

Rapid Proliferation of Prostatic Epithelial Cells in Spontaneously Hypertensive Rats: A Model of Spontaneous Hypertension and Prostate Hyperplasia

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ABSTRACT: Spontaneously hypertensive rats (SHRs), a commonly used model of genetic hypertension, exhibit features of glandular hyperplasia of the ventral prostate, including the narrowing of acini with epithelial protrusions into the lumen and the piling up of epithelial cells. These rats also have frequent urinary voiding. In order to define the fundamental processes that lead to prostatic hyperplasia in SHRs, we compared the proliferation rate of their prostatic epithelial cells (PECs) in primary culture and in vivo to that of Wistar-Kyoto rats (WKYs), their normotensive controls. In vitro, primary cultures of SHR PECs had a shorter doubling time than those of WKY (3.3 vs 8.0 days) and showed higher levels of bromodeoxyuridine

(BrdU) incorporation into DNA. In vivo, the BrdU incorporation seen 48 hours following injection was observed primarily in areas of epithelial piling up, which are seen in SHRs but not in WKYs. We concluded that prostate hyperplasia in SHRs results from a genuine increase in the proliferation rate of PECs and that this rapid proliferation is a fundamental feature of SHR PECs, maintained both in vivo and in vitro. Thus, SHRs can serve as a model for glandular hyperplasia of the prostate, resulting from a genetic tendency for an increased rate of cell proliferation.

Key words: Bromodeoxyuridine, cell culture, Alamar Blue, growth. *J Androl* 2003;24:263–269

During the past decades, the increase in life expectancy has enhanced the incidence of 2 unrelated abnormalities in the growth of prostatic epithelial cells (PECs): benign prostate hyperplasia and prostate cancer. Much scientific effort has been directed toward the study of the regulation of prostate epithelial and stromal cell growth. It is clear that the growth and function of the prostatic epithelium depend upon endocrine, paracrine, autocrine, and neural factors (Peehl, 1996; Golomb et al, 1998; Foster, 2000). However, it is highly likely that there are endogenous genetic factors in cells that determine their growth rate and responsiveness to growth stimuli. Such factors might significantly modulate susceptibility to the development of glandular prostate hyperplasia. An appropriate experimental model to identify the intrinsic cellular factors that promote enhanced growth of prostate epithelium would be an animal that develops spontaneous glandular prostatic hyperplasia without any treatment and whose PECs show enhanced proliferation both in vivo and in vitro.

Spontaneously hypertensive rats (SHRs), a commonly used model of genetic hypertension, have been found to

exhibit hyperplastic morphological abnormalities in the ventral prostate that progress with age (Nakamura et al, 1991; Furukawa et al, 1994; Golomb et al, 2000). These consist of the narrowing of the acinar lumen and papillary protrusions composed of tall columnar epithelial cells. SHRs also show disturbances in bladder innervation and frequent urination (Clemow et al, 1998). The occurrence of glandular prostatic hyperplasia in the SHR, which is absent in its original normotensive strain, the Wistar-Kyoto rat (WKY), suggests that the SHR is an appropriate model to study the genetic component of prostate hyperplasia.

The etiology of prostatic hyperplasia in this model is unclear. Prostatic hyperplasia in SHRs does not result from the characteristic hypertension in this model: treatment with the calcium antagonist efonidipine normalized blood pressure in these rats but did not affect their prostatic abnormalities (Furukawa et al, 1994). Since SHRs are known to exhibit increased sympathetic activity (Julius, 1996), excessive adrenergic stimuli have been implicated in the development of prostatic hyperplasia in SHRs. This suggestion is supported by the finding that alpha-adrenergic inhibitors attenuate SHR prostatic abnormalities at an early age (Nakamura and Itakura, 1992).

