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Histochemical study of apoptotic epithelial cells depending on testosterone in primary cultured rat prostatic tissues

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Summary. To clarify whether apoptosis can be induced in cultured rat prostatic epithelial cells, they were investigated at various time points, depending on different concentrations of testosterone. Ventral lobes of rat prostates were cultured as small pieces of tissues up to 14 days. They were examined by anti-Fas antibody immunostaining and also compared to findings revealed by in situ end-labelling (ISEL) technique. To clarify apoptotic nuclei at high resolution, the quick-freezing and deep-etching (QF-DE) method was also used, as reported before. The localization and appearance of Fas-positive cells were detected more widely and earlier than those of ISEL-positive cells, but both label-positive localizations were closely related to each other. In addition, they were detected more often in epithelial cells cultured with low testosterone concentrations. By the QF-DE method, chromatin fibers were found to be broken in spotty parts of apoptotic nuclei. We could control the concentration of testosterone in culture medium and detect the appearance of Fas antigen in cultured prostatic epithelial cells, followed by apoptotic changes. So, Fas and Fas-ligand system is one candidate for apoptosis in the prostate glands, depending on removal of hormonal testosterone.

Key words: Fas antigen, In situ end-labelling, Prostate, Primary culture, Quick-freezing and deep-etching method

Introduction

It is known that the volume of mammalian prostatic glands is dramatically reduced with androgen withdrawal after castration of male animals and that some epithelial cells become apoptotic (Isaacs, 1984; Kyprianou and Isaacs, 1988). Moreover, prostatic epithelial cells were proliferated or degenerated, depending on the concentrations of serum testosterone (Furuya and Isaacs, 1993). Morphologically, the detection of programmed cell death or apoptotic changes has often been performed by the in situ end-labelling (ISEL) technique at light microscopic level (Gavrieli et al., 1992). Recently, various factors participating in the apoptotic death signaling have been reported for analyzing the molecular mechanism of apoptosis (Yonehara et al., 1989; Oehm et al., 1992; Suda et al., 1993; Nagata and Goldstein, 1995; Abasatado, 1996; Nagata, 1997). The Fas antigen, also called APO-1 or CD 95, is a cell surface receptor belonging to the nerve growth factor receptor family (Itoh et al., 1991). Functionally, it transduces the intracellular apoptotic death signal through Fas-ligand (Itoh et al., 1991; Suda et al., 1993). The interaction between Fas and Fas-ligand induces apoptosis of the antigen-expressing cells, and transmits its death signal to their nuclei. In the past decade, many studies about apoptosis have been morphologically performed by the ISEL technique as apoptotic detection for DNA fragmentation. Recently, Fas antigen was accepted as a surface marker of apoptosis among cell receptors (Gavrieli et al., 1992), and anti-Fas antibody immunostaining has become a popular technique for detecting the apoptotic cells. However, there is still little information about morphological changes revealed with the anti-Fas antibody immunostaining, as compared with those by the ISEL technique (Koji, 1994). It is accepted that binding of Fas ligand with Fas or cross-linking Fas antigens with agonistic antibodies usually induces an early stage of apoptosis (Nagata, 1997). When anti-Fas antibody immunostaining is used to clarify the localization of Fas antigen in prostatic epithelial cells as an apoptotic marker, we could probably detect apoptotic epithelial cells earlier than the ISEL technique. So, we have investigated primary cultured cells of rat prostates with both Fas immunostaining and the ISEL technique on serial sections of paraffin-embedded specimens.

Our previous study with the quick-freezing and deep-etching (QF-DE) method demonstrated apoptotic changes of fibrous chromatin structures in nuclei of rat prostatic epithelial cells after castration (Kubo et al., 1998). The QF-DE method is one of the advanced

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techniques in the field of electron microscopy, and has an advantage for examining three-dimensional ultrastructures of cells and tissues at high resolution. We have already reported ultrastructural findings in several animal tissues and cells by using the QF-DE method (Ohno and Takasu, 1989; Ohno and Fujii, 1990, 1991). So, another purpose of the present study was to check whether apoptotic changes in cultured prostatic cells can be detected at an early stage by the QF-DE method and regulated by different concentrations of testosterone in the culture medium.

Materials and methods

Primary cultured specimens

Adult male Wistar rats housed in an air-conditioned room with food and water ad libitum were used for the present experiment. Under anesthesia with sodium penthobarbital, prostatic ventral lobes were obtained from 11-to 13-week-old rats. Their capsules and other tissues were carefully removed, and remaining prostatic tissues were cut into small pieces, about $1mm^3$ in size, with razor blades. Then the most central parts of the prostate near the urethra were discarded, since they consist mainly of ducts and connective tissues. The tissue pieces were placed on 18 mm collagen-coated cover glasses. Four to six pieces of the tissues were usually put on each cover glass in a 35 mm diameter plastic dish (Becton, Dickinson Co., Ltd, Franklin Lakes, NJ, USA).

Medium and growth factors

The medium used contained IMDM (Iscov's Modified Dulbecco's Medium) and Ham F-12 (3:1) and fetal bovine serum (10%) treated with dextran coated with charcoal to remove testosterone (O'Connor and Shinha, 1985). In addition, several kinds of growth factors and chemicals were added as described as follows (Mckeehan et al., 1984; Martikainen, 1987; Martikainen et al., 1987; Chang and Chung, 1989); adenine (25 μg/ml), insulin (5 μg/ml), cholera toxin (10 ng/ml), epidermal growth factor (10 ng/ml), transferrin (5 μg/ml), hydrocortisone (400 ng/ml), prolactin (10 ng/ml). To prevent bacterial contamination, both penicillin (100 U/ml) and streptomycin (0.1 mg/ml) were also added to the medium.

Testosterone treatment

Serum levels of testosterone in examined rats were measured at the time when prostate glands were dissected. The mean level of testosterone concentrations was 2.3 ng/ml (0.9~10.5 ng/ml). Moreover, the serum levels of testosterone, which were measured two days after the castration, were less than 0.1 ng/ml. So, various concentrations (0, 10, 100 ng/ml) of testosterone were added to the culture medium. The cultured prostatic tissues were incubated in a humidified atmosphere of 5% carbon dioxide and 95% air at 37 °C up to 14 days. The culture media were renewed on days 1, 2, 4, 7, 10 and 13.

Paraffin sections for light microscopy (LM)

The cultured prostatic tissues were fixed with 2% paraformaldehyde (PF) in 0.1M phosphate buffer, pH 7.4, (PB) for 1 h and routinely embedded in paraffin. Paraffin sections were serially cut at 3 μm thickness and mounted on gelatin-coated glass slides. One section on a glass slide was stained with hematoxylin-eosin (HE), and another unstained section was used for other stainings, as described below.

