Histol Histopathol (2001) 16: 453-462

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

Intravesicular Fas localization in epithelial cells of castrated rat prostate glands

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Summary. Androgenic steroids regulate the development and size of mammalian prostate epithelial cells. To evaluate the relationship between Fas-Fas ligand system and apoptosis in prostate epithelial cells of the castrated rats, we have examined immunocytochemical localization of Fas antigen in the castrated rat prostate glands at a series of different times. We used a rabbit polyclonal anti-Fas antibody with a streptavidin-biotin method and confocal laser scanning method or an immunogold method. Fas immunolocalization was examined in ventral lobes of prostate glands taken from intact or castrated adult male Wistar rats on day 1, 2, 3, 4 and 5 by light or electron microscopy. At a light microscopic level, the castrated prostate epithelial cells showed mostly Fas immunolocalization in their apical parts of cytoplasm on day 2 after the castration. In addition, their extent of the Fas expression was expanded throughout the cytoplasm in proportion to the androgen ablation periods, and later the Fas expression was detected at luminar or basolateral sides of the epithelial cells. Both immunogold labeling with ultrathin sections and immunoperoxidase technique with cryostat sections demonstrated that Fas was localized mainly in secretory granules of the castrated prostate epithelial cells and some parts of their cell membranes at later stages. Our immunocytochemical findings showed that Fas expression was time-dependently induced in most of the prostatic epithelial cells after castration of rats. The rate of Fas-expressing epithelial cells was too high and inconsistent with the previously reported rate of TUNEL-positive ones. The membrane-associated Fas may have little effect on the apoptosis in the present case, bacause a lot of soluble Fas was secreted from the prostatic epithelial cells. A further study is needed to clarify some significance of the secretory Fas in the prostatic epithelium after the rat castration.

Key words: Apoptosis, Fas, Immunocytochemistry, Prostate, Castrated rat

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Introduction

It has been well-known that postpuberal development of rat prostate glands depends upon sufficient amounts of circulating androgen hormone (Isaacs, 1984; Kyprianou and Isaacs, 1998), and that the androgen ablation causes a rapid atrophy of epithelium in their ventral lobe (Sanford et al., 1984; Banerjee et al., 1995). Morphologically, the androgen ablation with rat castration reduces the epithelial cell height and the number of apical vesicles in cytoplasm, and finally induces a typical apoptosis of the epithelial cells (Isaacs et al., 1984; Sanford et al., 1984; Banerjee et al., 1995; Kyprianou and Isaacs, 1998). The apoptosis is different from necrosis in respect to morphological changes and cell death processes. The apoptotic cells usually show an aggregation of nuclear chromatin near nuclear envelopes and its rapid condensation in their cytoplasm. Other morphological features are cytoplasmic aggregation without alteration of cell organelle integrity and then final fragmentation into apoptotic bodies (Kerr et al., 1972; Gaffney et al., 1995; Julliard et al., 1996). Recent extensive studies on apoptosis have already disclosed such molecular mechanisms that play an important role in the homeostasis of multicellular organisms, genetic regulation, immune system mechanism including autoimmune diseases, and cancers (Cummings et al., 1997; Leach, 1998; Schmitt and Lowe, 1999).

In addition, various apoptotic death signals have been recently identified to initiate apoptosis in animal cells (Nagata and Golstein, 1995). The Fas is a type I membrane protein of 45 kDa which belongs to the tumor necrosis factor(TNF)/nerve growth factor receptor family. Functionally, it is a cell surface receptor that is capable of initiating an apoptotic signaling pathway when bound to Fas ligand (Itoh et al., 1991; Oehm et al., 1992; Suda et al., 1993). In animal bodies, it is usually expressed in cytotoxic T cells, B cells, and several other cells in livers, hearts, kidneys, ovaries, and thymus (Oehm et al., 1992; Suda et al., 1993; Krammer et al., 1994; Schmitt and Lowe, 1999). Additionally, Fas and Fas ligand have been found to be expressed in a few epithelial tissues, which are marked by the apoptotic cell turnover, such as the prostate and uterus (Leithauser et

al., 1993; French et al., 1996; Xerri et al., 1997). Both of these tissues are steroid hormone-dependent and usually undergo apoptosis within 24-48h after the hormone depletion (Isaacs, 1984; Rotello et al., 1992). Interestingly, the apoptosis that occurres in response to the steroid hormone depletion has been recently shown to require sufficient Fas expression in epithelial cells (Suzuki et al., 1996). These data support a role for the Fas signaling pathway in the normal renewal of the prostate and uterine epithelium. In humans, at least five Fas isoforms can be generated by alternative splicing of a Fas gene, and four of them lack transmembrane domains, resulting in soluble forms (Cheng et al., 1994; Owen-Schaub et al., 1995). Some in vitro studies have suggested that the soluble Fas inhibits cell death through the previous binding with Fas ligand in the extracellular matrix (Cheng et al.,1994; Owen-Schaub et al, 1995). Several tumor cell lines themselves synthesize the soluble Fas and may be protected from the immune reaction system (Lee et al., 1998). Recently, the existence of soluble Fas was also reported in rats (Kobayashi and Koike, 1996).