Another potential explanation for prostate hyperplasia in SHRs involves a higher rate of proliferation of prostatic cells. Different cells derived from SHRs have been found to proliferate in primary culture more rapidly than those

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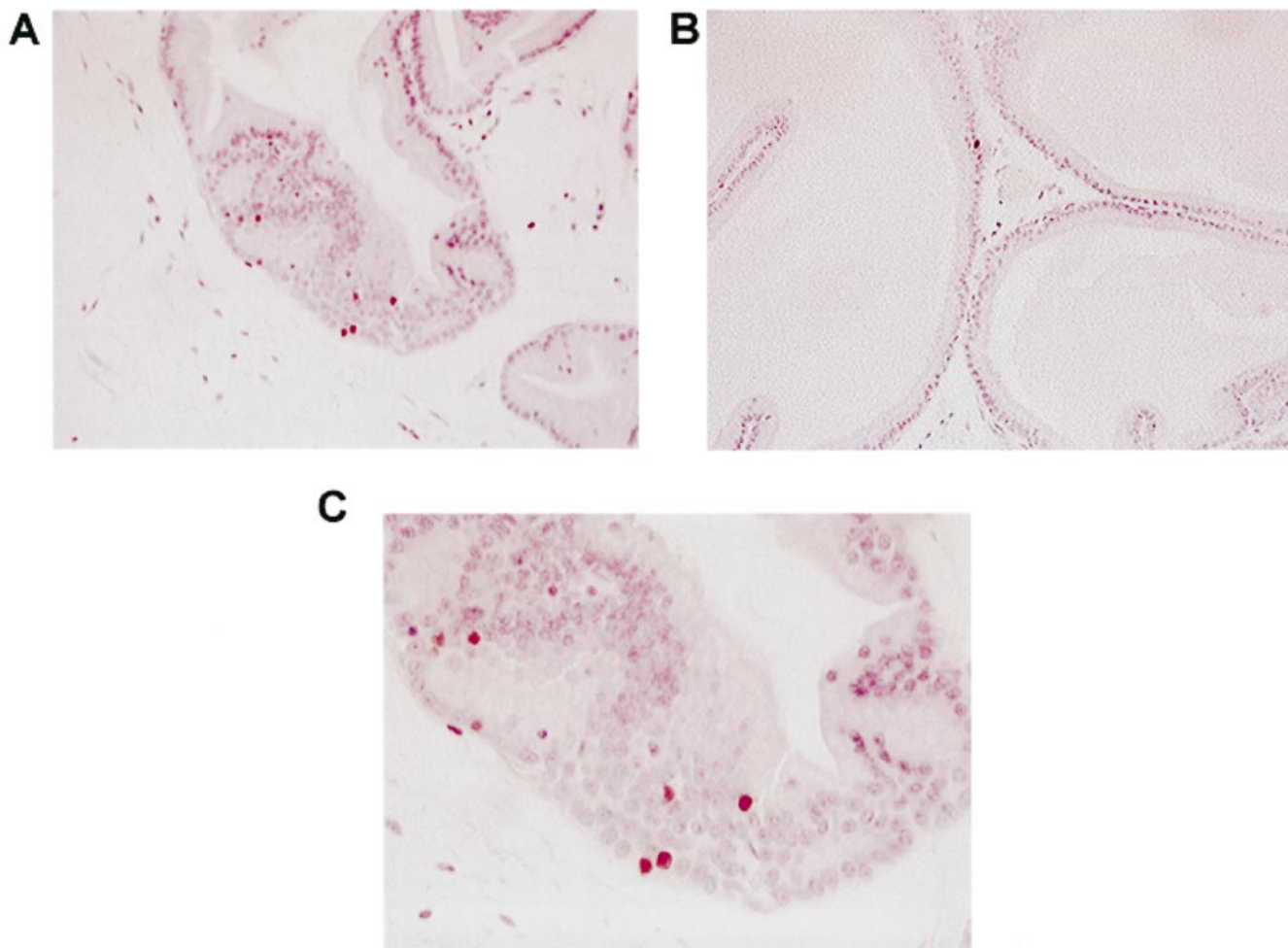


Figure 1. Bromodeoxyuridine (BrdU) labeling of prostatic epithelial cells in spontaneously hypertensive rats (SHRs) at (A) 100 \times and (C) 400 \times and in Wistar-Kyoto rats (WKYs) at (B) 100 \times . BrdU was injected intraperitoneally at 50 mg/kg and 10 mg/mL; 48 hours later, the prostate was excised and immunostained with anti-BrdU antibody.

derived from their normotensive counterpart strain, WKYs (Scott-Burden et al, 1988; Guicheney et al, 1991; Golomb et al, 1994). These include smooth muscle cells and fibroblasts from different tissue origins. To the best of our knowledge, the growth rate of epithelial cells has not been reported to differ between SHRs and WKYs.