The ISEL technique

To examine DNA strand breaking at 3'-OH as an apoptotic change of cultured epithelial cells, we used commercially available ApopTag, In Situ Apoptosis Detection Kit (Oncor, Gaitherburg, MD, USA), which was based on the method developed by Gavrieli et al. (1992). Paraffin sections were deparaffinized by a routine xylene-ethanol series and washed in PB containing 0.15M sodium chloride (referred to as PBS). They were first treated with 5 μg/ml proteinase K (E. Merck Darmstadt, Darmstadt, Germany) in PBS at 37 °C for 10 min. After washing in distilled water, they were quenched with 0.1% sodium azide in PBS to block inert peroxidase. They were immersed in equilibration buffer solution after washing in PBS and reacted with terminal deoxynucleotidyl transferase (TdT) enzyme containing TdT and digoxigenin-labelled deoxyuridine triphosphate, at 37 °C for 1 h. After their immersion in working strength stop/wash buffer, they were finally reacted with anti-digoxigenin-peroxidase for 30 min at room temperature and routinely visualized with 0.05% diaminobenzidine (DAB) solution. For counterstaining after visualization of DAB, 0.5% methyl green was usually used. For negative labelling controls, the TdT treatment was omitted from the labelling mixture solution (data not shown).

The rate of ISEL positiveness on sections was counted as percentages of ISEL-positive epithelial cells to total counted epithelial cells. The nuclei of apoptotic epithelial cells were heavily or slightly stained as brown colors of DAB deposits, which were regarded as ISEL positiveness. Many cell clumps located in glandular lumens, which were surrounded with some regenerative epithelial cells, were randomly photographed. Then, the positive rates of the detected epithelial cells were counted on 10 microscopic photographs, and morphometric data were expressed as mean ± standard deviation in some groups (three testosterone concentrations; 0, 10, 100 ng/ml) on several culture days. Statistical difference among the experimental groups was determined with Scheffe's F-test. A level of $p<0.01$ was accepted as statistical significance.

Fas immunostaining for LM

To examine the immunolocalization of Fas antigen, anti-Fas antibody immunocytochemistry was used for paraffin sections. Two kinds of rabbit or goat anti-Fas antibodies (referred to as M-20 and X-20), were commercially purchased (Santa Cruz Biotechnology Inc. California, U.S.A).

Deparaffinized sections were treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase and were also preincubated with both 200 μg/ml goat IgG and 2% BSA in PBS at room temperature for 1 h in order to inhibit nonspecific binding of antibodies. Then, they were immunoreacted with 100 μg/ml anti-Fas antibody (X-20, 1:2000) diluted with PBS at room temperature in a humidified atmosphere for 1 h. As a second antibody, biotin-labelled goat anti-rabbit IgG antibody was used. After processing by the streptavidin-biotin method (Guesdon et al.,1979) (Histofine, SAB-PO(R) Kit, Nichirei Co., Tokyo, Japan), the routine DAB reaction was used to detect the antigen-antibody binding, followed by counterstaining with 0.5% methyl green.

PCNA immunostaining for LM

To examine whether some parts of cultured epithelial cells synthesized DNA for their division, proliferating cell nuclear antigen (PCNA) immunostaining was used. The PCNA was identified as a proliferation-associated antigen by Miyachi et al. (1978). The level of PCNA, which is an acidic nuclear protein, shows a marked increase in both late G1 phase and early S phase of the cell cycle (Mathews et al., 1984). Paraffin sections were deparaffinized for the PCNA immunostaining. After blocking endogenous peroxidase activity with 0.3% H_2O_2, they were incubated with purified mouse anti-PCNA monoclonal antibody (MBL Co. Ltd., Nagoya, Japan) in PBS at 1:500 dilution for 1 h. As a second antibody, biotin-labelled anti-mouse immunoglobulin antibody was used, and then prepared by the streptavidin-biotin method, as described in a previous section. For counterstaining, 0.5% methyl green was used. PCNA-positive nuclei of epithelial cells were counted inside glandular acini. The positiveness evaluation was performed in the same way as the ISEL technique. More than 10 microscopic photographs were taken and serially checked in each group. Thus, statistical analyses were done between groups and also between culture days. The data were expressed as mean±standard deviation, and statistical difference between them was determined by Scheffe's F-test. The level of significance was fixed at $p<0.01$.

Conventional electron microscopy

Some prostatic cultured tissues were fixed with 2.5% glutaraldehyde in PB at 4 °C for 1 h. An additional postfixation was performed with 1% osmium tetroxide for 1 h, and then the specimens were routinely dehydrated and embedded in Quetol 812. Ultrathin sections (80 nm thick) were cut and stained with uranyl acetate and lead citrate. They were examined in a Hitachi H-600 electron microscope.

QF-DE method

Other cultured prostatic tissues were fixed with 2% PF in PB for 30 min, cut into small pieces with razor blades and washed in PB for 10 min, as reported before (Kubo et al., 1998). Then they were treated with 1% saponin in PB for 1 h and also 5 μg/ml proteinase K in PBS for 30 min to remove soluble proteins. They were postfixed with 0.25% GL in PB for 30 min, rinsed with 10% methanol in distilled water, and quickly frozen in JFD-RFA quick-freezing apparatus cooled with liquid nitrogen (-196 °C). Some tissue surfaces of the frozen specimens were freeze-fractured with a scalpel in liquid nitrogen, as reported before (Ohno and Takasu, 1989; Ohno and Fujii, 1990, 1991). They were deeply etched at - 95 °C under vacuum conditions of $2\text{-}6\times10^{-7}$ Torr in an Eiko FD-3AS machine (Eiko Engineering Co., Hitachinaka, Japan) for 15-20 min and rotary shadowed with platinum at an angle of 25° and finally with carbon. The replica membranes with the specimens were taken out and immediately coated with 2% collodion, as reported before. The prostatic tissues were dissolved in household bleach. All replica membranes were put on Formvar-filmed grids and immersed in amylacetate solution to dissolve the collodion. They were observed in the electron microscope.

Results

HE staining for LM

The prostatic epithelial cells were easily detached from the basement membrane by manipulating mechanical forces, when they were cut into small pieces with razor blades (Figs. 1, 2). So, they were aggregated in central parts of the acinar space (Figs. 1-4). Some epithelial cells survived for more than 14 days, regardless of different testosterone concentrations. Other regenerative epithelial cells grew along the basement membranes within 2 to 4 days. Stromal cells in interacinar tissues were especially increased in less amounts of testosterone (Fig. 1). By light microscopy with HE, peripheral cell clumps were often seen to be fragmented and appeared to be apoptotic, and some apoptotic bodies were detected after 2 days of culture (Fig. 2a,d).