It has been assumed that the Fas-Fas ligand system may play an important role in mediating apoptosis of prostate glands after castration (Isaacs et al., 1992). Some previous studies reported that apoptosis occurred in the prostate epithelium of mice following their castration, and that Fas upregulation has also been detected in the castrated prostate (Lee et al., 1998). Our previous study demonstrated that Fas upregulation has been detected under various concentrations of testosterone in cultured prostatic epithelial cells of rats (Furuya et al., 2000). However, there has been no report as to immunolocalization of Fas in the prostate epithelium of castrated rats at an electron microscopic level. In the present study, to address whether castration induces the apoptosis of prostate epithelial cells through the Fas-Fas ligand system, we have examined immunocytochemically the Fas expression in the castrated rat prostate glands at a series of different times.

Materials and methods

Animals and tissue preparations

A total of 30 adult male Wistar rats at ages of 11-15 weeks were housed in an air-conditioned room with food and water ad libitum. Some rats were castrated under intraperitoneal anesthesia with sodium pentobarbital (10mg/kg body weight), and used for the present experiment on day 1, 2, 3, 4, and 5, as described before (Kubo et al., 1998). Anesthetized rats with the sodium pentobarbital after weighing the bodies were perfused with 2% paraformaldehyde (PF) or 2% PF mixed with 0.05% glutaraldehyde (GL) in 0.1M phosphate buffer (PB), pH7.4, via left cardiac ventricles. Ventral lobes of prostates were isolated under observation with a dissection microscope, and additionally fixed in the same fixative for 1h. Animal experimental protocols were approved by the Animal Use Committee of Yamanashi Medical University.

Weight measurement of ventral lobes

Isolated ventral lobes were blotted onto filter paper and their wet weights were checked, as reported before (Kubo et al., 1998). A relative weight of the ventral lobe to the body weight was calculated and expressed as a percentage. Means and standard deviations were obtained from 4 rats in each group (data not shown), indicating that they were almost similar to those as reported before (Kubo et al., 1998).

Antibodies

We used rabbit polyclonal anti-Fas antibody raised against a peptide corresponding to amino acids 295-314 mapping at the carboxy terminus (X-20, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), which was used at 1:1000 dilution.

Immunoperoxidase technique

Prostatic tissues fixed with 2% PF were routinely dehydrated in a graded series of alcohol and embedded in Paraffin, cut at 4 μm thickness, and finally mounted on gelatin-coated glass slides. After being deparaffinized in a routine xylene-methanol series, the sections on the glass slides were treated with 0.3% hydrogen peroxide in methanol for 15 min and washed in PBS. They were then incubated with 10% normal goat serum for 30 min to block non-specific reactions, and with primary anti-Fas antibody in PBS containing 2% bovine serum albumin at 4 °C overnight. After being rinsed in PBS, they were incubated with biotin-labeled anti-rabbit IgG antibody for 10 min, and then with peroxidase-labeled streptavidin for 5 min according to the manufacture's protocol (Histofine, Nichirei, Tokyo, Japan). Subsequently the immunostained sections were treated with a metal-enhanced diaminobenzidine technique (Pierce, IL, USA) and methylgreen for counterstaining.

Confocal laser scanning microscopy

Paraffin sections at 4 μm thickness were treated with 0.3% hydrogen peroxide in methanol for 15 min and washed in PBS. They were incubated with 10% normal goat serum for 30 min, the primary antibody at 4 °C overnight, and biotin-labeled anti-rabbit IgG antibody for 10 min. Subsequently, they were incubated with Alexa 488-labeled streptavidin (1:500, Molecular Probes, OR, USA) and propidium iodide at 1:1000 dilution for 1h. After embedding with anti-fading mounting medium (Bio Rad Laboratories, CA, USA), they were routinely observed with a confocal laser scanning microscope (TCS4D: Leica, Heidelberg, Germany).

Conventional electron microscopy

Some castrated or normal rats were perfused with 2.5% GL in 0.1M PB via hearts. The prostate tissues were additionally immersed in the same fixative for 1h, postfixed with 1% osmium tetroxide in 0.1M PB for 1h, dehydrated in a graded series of ethanol and embedded in Quetol 812 (Nishin EM, Tokyo, Japan). Ultrathin sections at 75 nm thickness were cut with an ultramicrotome, counterstained with uranyl acetate and lead citrate, and finally examined in a Hitachi H-7500 electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV.