The aim of the present study was to examine whether the PECs of SHRs exhibit genuine hyperplasia (ie, whether the morphological abnormalities in their ventral prostate are associated with an increased rate of proliferation and whether such a tendency for increased proliferation is maintained *in vitro* in a cellular environment that is independent of the local, endocrine, and neural control mechanisms operating in the whole organism). For this purpose, we explored whether DNA synthesis *in vivo* is enhanced in SHRs, compared to WKYs, by comparing *in situ* bromodeoxyuridine (BrdU) incorporation. We then cultured primary epithelial cells from the ventral prostate of SHRs and WKYs and compared cell numbers, both by

counting and by Alamar Blue staining, and the rate of DNA synthesis by BrdU incorporation.

Methods

Animals

Male, 13- to 14-week-old SHRs and WKYs ($n = 10$ per experiment for each strain) were used. They were housed in plastic cages, with free access to standard rat chow pellets and tap water, in rooms with standard controlled humidity and temperature (24 $^{\circ}$) and a 12-hour light-dark cycle. Rats were sacrificed by a lethal dose of chloral hydrate intraperitoneally (IP).

BrdU Incorporation *In Vivo*

5-Bromo-2-deoxyuridine (Sigma Chemical Company, St Louis, Mo) was injected at 50 mg/kg and 10 mg/mL IP. Forty-eight hours later, the rats were sacrificed, and the prostate was excised and fixed in a 4% formaldehyde solution in phosphate-buffered saline (PBS). The tissue was then paraffin embedded and cut

into serial 6- μ m-thick sections. Consecutive sections were stained with hematoxylin-eosin (H&E), immunostained for BrdU, and counterstained with hematoxylin. A standard immunostaining protocol was used consisting of deparaffinization, dehydration, quenching of endogenous peroxidase activity with 0.3% H₂O₂, rehydration, washes with trypsin in PBS, exposure to HCl 2 N, and washes with PBS. The tissue sample was then blocked with rabbit serum in a humid chamber, washed with PBS, and exposed to the anti-BrdU antibody (1:250; Dako Corporation, Copenhagen, Denmark) for 1 hour at 37°C. Biotinylated rabbit anti-mouse immunoglobulins (Dako) were added for a 30-minute incubation period, followed by a 20-minute incubation with peroxidase-labeled streptavidin (Dako). The procedure was terminated by the addition of 3-amino-9-ethylcarbazole (Zymed, San Francisco, Calif) for 5 minutes at room temperature, which produced a red-brown-colored deposit upon reaction with peroxidase.

TUNEL Assay

The TUNEL assay was performed using a commercial kit (ApopTag, Oncor, Gaithersburg, Md) according to the manufacturer's instructions. Methyl green was used for counterstaining. The TUNEL-stained specimens were thoroughly scanned for apoptotic nuclei. Prostate tissue sections from castrated rats served as a positive control for the assay.

Culturing of Ventral Prostate Epithelial Cells

The preparation of cultured ventral prostate epithelial cells was performed using slight modifications of the methods described by McKeehan et al (1984, 1987) and Ilio et al (1995). For each culture experiment, prostates from 13- to 14-week-old male SHRs and WKYs (10 rats of each strain per experiment) were removed aseptically. The ventral lobes were carefully dissected, washed with PBS, and placed in RPMI-1640 (GIBCO, Paisley, Scotland). The harvested ventral prostates were minced into 1-mm³ pieces and then placed in 1 mM dithiothreitol (Sigma) in PBS for 30 minutes in a 37°C rotating bath. After a brief settlement period, the tissue pellet was exposed to 0.3% DNase type I/0.1% collagenase type I (Sigma) in RPMI-1640 plus 10% fetal calf serum (FCS; Biological Industries, Bet Haemek, Israel) for 30 minutes in a rotating bath. Then, the suspension was passed through a 75- μ m nylon mesh. The tissue pieces that did not dissociate were subjected to another treatment with the enzyme solution as described. Then, the cell suspensions were combined, washed, resuspended in RPMI-1640 with 10% FCS, and counted as described below. Cell death was determined by trypan blue staining (Biological Industries).