The ISEL technique

In cultured epithelial cells at day 2 (Fig. 2), ISEL-positive nuclei appeared chiefly in peripheral parts of the epithelial cell clumps (Fig. 2b,e), which were detached from the basement membrane and aggregated in the

acinar space. However, ISEL-positive cells were distributed into central parts of the cell clumps at later stages (Figs. 3, 4). At each culture day of 2, 4, 7 and 14 days, the highest positive rate was seen in such cultured cells as incubated in the culture medium without testosterone (Figs. 2b, 3a, 4a). The 10 ng/ml testosterone concentration caused a middle rate of ISEL positiveness, and the 100 ng/ml testosterone concentration caused a lowest rate (Fig. 5). With each testosterone concentration, the positive rate was gradually increased up to 14 days. The minimal ISEL-positive rate was 6.5% at 100 ng/ml testosterone concentration on day 2 (Fig. 5). However, a significant difference of statistical analysis was not seen between various testosterone concentrations on day 2. The maximal ISEL-positive rate was 42% at 0 ng/ml testosterone concentration on day 14. The ISEL-positive rate at 100 ng/ml testosterone concentration was significantly lower by statistical analysis than the other concentrations of testosterone on day 4, 7 and 14 (Fig. 5).

Fas immunostaining

Some serial sections were already labelled by the ISEL technique, as shown in Figs. 2-4. In other sections, DAB-positive parts with Fas immunostaining were restricted to peripheral areas in the cell clumps, which were seen as brown color of DAB deposits (Figs. 2-4). The nuclei were all stained with methyl green in both Fas-positive and negative cells. Fas-positivity appeared slightly on day 2 with both 100 ng/ml and 0 ng/ml testosterone concentrations (Fig. 2c,f). However, the level of positivity was apparently higher in the latter concentration. The Fas immunostaining on serial paraffin sections was performed to see that immunopositive cells were serially detected in the same regions (Fig. 6c). The similar labelling was performed in the case of ISEL-serial paraffin sections (Fig. 6d-f). When some sections were immunoreacted with normal rabbit serum instead of anti-Fas antibody, no immunostaining was observed (data not shown). The cultured cells at 100 ng/ml and 0 ng/ml testosterone concentrations were strongly immunostained on day 4 (Fig. 3b,d) and weakly on day 7 (Fig. 4b,d). The localization of Fas-positive cells was detected less on day 7, as compared that with the ISEL technique. It was examined whether epithelial cells were stained on serial paraffin sections by both Fas immunostaining and the ISEL technique (Figs. 2-4).

However, there appeared to be no labelling in an identical cell. At earlier periods, the positive parts of Fas immunostaining were detected more broadly than those revealed by the ISEL technique (Figs. 2-4). Some of the ISEL-positive cells in an identical area appeared to be immunostained with anti-Fas antibody.

PCNA immunostaining

PCNA-positive cells were located along the basement membrane in the glandular body (Fig. 7). The immunopositive nuclei on day 2 were detected more often in the 0 ng/ml testosterone concentration than in the 100 ng/ml concentration (Fig. 8). The immunopositive rate in the former reached a peak on day 4, though it gradually increased in the latter. However, PCNA-positive immunostaining was not detected in nuclei of cell clumps under any culture conditions. So, aggregated epithelial cells in the cell clumps, which

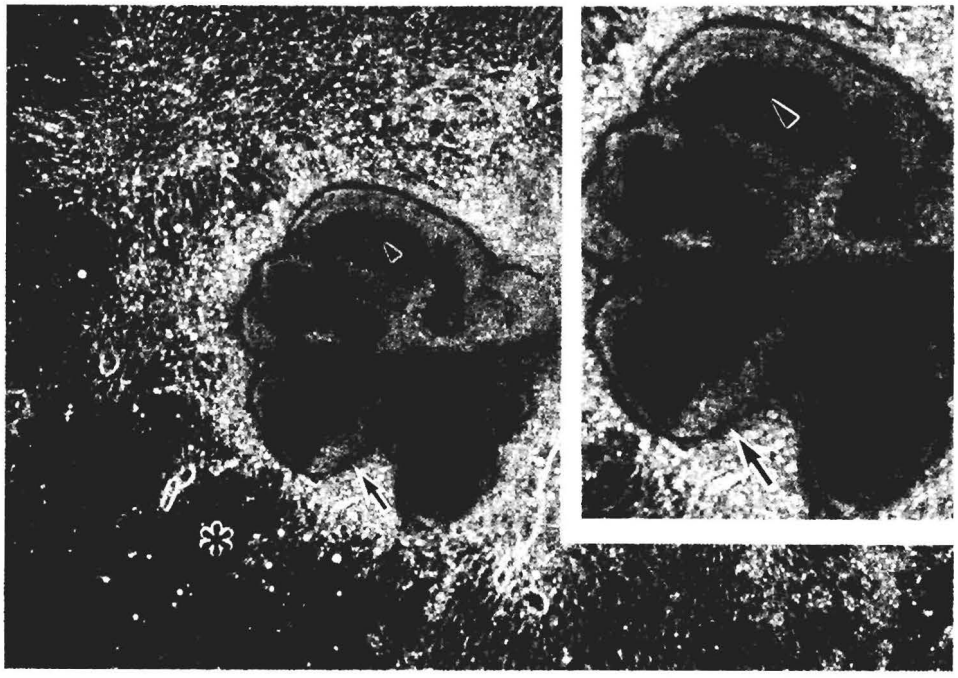

Fig. 1. Phase-contrast micrographs of cultured ventral prostatic tissues (arrow) and migrating stromal cells (*) on plastic dishes (day 7, testosterone 10 ng/ml). x 90. The prostatic tissues are surrounded by spindle-shaped fibroblasts. Inset; higher magnification. x 150. Arrowheads: cell clump.

were detected positively by ISEL and Fas stainings, did not make any DNA synthesis.

Conventional electron microscopy

Apoptotic epithelial cells were seen under any testosterone concentration after day 2 in the present study. They appeared mainly at peripheral parts of the cultured cell clumps. They had typically condensed chromatin, shrinkage of the cell size and some fragments of nuclei (Fig. 9a,b). Electron-dense apoptotic bodies were also recognized in some epithelial cells (Fig. 9c). After culturing for 7 days, such apoptotic bodies were more often seen in low testosterone concentrations. Some epithelial cells cultured on day 14 had normally appearing nuclei with dispersed euchromatin and condensed heterochromatin (Fig. 9d,e). Apparently, such nuclei of epithelial cells were always seen up to 14 days in 100 ng/ml testosterone concentration.