Immunoelectron microscopy

Pre-embedding immunostaining

Under anesthesia with sodium pentobarbital, other castrated or normal rats were perfused with 2% paraformaldehyde (PF) in 0.1M PB via the left cardiac ventricle. Afterwards, the prostate ventral lobes were removed and pieces of the prostatic tissues were immersed in 30% sucrose, embedded in Tissue Tek (Miles Inc. IN, USA) and frozen in liquid nitrogen. Several serial 4 μm sections were obtained from each sample and mounted on gelatin-coated glass slides. They were washed in PBS and treated with 0.3% hydrogen peroxide in methanol for 10 min. After being rinsed with PBS, they were incubated with 10% normal goat serum for 10 min to block non-specific antibody binding reactions. They were immunoreacted with the primary anti-Fas antibody (X-20) diluted at 1:500 in PBS containing 2% bovine serum albumin (BSA) at 4 °C for 18h. After being rinsed with PBS, they were incubated in biotin-labeled anti-rabbit IgG antibody for 10 min. They were immersed in peroxidase-labeled streptavidin for 5 min. They were fixed with 0.25% GL in 0.1M PB for 15 min and reacted with metal-enhanced diaminobenzidine (Pierce, IL, USA) solution for 3 min. They were post-fixed with 1% osmium tetroxide in 0.1M PB for 60 min and dehydrated in a graded series of ethanol for a few minutes. They were embedded in Quetol 812 by an inverted gelatin capsule method. Ultrathin sections were cut with an ultramicrotome, mounted on copper grids and observed with an H-7500 electron microscope at an accelerating voltage of 80kV.

Immunogold method

Some anesthetized rats were perfused with 2% PF and 0.05% GL in 0.1M PB via hearts. Ventral lobes of their prostates were removed, cut into small pieces and immersed in the same fixative at 4 °C for 30 min. They were dehydrated in a graded series of dimethylformamide (DMFA, Kanto-Chemical Co., Tokyo, Japan), embedded in Lowicryl K4M (Polyscience, Inc, Washington, USA) and polymerized under ultraviolet light at -20 °C for 36h. Ultrathin sections at 80nm thickness were cut and mounted on Formvar-coated nickel grids. They were blocked with 2% BSA and 5% normal goat serum in PBS for 30 min and incubated with the primary anti-Fas antibody at 1:1000 dilution in PBS containing 2% BSA at 4 °C overnight. Subsequently, they were incubated with goat anti-rabbit IgG antibody conjugated with 10nm colloidal gold (1:40; British Bio Cell International, Cardiff, UK) at room temperature for 1h. After being fixed with 2% GL in 0.1M PB for 10 min, they were counter-stained only with uranyl acetate and coated with carbon. The immunostained sections were routinely observed with an H-7500 electron microscope.

Results

Light microscopic immunoperoxidase technique

Immunohistochemical changes of prostates were found in apical sites of the epithelium at early stages in castrated rats (Fig. 1b), as compared with those in normal rats (Fig. 1a). Immunoreaction products of Fas were recognized in granular patterns mainly at apical sites of the prostate epithelium and Golgi areas (Fig. 1b, inset), and additionally localized in deep cytoplasm of the epithelial cells at later stages (Fig. 1c). On day 2 after the castration, Fas-immunopositive epithelial cells were found in more than half of the whole prostate epithelium (Fig. 1b). Furthermore, the Fas-immunopositive cells were spreading throughout the polarized epithelium on day 3. On day 4, many epithelial cells were positively immunostained not only in apical cytoplasmic parts, but also lateral and basal ones (Fig. 1c). On the contrary, no immunopositive reaction was found in sections obtaind from uncastrated rats (Fig. 1a) and in immunocontrol sections (data not shown).

Confocal laser scanning microscopy

The similar immunocytochemical changes which were obtained by the immunoperoxidase technique for the prostates, were also found in apical sites of the epithelium on day 2 (Fig. 1d). Immunoreaction products of Fas were recognized in granular patterns mainly at the apical cytoplasm and supranuclear regions of the prostate epithelium, and additionally localized on secretory vesicles in not only apical but also deep cytoplasm of the epithelial cells at later stages (Fig. 1e).

Pre-embedding immunostaining

At an electron microscopic level, the pre-embedding immunoperoxidase technique with cryostat sections demonstrated that Fas-immunoreactive products were localized mainly in vesicular structures along apical cell membranes and Golgi areas in supranuclear cytoplasm of prostatic epithelial cells on day 2 after castration (Fig. 2a,b). Most of the immunopositive epithelial cells were lining along the apical side of the epithelium.

