Subcellular Concentrations of Calcium, Zinc, and Magnesium in Benign Nodular Hyperplasia of the Human Prostate: X-Ray Microanalysis of Freeze-Dried Cryosections

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ABSTRACT
Biopsies from human prostates were obtained from normal and hyperplastic glands. The intracellular concentrations of calcium, zinc, and magnesium were analyzed using X-ray microanalysis of freeze-dried cryosections. Two prostate biopsies were obtained from kidney donors, ages 19 and 50 years, without any sign of benign nodular hyperplasia. The normal tissues were frozen within 15 min after circulatory arrest. The central part of biopsies from eight elderly men suffering from benign nodular hyperplasia were frozen within 30 s after excision. Adjacent tissue was processed for light microscopy and histopathological diagnosis. All samples were fresh-frozen using liquid nitrogen cooled pliers, without the use of any freeze-protection, fixation, or staining. In both the normal and the hyperplastic prostates high concentrations (up to above 100 mmol/kg dry weight) of zinc were present in electron dense bodies in the cytoplasm of the epithelial cells. Together with zinc, about equal concentrations of magnesium were found. Calcium was detected in 4 to 8 times the concentration of zinc. Significant, positive correlation between calcium and zinc as well as between calcium and magnesium in the cytoplasm was a typical finding in both normal and hyperplastic glands. In six of eight patients, older than 60 years of age, high levels of calcium (17.0–38.8 mmol/kg dry weight) were present in electron dense bodies in the cytoplasm of the epithelial cells. While very low values were found in the remaining two. In the two younger cases (19 and 50 years of age), the nuclear calcium level in prostatic epithelium was relatively low (about 10 mmol/kg dry weight). These observations suggest that an increase of intranuclear calcium with advancing age may be of pathogenetic significance to growth disturbances in the prostate.

INTRODUCTION
Zinc, a trace element essential to most cells, is present in high concentrations in the prostate, mainly located in the prostatic fluid and the epithelial cells lining the acini (1, 2). On the subcellular level information concerning elemental distributions is more scanty and conflicting. Analysis of subcellular fractions from human and rhesus monkey prostates has revealed high zinc concentrations in the nuclear sediments but also significant contents in the mitochondrial and microsomal fractions (3–5). Combined histochemistry and X-ray microanalysis has demonstrated zinc only in the secretory vacuoles within epithelial cells of human prostate (6). In the lateral prostate of the rat and in the dog prostate, zinc was also found within the nuclei and the lysosomes when the pyroantimonate method was combined with crystal spectrometer X-ray microanalysis (7,8).

Intracellular zinc concentrations of the prostate are particularly interesting because of a possible relationship to growth disturbances with advancing age. Several workers have found tissue zinc concentrations to be increased in hyperplasia and decreased in carcinoma of the human prostate (1,9–11). Recently, tissue zinc concentrations have been correlated to the degree of differentiation in prostatic tumors (12).

However, other divalent ions, e.g., calcium and magnesium, are also secreted into the prostatic fluid. Compared to zinc and magnesium, calcium shows about 4 times higher concentration (2,13). This element, believed to be an important intracellular messenger (14), has recently also been associated with the regulation of cell proliferation (15). Furthermore, it is known that zinc will strongly antagonize calcium in some biological systems (16, 17). Thus, the interrelation of zinc and calcium may be of importance for normal growth and function of several accessory reproductive organs.

The discrepancies in the results reported earlier may be due to various factors. If the zinc in the gland exists, at least partly, as soluble compounds, different preparation procedures may affect the zinc distribution to a variable extent. Secondly, there seem to exist biological differences among the species (13,18,19). Consequently, animal models may be of limited value in the study of human prostatic hyperplasia and neoplasia.

X-ray microanalysis of freeze-dried cryosections represents a new approach in the study of diffusible elements and may provide insight into the role of ions and elements in normal and pathological processes in the cell (20). When freeze-dried cryosections are prepared from fresh frozen tissue samples, energy dispersive microanalysis can reveal local elemental concentrations of several elements simultaneously. Furthermore, freeze-fixation and freeze-drying preserve the concentration gradients within the specimen.

Growth disturbances of the prostate are evident at the microscopic level in most elderly men. Specimens to be used as controls should ideally be the prostates from young men. However, quickly frozen normal prostate from a young man is rarely obtainable. In this report analytical results from old and young human prostates are presented for the first time.

MATERIALS AND METHODS
Tissue Preparation. Prostatic tissue samples were obtained from 10 patients admitted to The Department of Urology, Regionsykehuset i Trondheim. Normal prostatic tissue was collected from two brain-dead kidney donors, 19 and 50 years of age. Ten transrectal needle biopsies were taken from prostatic glands suspected to be malignant. Three of these 10, which showed no sign of malignancy but had fully developed BPH,2 were included in the study. Five needle biopsies were obtained during open prostatectomy, before enucleation.