The isolated prostatic cells consisted of a mixed cell population of stromal (mostly fibroblasts) and epithelial cells. Aliquots (2 mL) of the mixed cell suspension (200 000 cells/mL) were seeded onto 35-mm petri dishes (Corning, Ithaca, NY). Twenty-four hours after seeding the cells, the supernatant was removed and centrifuged, and the cell pellet was suspended in WAJC-404 medium (prepared as a custom order by Biological Industries) containing additives and 10% FCS (McKeehan et al, 1984, 1987; Ilio et al, 1995). The additives included insulin (5 μ g/mL), transferrin (5 μ g/mL), selenious acid, penicillin, streptomycin, amphotecrin (Biological Industries), 5 μ g/L of epidermal growth

factor, and 50 mg/L of bovine pituitary extract (GIBCO). Since stromal cells attach faster than epithelial cells in RPMI-1640 plus 10% FCS, this procedure separated the epithelial cells from the fibroblasts. The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. The epithelial cells were then seeded at a concentration of 30 000 to 40 000/well in 1 mL of media into 24-well plates or petri dishes for a 48-hour incubation. At this stage, the epithelial cells have attached to the plate surface and have been treated according to the assay protocol. Media were changed to WAJC-404 plus 5% or 2.5% FCS.

To verify that the cells obtained by this procedure were indeed PECs and to further characterize them, the cells were stained with antibodies against cytokeratins 8 and 18, characteristic of prostatic luminal cells. For this purpose, the cells were grown in the petri dish on a coverslip and then fixed in cold methanol for 10 minutes at 5°C, followed by 10 minutes with cold acetone at 5°C. Following blocking of nonspecific binding sites by normal goat serum, primary antibodies against cytokeratins 8 and 18 diluted 1:50 (Chemicon, Temecula, Calif) were added to the specimen for 2 hours. The secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa) was used for 2 hours at room temperature. Negative control slides were processed in an identical manner without the primary antibody. Results were analyzed by Confocal microscopy (Model LSM310, Zeiss, Oberkochen, Germany).

Cell Proliferation Assays

Cell counts were carried out using different approaches: cell counting, the Alamar Blue assay, and BrdU incorporation tests.

Cell counts were performed using a hemocytometer (Haussen, Berlin, Germany). Cell viability was determined by the trypan blue (Biological Industries) exclusion test. The Alamar Blue assay was performed using a slight modification of the method described by de Fries and Mitsuhashi (1995). Alamar Blue (10% culture volume, Serotec, Oxon, United Kingdom) was applied for an incubation period of 3 hours. Fluorescence excitation wavelength was measured at 560 nm, and emission wavelength was measured at 590 nm.

Assays for BrdU included both immunostaining (and the subsequent counting of positive nuclei—500 cells counted per sample) and a colorimetric assay performed in 96-well plates. Both were performed using specific kits (Roche, Indianapolis, Ind), according to the instructions of the manufacturer.

Results

Prostate Hyperplasia in SHRs

SHRs showed morphological features of glandular hyperplasia of the ventral prostate, characterized by a piling up of epithelial cells, with papillary formations, as reported earlier (Nakamura et al, 1991; Furukawa et al, 1994; Golomb et al, 2000). Prostate weights did not significantly differ between SHRs and WKYs and even tended to be slightly lower in SHRs than in WKYs (793 \pm 36 vs 827 \pm 35 total wet prostate weight and 517 \pm 12