Immunopositive apoptotic bodies were seen in some parts of the epithelium on day 2 after the castration (Fig. 2c). No immunopositive reaction was detected in sections obtained from uncastrated rats and immunocontrol sections (data not shown).

Immunogold method

As reported before (Kerr and Searle, 1973), pure ultrastructural examination showed that many secretory vesicles were localized in apical cytoplasm and around Golgi areas in the normal prostate epithelium (Fig. 3a). However, no immunopositive epithelial cells were detected by the Fas-immunogold labeling method (Fig. 3a, inset). Early ultrastructural changes of the epithelial cells after castration were found to be temporarily dilated cisternae of endoplasmic reticulum on day 1 after the castration, which were later followed by progressive

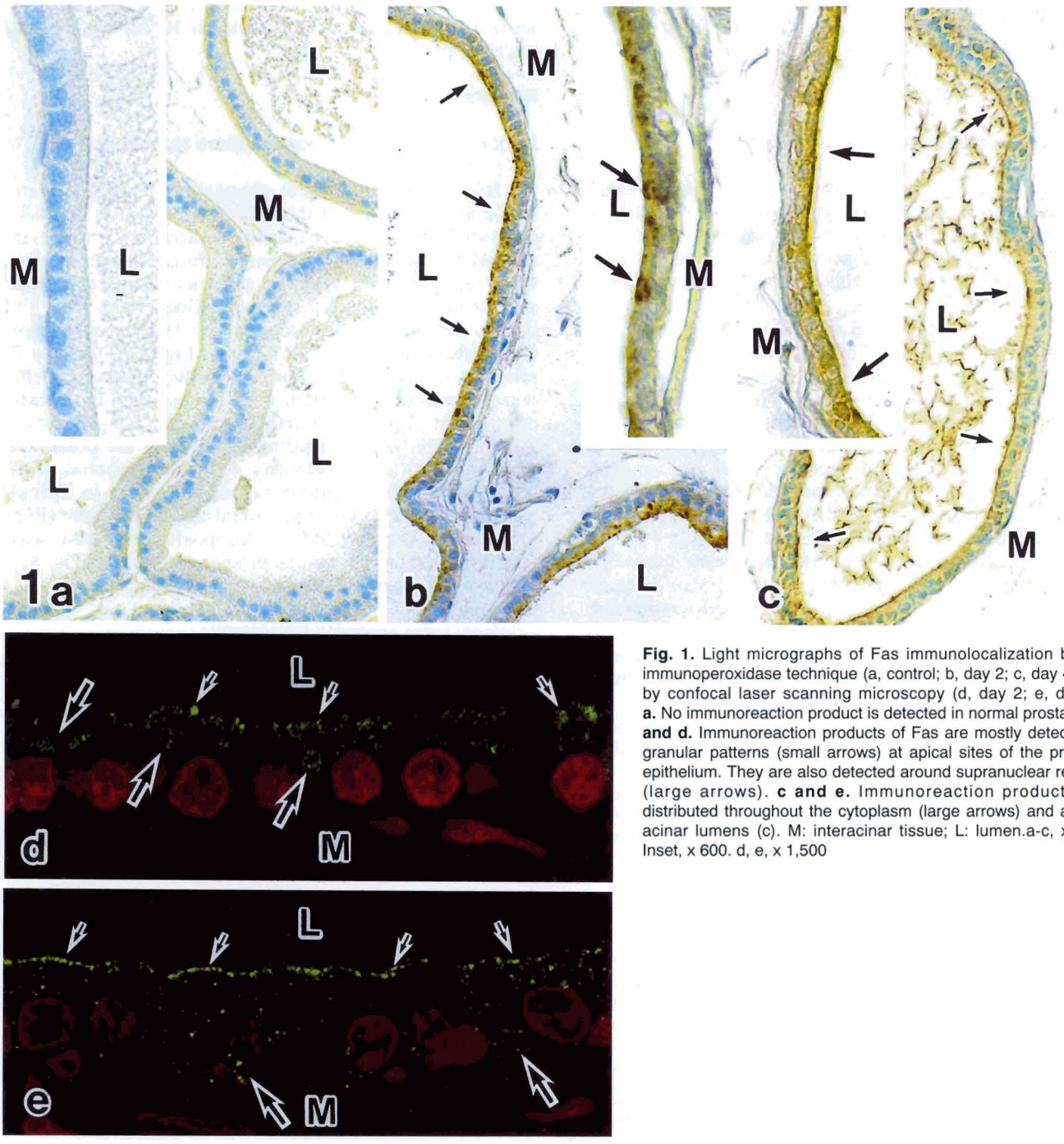

Fig. 1. Light micrographs of Fas immunolocalization by the immunoperoxidase technique (a, control; b, day 2; c, day 4) and by confocal laser scanning microscopy (d, day 2; e, day 4). **a.** No immunoreaction product is detected in normal prostates. **b and d.** Immunoreaction products of Fas are mostly detected in granular patterns (small arrows) at apical sites of the prostate epithelium. They are also detected around supranuclear regions (large arrows). **c and e.** Immunoreaction products are distributed throughout the cytoplasm (large arrows) and also in acinar lumens (c). M: interacinar tissue; L: lumen.a-c, x 480; Inset, x 600. d, e, x 1,500