Excised tissues from normal prostates were frozen by quickly clamping the specimen with a pair of pliers that had been precooled in liquid nitrogen. The time lapse between the circulatory arrest and the freezing of the specimens was about 15 min. Histopathological examination was performed on adjacent tissue samples. The biopsies from elderly patients (60–87 years old; mean, 70.4 years) were derived using Tru Cut disposable biopsy needles with a 20-mm specimen notch. A 2-mm central part of each tissue cylinder was excised, transferred to a Formvar film, and frozen by quickly clamping the specimen with a pair of pliers. The method is described in detail elsewhere.4 The freezing of the needle

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3 The abbreviation used is: BPH, benign nodular hyperplasia.
biopsies was accomplished within 30 s after excision. The remaining parts of the tissue cylinder were studied in order to confirm the histopathological diagnosis in the area immediately adjacent to the frozen specimen.

**Sectioning.** Frozen thin sections (0.35 µm) were cut on a Reichert-Jung Ultratoc/FCU cryosystem. All specimens were sectioned with glass knives without prior trimming. Specimen and knife stage temperatures were kept at about 150°K (panel meter readings). The temperature in the chamber close to the knife edge was about 140°K (thermocouple measurement).

The dry-cut sections were transferred to beryllium grids which had been glued on carbon retainers mounted in a transportable press situated close to the knife (21). Between the sections, 100 µm were trimmed off the specimen. The sections were pressed between two retainers and transferred in a cold atmosphere to a vacuum freeze-drier.

**Freeze-Drying.** The freeze-dried grid was desiccated and extesively flushed with cold nitrogen gas before and during the installation of the press. The specimen table was heated from 170°K to 190°K at 0.1 Pa during a period of 60 min. This temperature was maintained for 4 h and then gradually raised to room temperature over a period of 1.5 to 2 h. The sections were stored under vacuum until examination in the microscope.

**X-Ray Microanalysis.** Analysis was performed with a Kevev 7000 energy dispersive spectrometer in combination with JEOI 100 CX electron microscope with scanning attachment. The probe current was allowed to reach a stable level (usually 1–2.5 nA after 1 h) and control measurements were also performed during and after an analyzing sequence. The background was constructed using an automatized background modeling routine with preset modeling regions. From standard spectra correction factors were calculated in order to subtract K/β counts in the CaKα window and CuKβ in ZnKα. The total counts in the peak free 4.6–6.0 keV region of the spectra were measured. The background contribution from the specimen (W_b) was calculated as

$$W_b = N_t - N_f - (P_1 - P_2) \cdot r - W_f$$

where N_t is the total background measured on the section, N_f is the total background measured on the film, P_1 and P_2 are the copper net peaks originating from specimen and film, respectively, and r is the background:peak ratio for a pure copper standard (22). The amount of background from the beryllium-carbon support, W_f, was calculated from analyses of standard sections with known peak:background ratios. These ratios had been obtained from the standard sections on a nickel grid.

From spectra obtained by analysis of freeze-dried sections with known elemental compositions, the peak:background ratio, (P_1 - h)/W_b, for each element x, was calculated. The unknown concentrations in the specimen (sp) were calculated by the expression given by Hall et al. (23):

$$C_{x,sp} = C_{x,stan} \cdot (R_{x,sp}/R_{x,stan}) \cdot (Z_{x}^2/A_{x})(Z_{x}^2/A_{x})_{stan}$$

where C_x is the concentration (mass fraction) and R_x is the relative peak intensity of element x in the cell (sp) or standard (stan). Z is the atomic number and A is the atomic weight. Z^2/A is the average value of Z^2/A for all elements present in the specimen, weighted according to mass fraction.

**Analysis of the Nucleus.** In acini where distinct concentrations of zinc were present in the cytoplasm, the nuclei of all cells were analyzed by scanning a 0.5–1 µm^2 area covering several ice crystal cavities. All epithelial cells in the selected section were analyzed, except those closer to the grid bar than 20 µm. One analysis was performed in each cell with a well defined nucleus. The total number of analyses from each patient was never below 30, comprising two or more acini.

**Random Analysis in the Cytoplasm.** In order to evaluate statistically the correlation between calcium, zinc, and magnesium in the cytoplasm, some analyses were performed according to a separate protocol. The best section on the grid was chosen, and cells which could be clearly recognized as epithelial and had a well defined cell border were included in the study. All intracellular electron dense bodies, recognizable at X4000, were preselected for analysis. At X20,000, these spots were identified on the STEM display and analyzed with a focused beam, in the center of the body, and at 0.5, 0.7, and 1.1 µm distances from the center, respectively, according to a predrawn grid on the display.