The QF-DE method

To investigate apoptotic changes of nuclei in the cultured epithelial cells, the QF-DE method was used. Three-dimensional images of such nuclei could be easily obtained at higher resolution on replica membranes. In

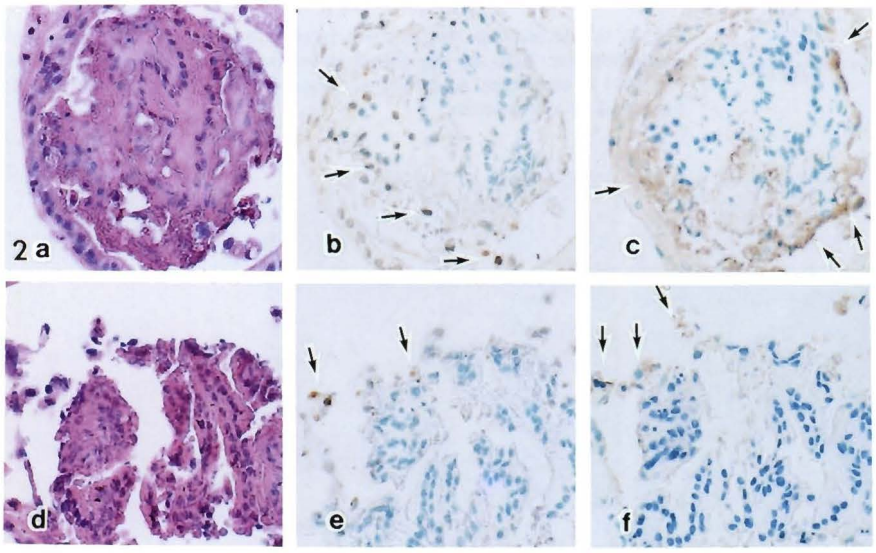

Fig. 2. Light micrographs of cultured ventral prostatic tissues on day 2. Serial sections; testosterone 0 ng/ml **(a-c)**, and 100 ng/ml **(d-f)**. They are stained with hematoxylin and eosin **(a, d)**, in situ end-labelling **(b, e)** and Fas immunostaining **(c, f)**. Positive cells of Fas immunostaining (arrows in c) appear more broadly than those revealed by in situ end-labelling (arrows in b). The level of each positivity is stronger in 0 ng/ml testosterone concentration than in 100 ng/ml concentration, chiefly at peripheral cell clumps. x 240

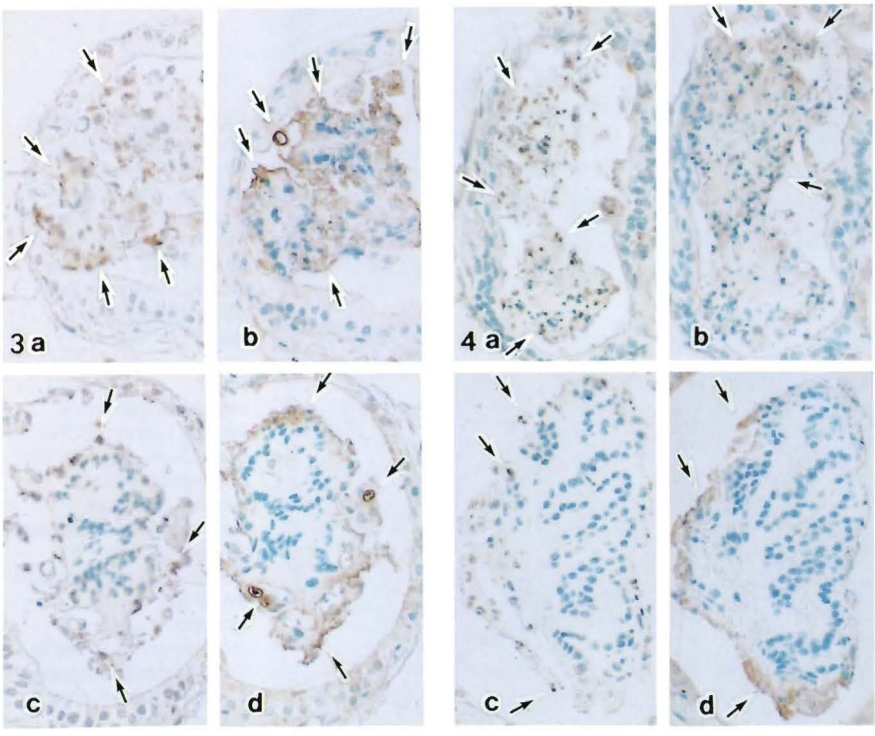

Fig. 3. Light micrographs of cultured ventral prostatic tissues on day 4. Serial sections; testosterone 0 ng/ml **(a, b)**, and 100 ng/ml **(c, d)**. They are stained by in situ end-labelling **(a, c)**, and Fas immunostaining **(b, d)**. Positive cells of Fas immunostaining (arrows in b and d) appear more broadly than in situ end-labelling positivity (arrows in a and c). The level of both positivities is high in peripheral parts of the cell clumps. x 240

Fig. 4. Light micrographs of cultured ventral prostatic tissues on day 7. Serial sections; testosterone 0 ng/ml **(a, b)**, and 100 ng/ml **(c, d)**. They are stained by in situ end-labelling **(a, c)**, and Fas immunostaining **(b, d)**. The level of positive cells with Fas immunostaining (arrows in b and d) appears to be decreased, but in situ end-labelling positivity (arrows in a and c) is still high. x 240

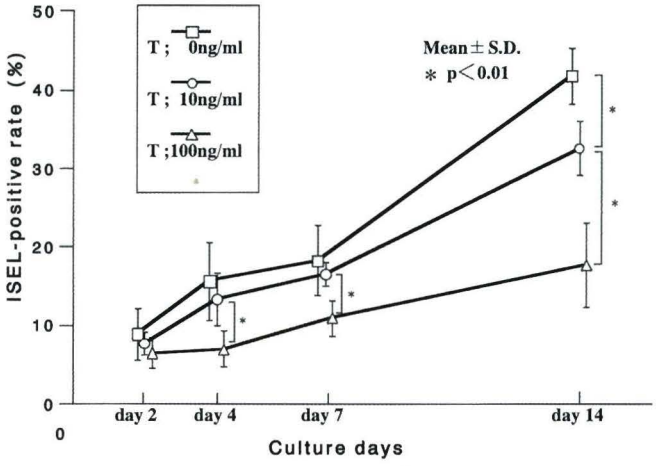

Fig. 5. In situ end-labelling positive rates due to different testosterone concentrations. Data are expressed as mean ± standard deviation (SD). *: $p<0.01$ is significant, as compared between 100 ng/ml testosterone and the others. T: testosterone.

the nuclei of normal epithelial cells, fibrous structures of chromatin formed continuous networks (Fig. 10a,b), as reported before (Kubo et al., 1998). However, in the apoptotic nuclei, filamentous networks were randomly broken, and spotty spaces were distributed throughout the nuclear matrix (Fig. 10c,d).

