplasia

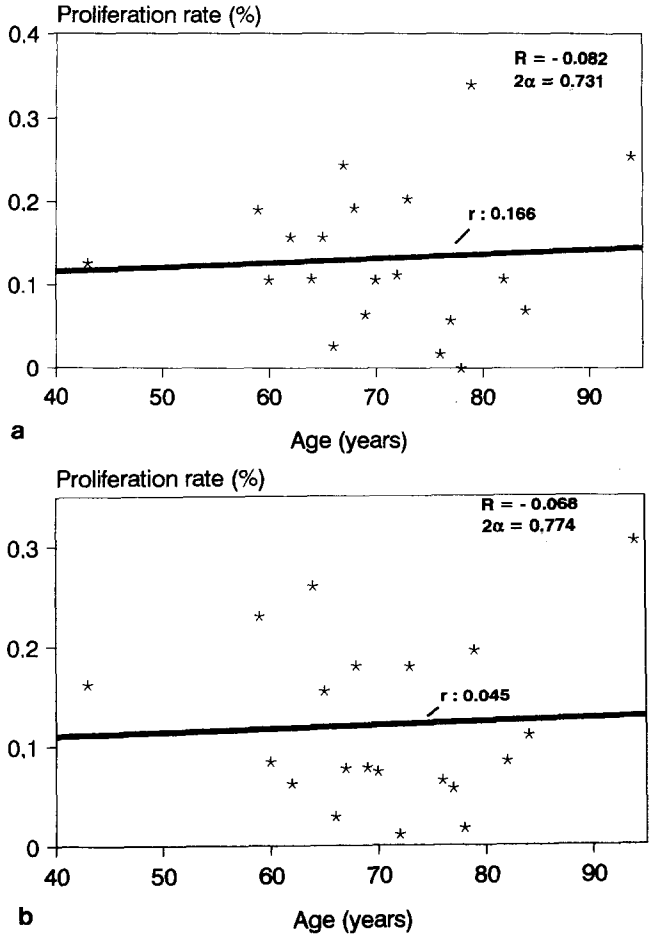

Fig. 2a, b. Correlation between patient's age and proliferation rate in epithelium (**a**) and stroma (**b**) in BPH. *R*, correlation coefficient; *2α*, significance; *r*, regression coefficient

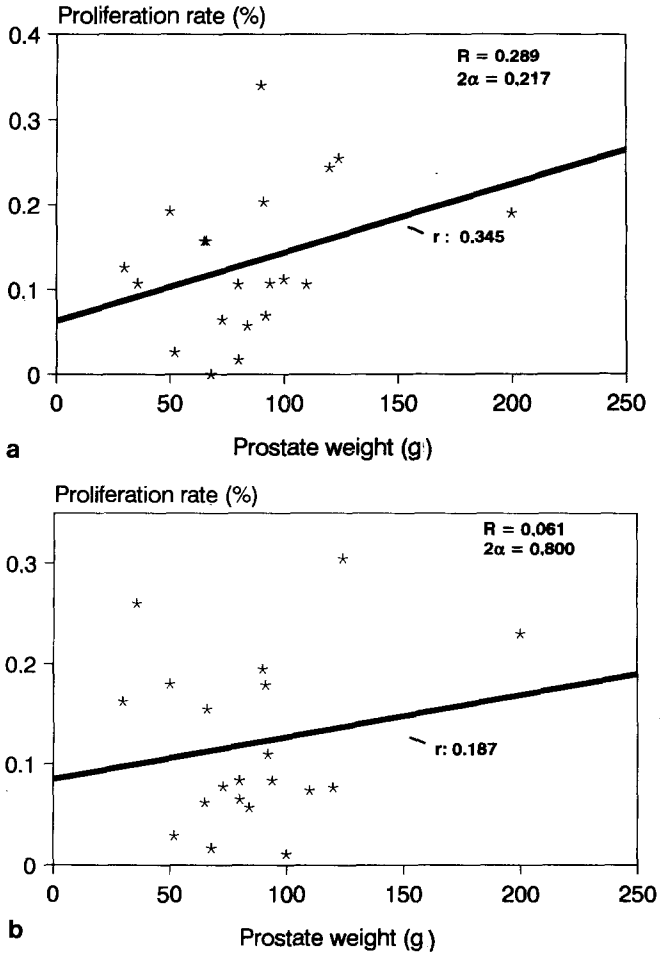

Fig. 3a, b. Correlation between prostate weight and proliferation rate in epithelium (**a**) and stroma (**b**) in BPH. *R*, correlation coefficient; *2α*, significance; *r*, regression coefficient