depletion of endoplasmic reticulum, a major cell organelle responsible for the protein synthesis. A slight increase in sizes of secretory vesicles was detected in conventional ultrathin sections on day 1 after the castration (Fig. 3b). Some secretory vesicles were immunolabeled by the Fas-immunogold method (Fig. 3b, inset), showing that secretory materials were immunopositive for Fas. Further immunoreaction products in secretory vesicles were apparently seen in other epithelial cells and localized around Golgi areas on day 1 (Fig. 3c,d). Pure ultrastructural examinations showed that epithelial cells on day 2 after the castration still contained many vesicles and Golgi areas (Fig. 4a,b). Immunogold particles for Fas were also found in the secretory vesicles (Fig. 4c), and appeared to be localized in both peripheral and central regions of the vesicles. Some immunogold particles were seen on outer surfaces of the epithelial cell membrane (Fig. 4c).

On day 4, the reduction in height of epithelial cells was clearly detected (Fig. 5a), and secretory vesicles were apically localized in the epithelial cells. Some of them appeared to be fused to each other to form larger secretory vesicles, which were positively immunolabeled by the immunogold method (Fig. 5b, c), showing Fas localization in some epithelial cells on day 4 after the castration.

Discussion

In this study, we have reconfirmed that androgen ablation causes a rapid atrophy of the epithelium, which is confined to the ventral prostate (Sanford et al., 1984; Banerjee et al., 1995). At an electron microscopic level, the androgen ablation reduced both epithelial cell height and number of apical vesicles containing secretory materials at later stages of day 4 and 5, but at an early stage of day 2, the epithelial cells showed an increased number of vesicles around Golgi areas and at apical cytoplasmic regions. In normal rats, the cell number in prostate ventral lobes is regulated by cell proliferation and apoptosis (Leach, 1998). Most of the columnar epithelial cells, making up 80-85% of the ventral lobe, are dependent on androgenic steroids, and so this accounts for their response to androgen-withdrawal (English et al., 1987). It is also reported that the collapse and loss of endoplasmic reticulum are most prominent in epithelial cells of prostate glands at late stages after the castration. The numbers of ribosomes and mitochondria gradually decreased, depending on periods after the castration. Some secretory vesicles were apically concentrated in residual epithelial cells, as described before (Kerr and Searle, 1973). Our present ultrastructural features were almost similar to those reported before (Kerr and Searle, 1973; Sanford et al., 1984; English et al., 1987; Banerjee et al., 1995; Leach, 1998).

We have already reported the Fas immunolocalization in cultured prostatic epithelial cells at different concentrations of testosterone (Furuya et al., 2000). In the previous study, the Fas expression was seen