Discussion

Prostatic epithelial cells do have androgen receptors as indicated by the epithelial cell regulation of PSA (Prostate specific antigen) production which is

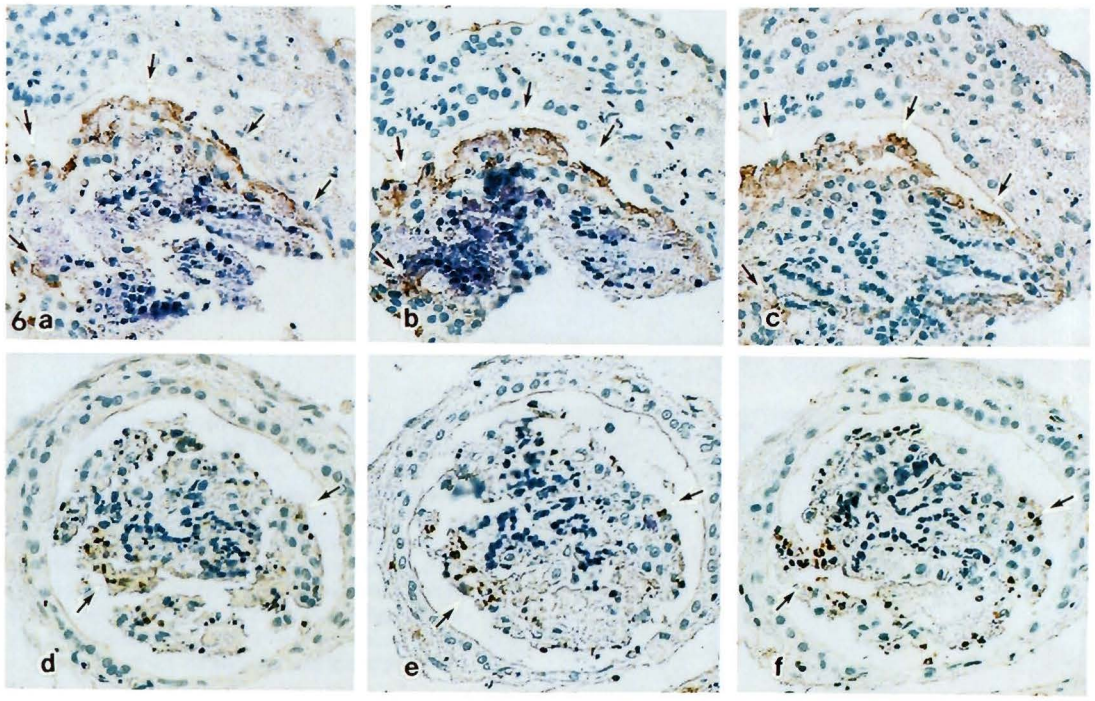

Fig. 6. Light micrographs of three serial sections labelled by anti-Fas antibody immunostaining (arrows, **a-c**) and in situ end-labelling technique (arrows, **d-f**) . They are detected almost in the same regions in each labelling. x 240

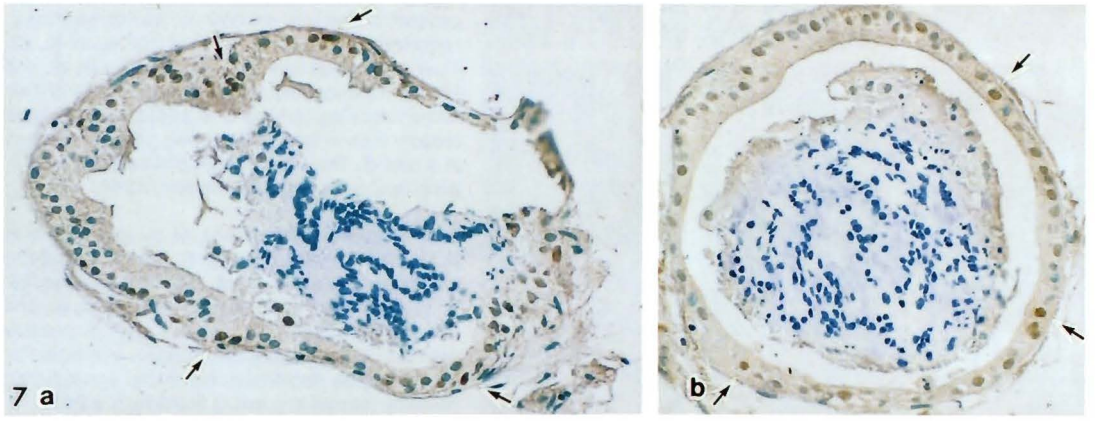

Fig. 7. Immunohistochemical staining with anti-PCNA monoclonal antibody. The prostatic tissues are cultured in 0 ng/ml testosterone concentration **(a)** and 100 ng/ml testosterone concentration **(b)** on day 4. The immunopositive nuclei (arrows) are observed in lining epithelial cells along the basal parts, but not in the cell clumps. x 240

androgen-sensitive (Wang et al., 1979), but some previous reports demonstrated that testosterone did not exert direct mitogenic action on prostatic epithelial cells, but on stromal cells (Chang and Chung, 1989; Nemeth and Lee, 1996), when the former cells were isolated from the latter ones. In the present study, however, we did not investigate the relationship of apoptotic changes between the epithelial cells and the stromal cells. It was also reported by using tissue culture system that isolated prostatic epithelial cells alone had no mitogenic activity even due to high testosterone concentrations (Mckeehan et al., 1984; Chang and Chung, 1989). When the rat prostatic epithelial cells were co-cultured with fibroblasts in higher testosterone concentrations, the number of the epithelial cells was increased, and their DNA synthetic activity was expressed at a higher rate (Chang and Chung, 1989), so androgen receptors were assumed to be located in the stromal cells, but not in the epithelial cells (Chang and Chung, 1989). The growth of prostatic epithelial cells was probably regulated via direct mitogenic action of testosterone on prostatic fibroblasts. If the epithelial cells alone had been cultured in the present study, the testosterone exerted no direct effect on them, in the similar way to those following after testosterone ablation in vivo (Colombel and Buttyan, 1995). So, in such a case, we might not be able to detect apoptotic cells under this experimental condition. That is a reason why we chose the explant culture system with co-culture of epithelial cells and stromal cells. Some epithelial cells were substituted by proliferating testosterone-independent cells, as shown in Figure 7a, when they were cultured without testosterone. However, some regenerative epithelial cells were probably derived from stem cells along the basement membrane, that seemed to be independent of testosterone concentrations. Because they were proliferated, even if they were cultured in the 0 ng/ml testosterone concentration.