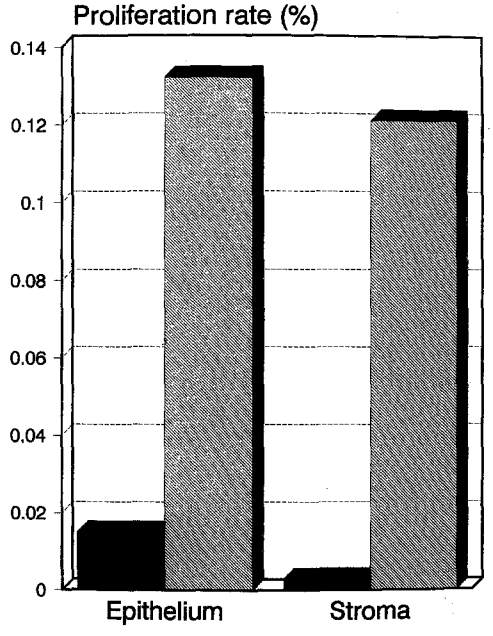

Fig. 4. Mean proliferation rates in epithelium and stroma in NPR (■) and BPH (▨). Values are shown in Table 1

The youngest organ donor (15 years old) had the highest proliferation rates of all normal prostates in both epithelium and stroma. But even these (probably puberty-associated) high proliferation rates were exceeded by the rates found in BPH, except for two cases in epithelium and one case in stroma.

Statistical comparison between NPR and BPH showed a significant difference ($P = 0.015$) in proliferation rate in epithelium. In stroma no statistical evaluation was possible, as in three of the four NPR samples no proliferating cells could be located at all.

Discussion

To the best of our knowledge immunohistochemical determinations of proliferation rates for epithelial and stromal BPH cells separately have never been reported before. Possible reasons are that stromal cells were considered negative for Ki-67 labelling [11] or that only epithelial cells were investigated [5].

Our findings revealed proliferating cells in epithelium as well as stroma. Nevertheless the proliferation rates obtained are not always in accordance with data reported by other authors. A mean Ki-67 index of about 1.4% for epithelium plus stroma was obtained by Oomens et al.

[14]. The range reported (0–3.8%) indicates that low proliferation rates, as found by us, were also present.

Another reason for the differences could be that proliferation rates reported by other authors were obtained by investigating tissue from biopsies [14] and transurethral resections [11], respectivity, which must have come from small-volume prostates. In our study we investigated BPH removed by suprapubic prostatectomy, the indication for which is the large volume of the gland. As there was no significant correlation between BPH weight and proliferation rate the enlargement of the prostate will result in lower relative proliferation rates, as the proliferating cells are more scattered.

Bonkhoff et al. [3] reported Ki-67 positivity in epithelial cells within a range of 0.5–1.7% in prostate ducts, 2.9–3.4% in post-atrophic hyperplasia, and 2.1–4% in basal cell hyperplasia. These high proliferation rates could result from a different calculation method. The subdivision of epithelial cells leads to a lower number of non-immunoreactive cells. Therefore the quotient of Ki-67-positive cells and the total number of cells could be higher as a consequence of a low total number of cells obtained.

Given that we used the same fixation method as other authors [5, 11, 14] – because fixation may influence the results [1] – the differences are otherwise difficult to explain. The proliferation rate determined in epithelium in our study was in better accordance with the values obtained by Gallee and co-workers [5]. They found that 0.3% of epithelial cell nuclei were reactive with Ki-67.

Poor immunoreactive cytoplasmic staining as described by other authors [5, 16] was not seen. The cytoplasm of some cells gave the appearance of being stained, though, because of the intense positive immunoreaction of the nuclei.

It was demonstrated that in BPH there was no significant correlation between proliferation rate and age in either epithelium or stroma. This result can be explained by the two-phase history of BPH postulated by Isaacs and Coffey [10]. The first, or pathological phase of BPH produces no symptomatic clinical dysuria. The second, or clinical phase of BPH involves the progression of pathological BPH to clinical BPH in which symptomatic dysuria develops in the patient. For this progession to occur clinical BPH prostatic enlargement is a necessary but not a sufficient condition. In our study we investigated only BPH with clinical symptoms, which occur in the second phase and which result from prostatic enlargement and additional factors (e.g. prostatitis, vascular infarct). Our data indicate that the onset of the second phase is different in individual patients, as there was no correlation between proliferation rate and age in BPH.

Enlargement of the prostate is associated with an increase in its weight, but there was no significant correlation in either epithelium or stroma between BPH weight and proliferation rate. Our data indicate that a large prostate volume is not always associated with a high proliferation rate.

In NPR there were much lower proliferation rates compared with BPH. The differences obtained were statistically significant in epithelium and even more distinct in stroma (37 fold, compared with ninefold in epithelium).

From these results the question arises as to whether proliferation plays a role in the development of BPH. Obviously the induction of BPH from NPR may be associated with a distinct increase in proliferation rate. In this phase the increase is even more pronounced in stroma than in epithelium, which is in accordance with the suggestion that BPH is a stromal disease [15]. However, our data indicate that the further increase of BPH volume in aging men is not correlated with a further increase in proliferation rate.

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