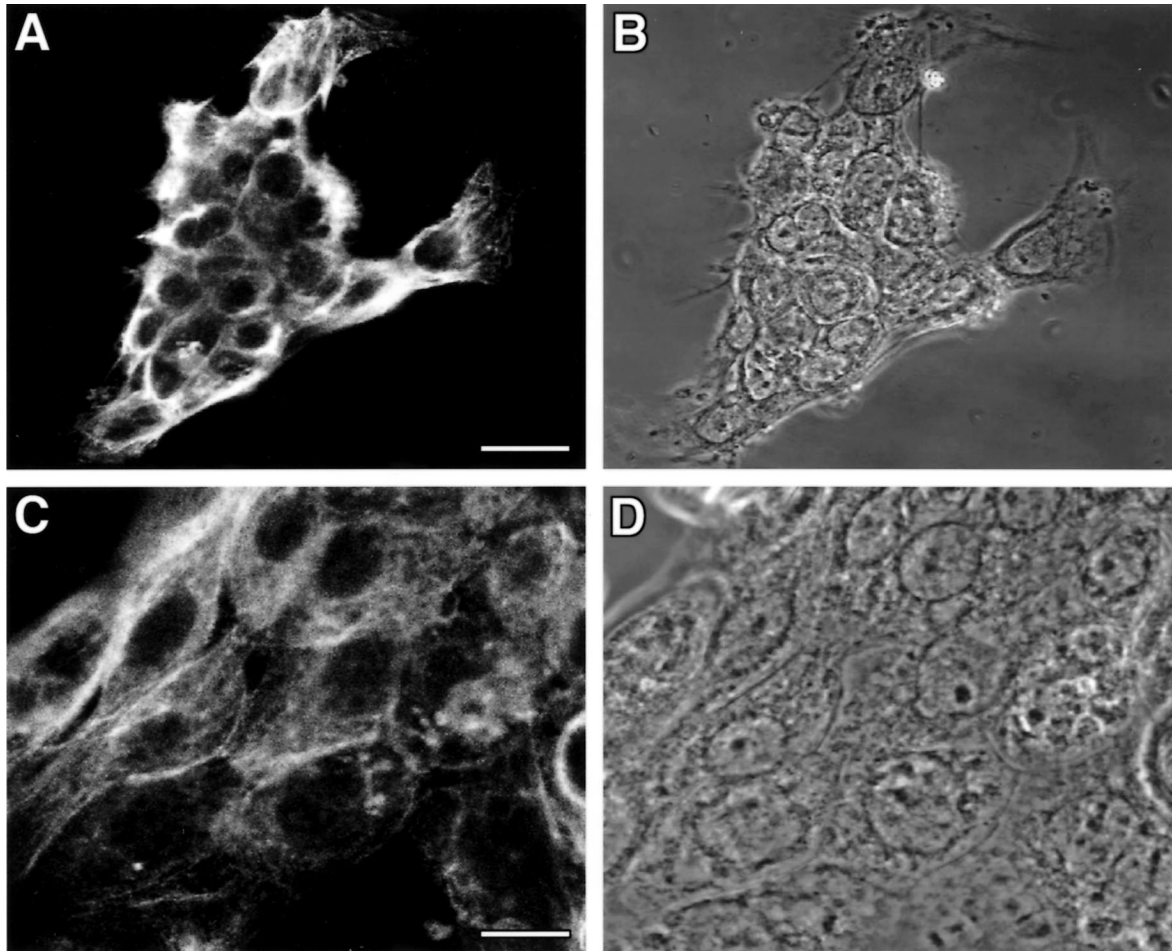


Figure 2. Fluorescent (A, C) and Nomarski (B, D) images of cultured prostatic epithelial cells, immunostained with antibodies against cytokeratins 8 and 18. The cells in this photo were derived from spontaneously hypertensive rats (SHRs). Scale bar = 25 μm in (A) and 8 μm in (C).

vs 526 ± 9 wet weight of ventral prostate of SHRs and WKYs, respectively).

BrdU-Labeled Cells in the Prostate

BrdU-labeled nuclei of epithelial cells were present in all prostatic specimens. The percentages of labeled cells were 0.38% plus or minus 0.12% in SHRs (range, 0.2%–0.7%) and 0.14% plus or minus 0.03% in WKYs (range, 0.10%–0.24%) ($P < .05$ by chi-square analysis, mean \pm SD). In the dorsal prostate, labeled nuclei were more abundant, and the percentage of BrdU-labeled cells did not differ between the strains: 1.2% plus or minus 0.3% in WKYs and 1.4% plus or minus 0.5% in SHRs. There were more BrdU-labeled cells in areas of infolding into the lumen in SHRs than in WKYs; there was also a piling of epithelial cells, which is more common in the ventral prostate of SHRs than in that of WKYs (Figure 1).

TUNEL Assay

The TUNEL assay did not reveal significant apoptotic endonuclease activity in either strain. TUNEL-positive

nuclei were not a significant finding in any of the specimens. Occasional single TUNEL-positive nuclei were encountered in both strains, but none of the specimens contained more than 1 TUNEL-positive nucleus. A positive control of prostate from castrated rats (3 days after orchidectomy) showed 13% TUNEL-positive cells.