**RESULTS**

With the precooled pair of pliers, freezing of needle biopsies was easily performed within 30 s. The light microscopic sections of the two pieces of tissue on each side of the frozen central part were used to establish the histopathological diagnosis as well as to guide the selection of the most suitable specimen whenever more than one biopsy was taken.

A great number of sections could be produced from each frozen tissue specimen. In the specimens from the hyperplastic glands, an epithelial component was present in less than 20% of the sections. The freeze-dried cryosections showed distinct ice crystal artifacts, particularly in the nuclei, the stroma, and the intraluminal secretion (Fig. 1A). Electron dense bodies were abundant in the cytoplasm, particularly in the supranuclear region (Fig. 1B). Inside such bodies, smaller, more electron dense spots were often present. Occasionally, cells with much larger and more electron dense compartments in the cytoplasm were found. The latter contained large amounts of sulfur. Variable amounts of zinc were present in the cytoplasm of the epithelial cells. Often minor compartments with high local concentrations (above 100 mmol/kg dry weight) of this element were clearly more electron dense than the adjacent cytoplasm. Magnesium and calcium were also present in the electron dense bodies (Fig. 2). The statistical analyses revealed a significant positive correlation between calcium and zinc (Fig. 3) and between calcium and magnesium (Fig. 4). The concentrations of magnesium were about equal to the concentrations of zinc, while calcium was found in concentrations 4 to 8 times those of zinc and magnesium. Commonly, local calcium concentrations above 700 mmol/kg dry weight were found. This intracytoplasmic elemental distribution was typical of all prostates, and no difference between old and young patients could be demonstrated.

Analyses of standard sections containing potassium at an intracellular concentration level showed that the potassium Kβ peak completely covered the calcium Kα peak when calcium concentrations were below about 10 mmol/kg dry weight, visually evaluated (Fig. 2). However, from statistical evaluation of several sets of standard sections the potassium Kα:potassium Kβ ratio was determined. This factor was used to subtract the potassium Kβ contribution from the total counts recorded in the calcium window. Analyses of standard sections containing potassium at concentrations close to intracellular levels showed that with the calculated factor the analyzed mean calcium concentration was in good agreement with the concentration as determined by the quantity of salt added (Fig. 5).

The mean intranuclear calcium concentrations of the epithelial cells from the normal prostates were 10.0 ± 1.7 (SE) and 11.1 ± 1.9 mmol/kg dry weight, respectively (Fig. 6). In six of eight biopsies from patients with BNH, the mean intranuclear calcium concentrations ranged from 17.0 to 38.8 mmol/kg dry weight. Very low mean intranuclear calcium concentrations were present in two biopsies showing BNH, at 1.8 ± 1.0 and 1.9 ± 2.0 mmol/kg dry weight, respectively. The mean intranuclear zinc concentrations of the epithelial cells from normal prostates were 11.1 ± 1.7 and 7.2 ± 1.2 mmol/kg dry weight,
respectively. In four of eight biopsies showing BNH, the mean intranuclear zinc concentration was higher, ranging from 12.6 to 16.5 mmol/kg dry weight. The remaining four revealed mean zinc concentrations between 3.1 and 9.1 mmol/kg dry weight. The mean intranuclear magnesium concentration ranged from 22.2 to 61.3 mmol/kg dry weight.

Analysis of variance showed that the differences in mean nuclear calcium among individuals were significantly larger than what might be expected from the variation among single analyses within individuals (P < 0.005). The same was found for zinc and magnesium. Thus, there are significant differences between individuals with respect to the concentrations of nuclear calcium, zinc, and magnesium.

There was an apparent overall increase in the level of intranuclear calcium with increasing age of the patients, the coefficients of correlation being 0.52 (Pearson) and 0.55 (Spearman). However, these correlations were not statistically significant at the chosen level (P = 0.1 when tested against a two-sided alternative).

DISCUSSION

Benign nodular hyperplasia of the human prostate is frequently encountered after the age of 40 years (24). Although endocrine factors are believed to play a significant role, it seems unlikely that age associated variations of hormones represent the only factor in the pathogenesis of BNH (25). The cellular response to endocrine factors is not known on a molecular level.

Spontaneous development of prostatic hyperplasia is unique to humans and dogs, but the hyperplasia encountered in the dog may appear in younger individuals and is also of a cystic type (18). In addition, the unusual exocytic action recently demonstrated in the human prostate (13, 19) and our novel analytical data rather suggest that some aspects of the prostate physiology may also be unique to humans. It therefore seems that adequate control specimens for the human type of prostatic hyperplasia hardly exist apart from the prostates of younger men.