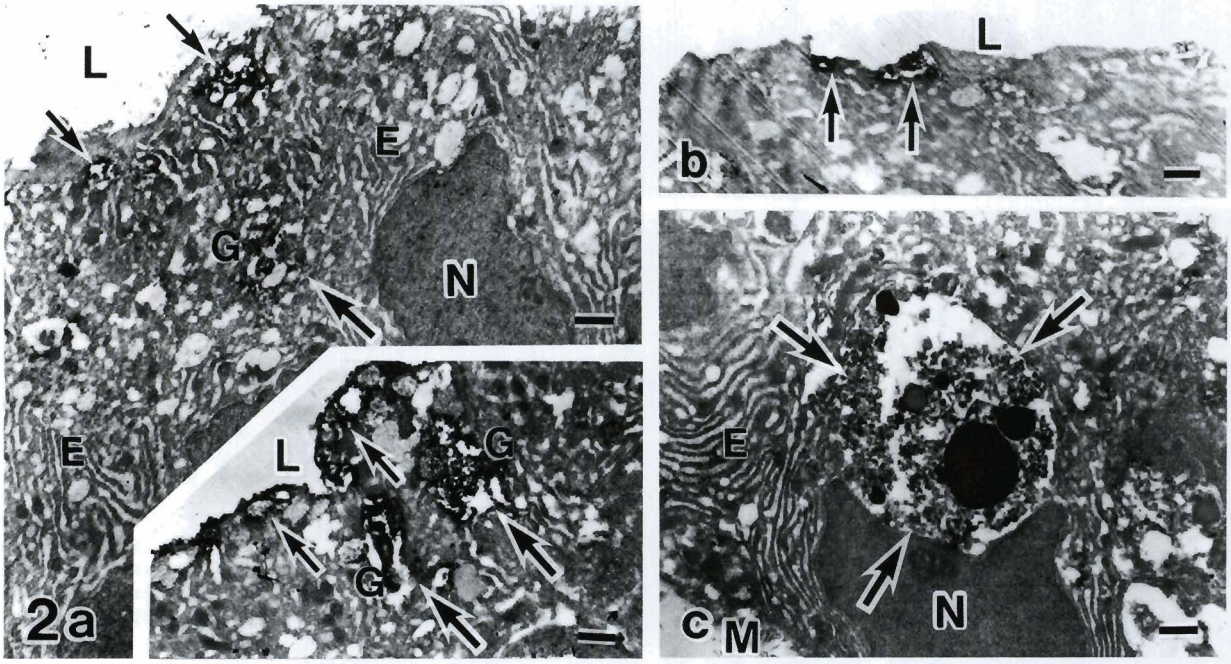

Fig. 2. Electron micrographs of Fas immunolocalization in the epithelium on day 2, as revealed by the pre-embedding immunoperoxidase technique. **a and b.** Immunoreaction products are detected at apical secretory vesicles (small arrows) and Golgi areas (G, large arrows) in some epithelial cells. Inset: Some parts of the surface cell membrane are also immunolabeled (small arrows). **c.** Apoptotic bodies are also immunopositive in cytoplasm of another epithelial cell (large arrows). N: nucleus; L: lumen; G: Golgi area; E: endoplasmic reticulum; M: interacinar matrix. Bar: 1.0 μm. x 5,000

under the culture condition of low dosage (10 ng/ml) of testosterone. In the present study, the immunocytochemical analyses confirmed the Fas expression in the prostatic epithelial cells of castrated rats. Most of the immunostained epithelial cells contained various sizes of secretory vesicles in their apical cytoplasm. However, a low amount of Fas was detected on the membrane surface of some epithelial cells, as revealed by electron microscopic immunocytochemistry. One probable reason might be a technical limit of the post-embedding