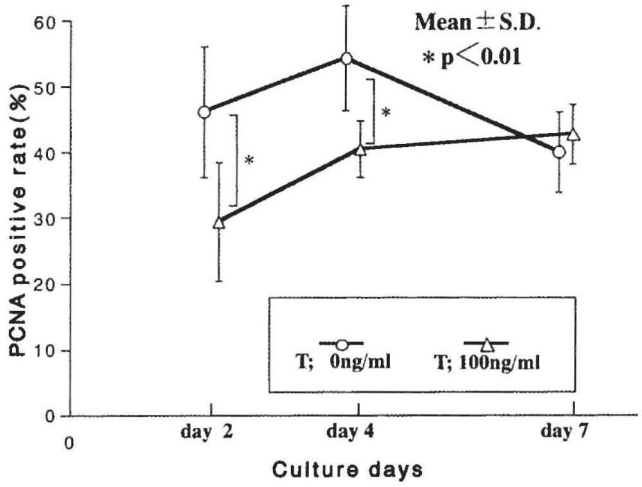

Fig. 8. Morphometric analysis on effect of testosterone concentrations for PCNA immunostaining. The rates of immunopositive nuclei with 0 ng/ml and 100 ng/ml testosterone concentrations are separately indicated. Data are expressed as mean ± standard deviation (SD). *: $p < 0.01$ is significant, as compared between 100 ng/ml and 0ng/ml concentrations. T: testosterone.

We could control the appearance rate of apoptosis in cultured prostatic epithelial cells by changing the testosterone concentrations, although the culture system of different testosterone concentrations may have residual endogenous testosterone. There were some differences of apoptosis between in vivo epithelial cells and in vitro ones. First, the rate of apoptosis was at least 6% per day in vitro, even if good culture conditions were kept, while that in vivo was less than 2% per day (Martikainen and Isaacs, 1990). Secondly, the labelling rate of ISEL-positive cells were gradually increased up to 14 days, regardless of the testosterone concentrations, as shown in Figure 5. However, ISEL-positive cells reached a peak of labelling in vivo on day 2, which then gradually decreased, as reported before (English et al., 1989; Kubo et al., 1998). The rate of the positiveness in vitro was higher on day 4 than that of the castrated epithelial cells in vivo. Although apoptotic epithelial cells in rat prostatic ventral lobes was reported to amount to 85% by 7 days in vivo after testosterone ablation (Kyprianou et al., 1988; English et al., 1989), we could not detect the same rate of apoptotic cells. That is probably due to the difference of cytokines, cell-mediated immunity and activity of phagocytic cells between in vivo system and in vitro one. It took a longer time for apoptotic cells to be phagocytosed and digested in vitro. Thus, the residual apoptotic cells were accumulated and more often seen in the cultured epithelial cells, regardless of testosterone concentrations. In the present study, they were easily detected, when apoptotic bodies remained in the epithelial cells, as revealed by electron microscopy (Fig. 9c).

Our previous study has revealed the organization of chromatin fibers in apoptotic nuclei at high resolution by the QF-DE method (Kubo et al., 1998). By using the replica membranes, we could clarify the destruction of these fibrous structures in early apoptotic nuclei, which would not be revealed by conventional electron microscopy. In the present study, we could also regulate apoptosis in cultured prostatic epithelial cells, which resembled those in vivo. They had often been investigated in terms of mitosis, depending on various testosterone concentrations, by previous researchers (Terracio and Douglas, 1982; Mckeehan et al., 1984; Martikainen, 1987; Chang and Chung, 1989). However, they were not concentrated on apoptotic changes of prostatic epithelial cells. Recently, there have been many reports about apoptotic pathways (Yonehara et al., 1989; Oehm et al., 1992; Suda et al., 1993; Nagata and Goldstein, 1995; Abasatado, 1996; Nagata, 1997), though little information has been obtained about a difference of labelling between Fas immunostaining and the ISEL technique. The Fas route is one of the apoptotic pathways that mediates death signal into the nuclei (Oehm et al., 1992; Suda et al., 1993; Nagata and

Goldstein, 1995; Abasatado, 1996; Nagata, 1997). Thus, the Fas immunostaining was often used as an apoptotic detection in addition to the ISEL technique. Since the Fas antigen appeared in the cytoplasm before some cells were changed to express apoptotic nuclei (Abasatado, 1996; Nagata, 1997), it could be recognized as an initial apoptotic marker. In the present study, we examined apoptotic epithelial cells on serial paraffin sections, which were stained with both Fas immunostaining and the ISEL technique. Some cultured epithelial cells were detected with each positive staining, which were generally located in an identical area of cell clumps, as shown in Figures 2-4. It is also probable that they had the same potentiality to express apoptotic changes. However, peripheral parts of the cell clumps got more nutrition and gas exchange from the culture medium. In addition, the transmission of death signal into nuclei has been reported to need active-energy-dependent processes