The freezing method used in this study allowed an almost instant freezing (within 30 s) which introduced smaller ice crystal artifacts compared to other methods tested in our laboratory, e.g., Freon 22 or nitrogen slush. The ice crystal artifacts are still obvious in the nuclei (Fig. 2) and much greater than we have obtained with liver tissues. This implies that the epithelial cells of the prostate contain more water available for crystallization than liver cells. The ice crystal artifacts are, however, an important indication of proper freeze-drying. They confirm that the phase separation introduced by freezing has been preserved through the freeze-drying procedure and during the transportation of the sections to the electron microscope.

Due to the low epithelium:stroma ratio in hyperplastic glands a great number of sections will contain no epithelial cells. Particularly in these cases, the freezing method used was advantageous, because the number of sections which could be produced from each frozen specimen was much increased compared to other freezing methods.

The finding of high local zinc concentrations in the cytoplasm of all the human prostates examined in this study supports the
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Fig. 2. Typical X-ray spectra of an epithelial nucleus and an intracytoplasmic dense body of normal and hyperplastic prostate. The copper peak is due to stray signals from the instrument, while silicon, present in some spectra, is due to contamination. A, nucleus of normal epithelial cell. B, dense body in normal epithelial cytoplasm. Note the high calcium, zinc, and magnesium peaks compared to the nuclei. C, epithelial nucleus of BNH. Note the broadening of the potassium Kβ peak. The calcium concentration was in this case calculated to be 17 mmol/kg dry weight. D, dense body in epithelial cytoplasm of BNH showing high calcium, zinc, and magnesium peaks.

Fig. 3. Scatterplot, showing correlation between calcium and zinc concentrations in the cytoplasm of a typical epithelial cell. d.w., dry weight.

Fig. 4. Scatterplot, showing the correlation between calcium and magnesium concentrations in the cytoplasm of a typical epithelial cell. d.w., dry weight.

observations previously reported from a combined histochemical and X-ray microanalytical study (6). However, we also recorded significant zinc concentrations in the nucleus. The reason for this discrepancy is most probably the fact that freeze-drying increases elemental concentrations 4–5 times compared to embedded specimens where water is replaced by resin. Furthermore, when properly carried out, freeze-drying also prevents redistribution and loss of elements during preparation. A strong positive correlation between zinc, magnesium, and cal-
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The concentrations of calcium calculated from the amount of salt added are plotted versus the concentrations as determined by X-ray microanalysis. The six points represent different standard sections (40 analyses in each). The concentrations of potassium from left to right are 711, 573, 752, 575, 196, and 48 mmol/kg dry weight (d.w.), respectively. r, coefficient of correlation.

Fig. 5. Results obtained with the procedure used for analyzing calcium. The concentrations of calcium in the nuclei of prostatic epithelial cells sequestrate a substantial quantity of calcium. Secreting epithelial cells of the human prostate suggest their prostatic origin (26). The high local calcium concentrations demonstrated in this study confirm that the human prostatic epithelial cells contain different zinc concentrations (1).

On the other hand, two of the patients showed very low intracellular zinc and magnesium. Consequently, redistribution of calcium as a result of improper preparation seems unlikely. The significance of increased intranuclear calcium concentrations in the majority of prostatic epithelial nuclei associated with advancing age and the development of BNH is uncertain.

An increase of intracellular free calcium has been suggested as a universal stimulus of cell growth (31). Calcium may mediate effects (e.g., through calcium calmodulin) on several important cellular functions (14). Zinc, on the other hand, may compete with the same binding sites, and often with antagonistic effects (16, 17). In view of this, it is interesting to notice that BNH and cancer develop in different lobes of the human prostate containing different zinc concentrations (1).

An elevation of free calcium in the aging prostate can be neither confirmed nor disproved through X-ray microanalysis with detectors currently in use because these do not distinguish between different binding states of the elements. However, a possible elevation of free calcium in the cytosol might mediate ionic diffusion into the nucleus where high affinity binding sites for divalent cations are present. X-ray microanalysis of mitotic chromatin in rapidly proliferating enterocytes has demonstrated calcium concentrations above 80 mmol/kg dry weight (28), and it has been suggested that calcium may be involved in chromosome condensation (32). It is not unlikely, therefore, that high intranuclear calcium in the human prostate may also be related to growth disturbances.

From the present study, it seems reasonable to conclude that in addition to zinc, calcium may also participate in the pathogenesis of growth disturbances in the aging prostate.

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