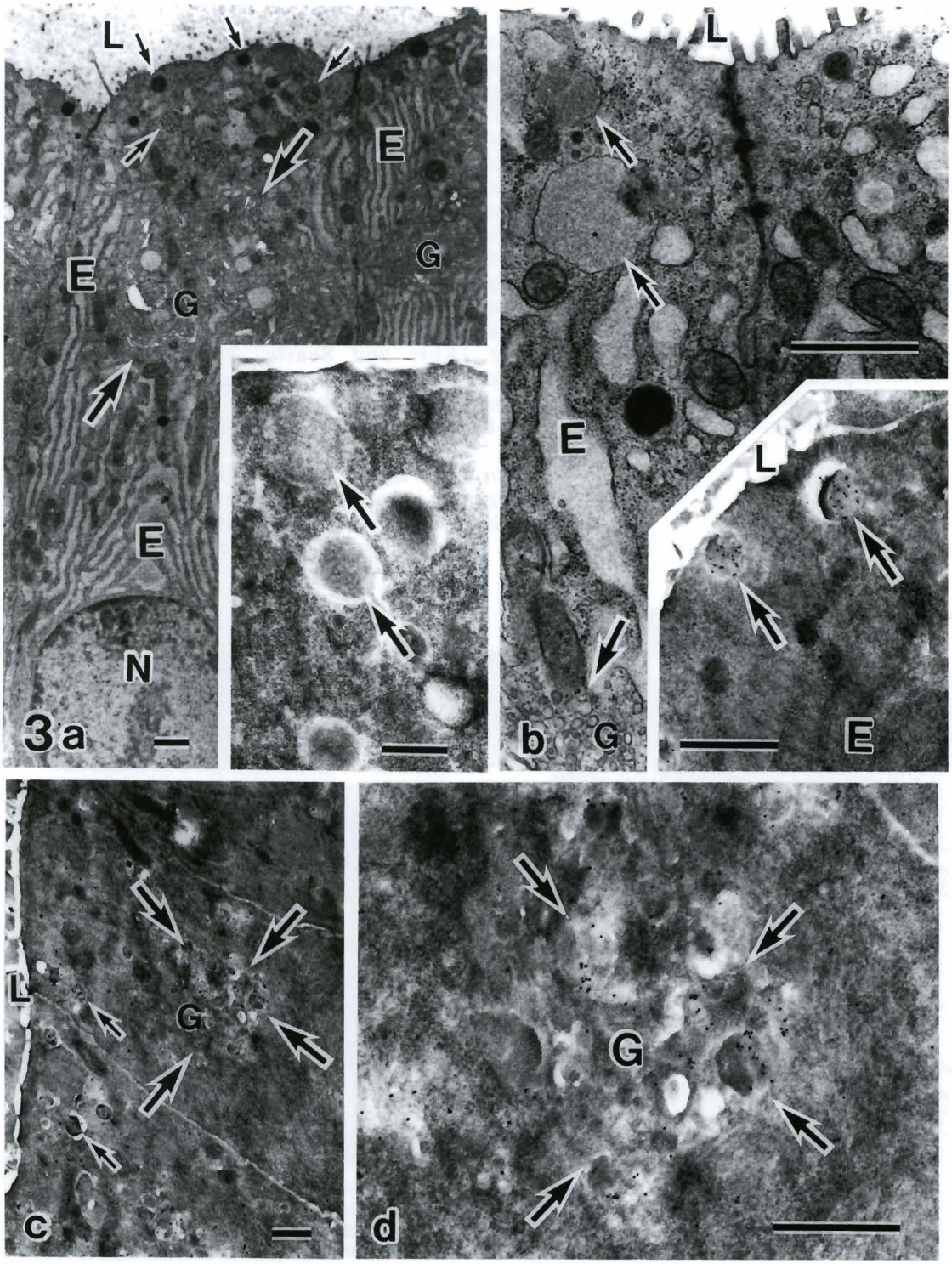

Fig. 3. a. Electron micrographs of prostate epithelial cells in normal uncastrated rats. Many vesicles are localized in apical cytoplasm (small arrows) and around Golgi areas (G, large arrows). Inset: No specific immunogold labeling is detected in normal epithelial cells (large arrows). N: Nucleus; G: Golgi area; E: endoplasmic reticulum; L: acinar lumen. Bar: 1 μm. x 5, 000. **b-d.** Electron micrographs of prostate epithelial cells on day 1 after the castration. b: Apical secretory vesicles are often seen (small arrows) and positively immunolabeled with immunogold particles for Fas (Inset, large arrows). Many small vesicles are localized around Golgi areas (G, large arrows). Bar: 1 μm. x 20,000. Inset: x 15,000. c: A Golgi area in supranuclear regions is also immunolabeled with immunogold particles for Fas. (G, large arrows). Small arrows indicate the immunogold labeling in apical secretoy vesicles. Bar: 1 μm. x 6,000. d: Higher magnification of the Golgi area shown in (c). Bar: 1 μm. x 20,000

immunogold method. However, it is hardly accepted in the present case, because many immunogold particles were actually seen inside secretory vesicles, as shown in Figures 3-5. Therefore, it is possible that a lower amount of the membrane Fas is localized along the vesicle membranes in comparison with the soluble Fas.

It was difficult to detect a clear relationship between Fas-positive epithelial cells and apoptotic cells. The Fas