Proliferation of Primary Cultured PECs

The adoption of the method for culturing rat PECs described by Ilio et al (1995) yielded a culture of cells with the typical appearance of epithelial cells, which were positively stained for cytokeratins 8 and 18, characteristic of luminal cells, in both strains. Examples of the cellular appearance and the cytokeratin immunostaining of SHR PECs are shown in Figure 2.

The time course of the growth, as expressed in cell number in live cultured PECs from SHRs and WKYs, is presented in Figure 3. Between days 2 and 6 in vitro, growth rate was maximal. At this interval, the doubling time of SHR PECs was 4 days (vs 9 days in WKY PECs). The percentage of dead cells in both cultures was similar

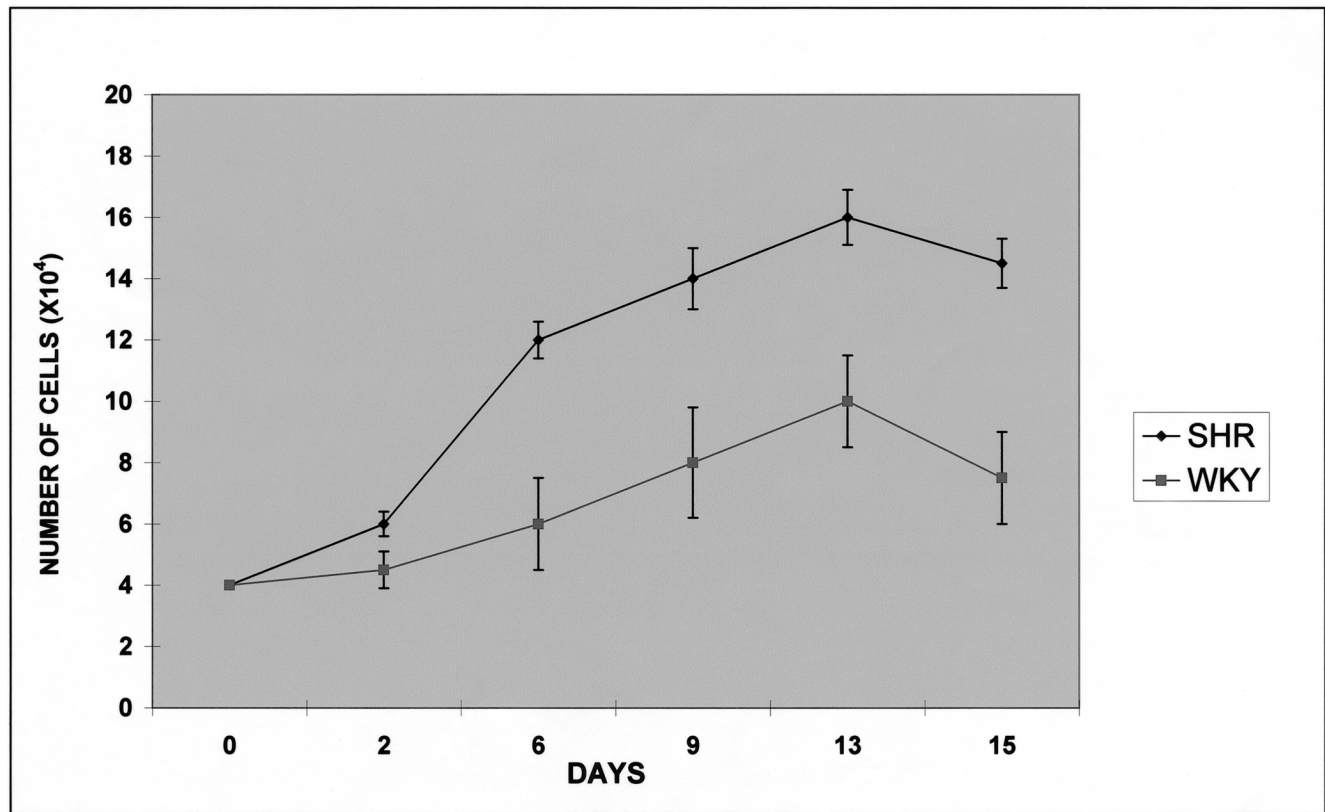


Figure 3. Growth rate of primary cultured epithelial cells from the ventral prostate of spontaneously hypertensive rats (SHRs) and Wistar-Kyoto rats (WKYs) grown in 24-well plates in WAJC-404 medium with 5% fetal calf serum and additives. Each time point represents the mean of 6 wells plus or minus the standard error of the mean. * Significant difference between SHRs and WKYs, $P < .05$.

in both groups (4%–7% in all cultures). Fluorescence readings at 560 nm after Alamar Blue staining were also higher in SHR cells than in WKY cells at all of the time points (Figure 4).