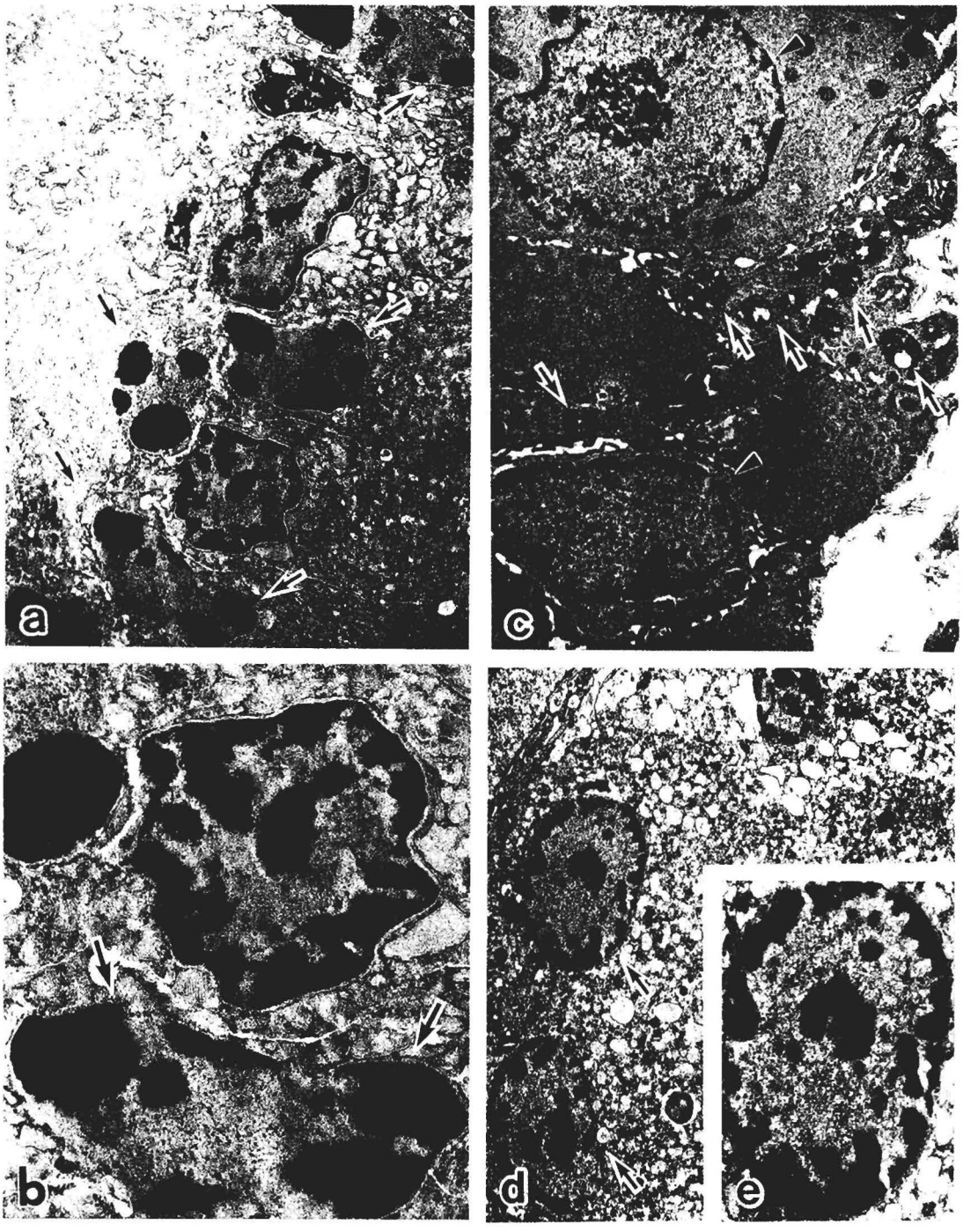

Fig. 9. Conventional electron micrographs of apoptotic or normal appearing cells. **a.** Typical apoptotic nuclei (arrows) are seen in cultured epithelial cells of 0 ng/ml testosterone on day 2. x 5,200. **b.** Higher magnification. x 12,000. **c.** Apoptotic bodies (arrows) are seen along the basal parts of regenerative epithelial cells (arrowheads) in 10 ng/ml testosterone on day 4. x 7,200. **d.** Normal appearing nuclei (arrows) are seen in 100 ng/ml testosterone on day 14. x 5,200. **e.** Inset; higher magnification of a nucleus. x 10,000

(Kerr et al., 1972). So the peripherally located cells were easily affected by culture environment and got earlier apoptotic changes.

On the other hand, it is not clear that Fas ligand was produced in cultured prostate glands, even though we could detect the appearance of their Fas antigen. The production of Fas ligand may be related to some mesenchymal cells, because they also had testosterone receptors (Chang and Chung, 1989). A previous study reported the Fas ligand expression in many kinds of human organs. The Fas ligand was strongly detected in human testis and placenta (Xerri et al., 1997), but its level in the prostate was detected to a lesser extent. It is probable that both Fas and Fas ligand were co-expressed

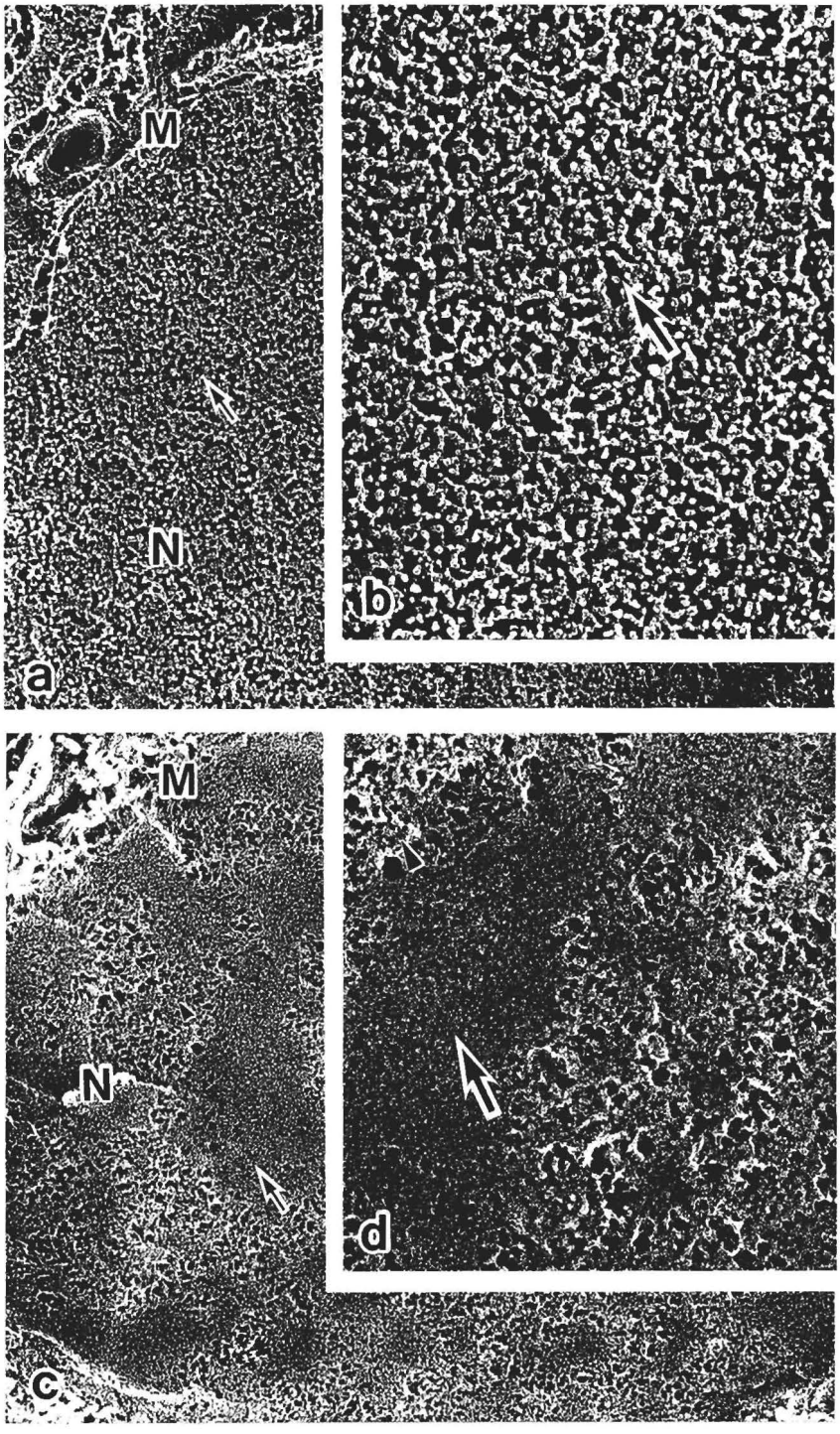

Fig. 10. Replica electron micrographs of normal or apoptotic nuclei. **a.** Normal nuclei are seen in 10 ng/ml testosterone on day 2. Filamentous network structures (arrow) are recognized. x 24,000. **b.** Higher magnification. x 50,000. **c.** Apoptotic nuclei are also seen. Filamentous networks are broken with spotty patterns in the nuclei. x 24,000. **d.** Higher magnification. x 50,000. Arrows: condensed networks; Arrowheads; spotty broken areas; N: nucleus; M: nuclear membrane.

in the cultured prostatic tissues, and the apoptosis in the prostate might be regulated by the Fas-Fas ligand pathway (Nagata and Goldstein, 1995; Abasatado, 1996; Hakuno et al., 1996; Nagata, 1997). Thus, the localization of Fas ligand in the cultured prostatic tissues should be examined under the present experimental conditions.