BrdU incorporation was detected in 16% of the SHR PECs compared to 7% of the cells from WKYs ($P < .01$ by chi-square analysis). Colorimetric analysis of BrdU incorporation yielded an even higher difference between the primary cultures derived from the different strains: the absorbance at 370 nm was 1.82 plus or minus 0.27 in SHR PECs vs 0.37 plus or minus 0.05 in WKY PECs ($n = 12$ in each group, $P < .001$).

Comparisons of cell proliferation rates were repeated 5 to 8 times. For each experiment, new primary cell cultures of rat ventral prostates were prepared.

Discussion

In this study, we showed that the characteristic prostatic irregularities of SHRs are associated with the proliferation of prostate epithelial cells and that the hyperplastic features of SHR PECs are maintained, at least partly, in culture, independent of local, neural, or endocrine control

mechanisms. To the best of our knowledge, this is the first model of spontaneous prostate hyperplasia in which hyperplastic features are exhibited both in vitro and in vivo. Therefore, it has the potential to provide insights into the intrinsic properties of hyperplastic epithelium in glandular prostate hyperplasia.

We showed that SHR ventral PECs divide faster than those of WKYs. The growth rate depends on a balance between cell division and programmed cell death. We could not determine whether the rate of programmed cell death differed between the strains: the turnover of cells in the prostate epithelium is slow, and almost all of the cells, at any given moment, are in a stationary state (G_0). Only a small portion of the cells are in active cell division. Since programmed cell death is a faster process than cell division, even a much smaller percentage of the cells are undergoing programmed cell death when an organ is in a steady-state condition. The use of BrdU enabled us to track cells that have undergone DNA synthesis during the 48 hours between the injection of BrdU and the sacrifice of the animal. However, since cells that undergo apoptosis rapidly disappear within 2 to 6 hours after the initiation of the process, the number of cells undergoing apoptosis is too small to enable the detection of differ-