We have investigated apoptotic changes of epithelial cells in rat prostatic organ cultures by using both Fas immunostaining and the ISEL technique. Our culture system is probably useful as an apoptotic cell model, depending on both testosterone concentrations and culture days.

References

Abastado J.P. (1996). Apoptosis: function and regulation of cell death. Res. Immunol. 147, 443-456.

Chang S. and Chung L.W.K. (1989). Interaction between Prostatic fibroblast and epithelial cells in culture: role of androgen. Endocrinology 125, 2719-2727.

Colombel M.C. and Buttyan R. (1995). Hormonal control of apoptosis: The rat prostate gland as a model system. Methods Cell Biol. 46, 369-385.

English H.F., Kyprianou N. and Isaacs J.T. (1989). Relationship between DNA fragmentation and apoptosis in the programmed cell death in the rat prostate following castration. Prostate 15, 233-250.

Furuya Y. and Isaacs J.T. (1993). Differential gene regulation during programmed death (apoptosis) versus proliferation of prostatic glandular cells induced by androgen manipulation. Endocrinology 133, 2660-2667.

Gavrieli Y., Sherman Y. and Ben-Sasson S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. 119, 493-501.

Guesdon J., Ternynck T. and Avrameas S. (1979). The use of avidin-biotin interaction in immunoenzymatic techniques. J. Histochem. Cytochem. 27, 1131-1139.

Hakuno N., Koji T., Yano T., Kobayashi N., Tsutsumi O., Taketani Y. and Nakane P.K. (1996). Fas/Apo-1/CD95 system as a mediator of granulosa cell apoptosis in ovarian follicle atresia. Endocrinology 137, 1938-1948.

Isaacs J.T. (1984). Antagonistic effect of androgen on prostatic cell death. Prostate 5, 545-557.

Itoh N., Yonehara S., Ishii A., Yonehara M., Mizushima S., Sameshima M., Hase A., Seto Y. and Nagata S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66, 233-243.

Kerr J.F.R., Wyllie A.H. and Currie A.R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26, 239-257.

Koji T. (1994). Immunohistochemical localization of Fas antigen in paraffin sections with rabbit antibodies against human synthetic Fas peptides. Acta Histochem. Cytochem. 27, 459-463.

Kubo M., Uchiyama H., Ueno A., Terada N., Fujii Y., Baba T. and Ohno S. (1998). Three-dimensional ultrastructure of apoptotic nuclei in rat prostatic epithelial cells revealed by quick- freezing and deep-etching method. Prostate 35,193-202.

Kyprianou N. and Isaacs J.T. (1988). Activation of programmed cell death in the rat ventral prostate after castration. Endocrinology 122, 552-562.

Kyprianou N., English H.F. and Isaacs J.T. (1988). Activation of a Ca^{2+}--Mg^{2+}-dependent endonuclease as an early event in castration-induced prostatic cell death. Prostate 13, 103-117.

Martikainen P. (1987). Maintenance of adult rat ventral prostate in organ culture. Anat Rec. 218, 166-174.

Martikainen P. and Isaacs J.T. (1990). An organ culture system for the study of programmed cell death in the rat ventral prostate. Endocrinology 127, 1268-1277.

Martikainen P., Harkonen P., Vanhala T., Makela S., Viljanen M. and Suominen J. (1987). Multihormonal control of synthesis and secretion of prostate in in cultured rat ventral prostate. Endocrinology 121, 604-611.

Mckeehan W.L., Adams P.S. and Rosser M.P. (1984). Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free, primary cell culture. Cancer Res. 4, 1998-2010.

Mathews M.B., Bernstein R.M., Robert F. Jr. B. and Garrels J.I. (1984). Identity of the proliferating cell nuclear antigen and cyclin. Nature 309, 374-376.

Miyachi K., Fritzler M.J. and Tan E.M. (1978). Autoantibody to a nuclear antigen in proliferating cells. J. Immunol. 121, 2228-2234.

Nagata S. (1997). Apoptosis by death factor. Cell 88, 355-365.

Nagata S. and Goldstein P. (1995). The Fas death factor. Science 267, 1449-1456.

Nemeth J. A. and Lee C. (1996). Prostatic ductal system in rats: Regional variation in stromal organization. Prostate 28, 124-128.

O'Connor T. and Sinha D.K. (1985). Characterization of rat ventral prostatic epithelial cells in collagen gel culture. Prostate 7, 305-319.

Oehm A., Behrmann I., Falk W., Pawlita M., Maier G., Klas C., Li-Weber M., Richards S., Dhein J. and Trauth B.C. (1992). Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily: sequence identity with the Fas antigen. J. Biol. Chem. 267, 10709-10715.

Ohno S. and Fujii Y. (1990). Three-dimensional and histochemical studies of peroxisomes in cultured hepatocytes by quick-freezing and deep-etching method. Histochem. J. 22, 143-154.

Ohno S. and Fujii Y. (1991). Three-dimensional studies of the cytoskeleton of cultured hepatocytes: a quick-freezing and deep-etching study. Virchows Arch. 418, 61-70.

Ohno S. and Takasu N. (1989). Three-dimensional studies of cytoskeletal organizations in cultured thyroid cells by quick-freezing and deep-etching method. J. Electron Microsc. 38, 352-362.

Suda T., Takanashi T., Golstein P. and Nagata S. (1993). Molecular cloning and expression of the Fas ligand: a novel member of the tumor necrosis factor family. Cell 75, 1169-1178.

Terracio L. and Douglas W.H.J. (1982). Densitometric and morphometric evaluation of growth in primary cultures of rat ventral prostate epithelial cells. Prostate 3, 183-191.

Wang M., Valenzuela L., Murphy G. and Chu T. (1979). Purification of a human prostate speficic antigen. Invest. Urol. 17, 159-163.

Xerri L., Devilard E., Hassoun J., Mawas C. and Birg F. (1997). Fas ligand is not only expressed in immune privileged human organs but is also coexpressed with Fas in various epithelial tissues. Clin. Mol. Pathol. 50, 87-91.

Yonehara S., Ishii A. and Yonehara M. (1989). A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J. Exp. Med. 169, 1747-1756.

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