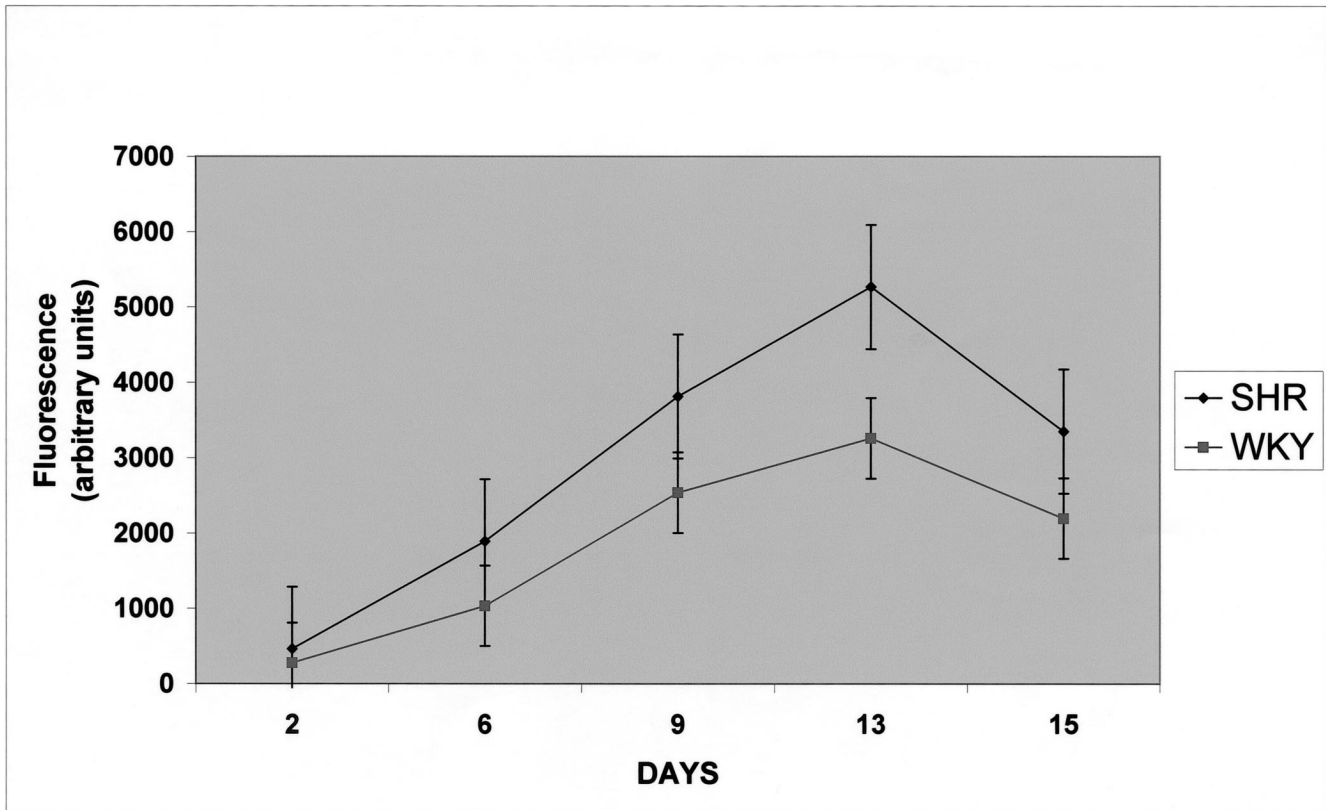


Figure 4. Proliferation of ventral prostatic epithelial cells as indicated by Alamar Blue staining. Ventral prostatic epithelial cells were seeded in 24-well plates at 40 000 cells/well and grown in plates containing WJJC-404 medium with 5% fetal calf serum and additives. Alamar Blue was applied in amounts equal to 10% of culture volume for an incubation of 3 hours. Fluorescence excitation wavelength was measured at 560 nm, and emission wavelength was measured at 590 nm.

ences between the strains. We could detect only a very small number of TUNEL-positive cells in both strains, and since no slide showed more than 1 TUNEL-positive cell, the contribution of apoptosis to the total growth could not be compared. We cannot rule out the possibility that programmed cell death is somewhat faster in WKYs than in SHRs, further contributing to the hyperplastic features in the latter strain.

SHRs have been derived from the WKY strain by the selective breeding of rats according to high blood pressure. Therefore, there is a high degree of similarity between these substrains. The dramatic difference in the proliferation rate of prostate epithelial cells between SHRs and WKYs can serve as a powerful tool to study genetic factors associated with prostate hyperplasia and PEC growth.

Although SHRs exhibit morphological features of prostate hyperplasia, their prostate weight was not increased. This result is not surprising: the lack of any correlation between prostate pathology and weight, especially in rats, has recently been thoroughly reviewed by Milman et al (2002). Furthermore, the rat prostatic weight is determined, to a large extent, by the content of prostatic se-

cretions in the acini. Adrenergic stimuli decrease the amount of secretions. Since SHRs exhibit enhanced tonic sympathetic activity, the amount of acinary secretions may be decreased. In H&E staining, the acinary lumen of SHRs actually looks less rich in secretions than that of WKYs.

Prostate hyperplasia is a common finding in the elderly population. The main focus in the research of its pathogenesis has been directed at the effect of androgen receptors and at the interaction between stroma and epithelium by paracrine factors. Although androgen ablation is an efficient tool in inhibiting hyperplastic changes, it should be borne in mind that responsiveness to androgens and other growth factors decreases with age. The finding in this study that a rat strain with spontaneous glandular prostate hyperplasia also shows an intrinsic tendency of epithelial cells to proliferate in vitro and in vivo suggests that basic features of the epithelial cells themselves may be associated with the tendency to hyperplasia rather than changes in levels of substances regulating their growth.

To summarize, SHRs exhibit spontaneous hyperplasia of the ventral prostate, which can be attributed, at least

in part, to the fundamentally high proliferation rate of its PECs maintained both in vivo and in vitro.

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