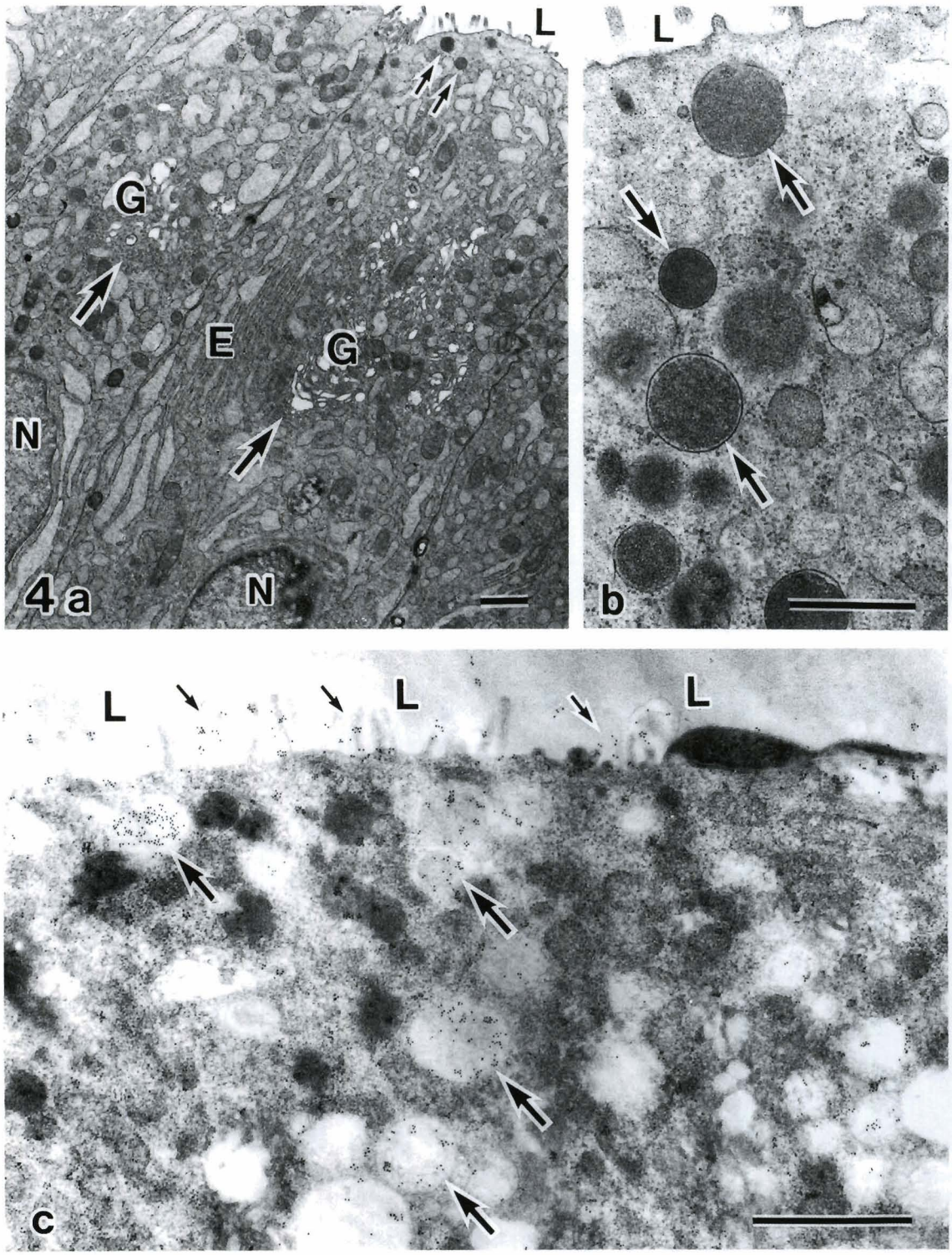

Fig. 4. Electron micrographs (a, b) and Fas immunogold labeling (c) of prostate epithelial cells on day 2 after castration. **a and b.** Some prostate epithelial cells still contain lots of cell organelles, including endoplasmic reticulum (E), Golgi apparatus (G, large arrows) and secretory vesicles, [small arrows in (a) and large arrows in (b). **c.** Many immunogold particles for Fas are scattered in some secretory vesicles (large arrows), and also detected along the apical cell surface of epithelial cells (small arrows). L: acinar lumen; N: nucleus. a, x 7,000; b, x 20,000; c, x 25,000. Bar: 1 μm.

in secretory vesicles seems not to be effective for inducing apoptosis, probably due to small amounts of the membrane Fas, as comparing a high percentage of the Fas-positive cells with the low percentage of apoptotic cells, as reported before (Kubo et al., 1998). If most of these vesicles contained large amounts of the membrane Fas, the number of apoptotic cells would be increased up to a similar percentage of the Fas-expressing epithelial cells. The present immunocytochemical data clearly indicate that Fas was localized mainly in secretory vesicles of the prostatic epithelium in castrated rats. Some previous studies have demonstrated that the prostatic epithelium expresses both Fas and Fas-ligand genes (Leithauser et al., 1993; French et al., 1996; Xerri et al., 1997), and an in vivo role of the Fas in the prostate was extensively studied by Suzuki et al. (1996). They used lpr (Fas mutant) mice which expressed very low levels of Fas due to a genetic mutation. In their study, the prostatic apoptosis was induced in normal mice on day 2 following castration, though the prostates of the lpr mice showed no sign of apoptosis even at 20 days after the castration. Therefore, it is suggested that Fas-Fas ligand system is one of the major pathways for apoptosis in the prostate epithelium after the castration. Such an in vivo role of Fas for apoptosis in rat prostates was also supported by an experiment using ethane-1,2-dimethanesulfonate (EDS) to induce androgen ablation in rats (Ian et al., 1998). In the study, the regression in weight of the EDS-treated rat prostates was evident in a time-dependent manner, and Fas expression was also detected after the androgen ablation. In the above-mentioned reports, it should be notified that the "Fas" means a membrane Fas for inducing the apoptosis of prostatic epithelial cells, not a soluble Fas. In the present study, however, it is demonstrated that lots of soluble Fas appeared to be secreted from the prostate epithelial cells in comparison with the membrane Fas.

Considering the appearance of the Fas-expressing cells and the apoptotic cells, our immunocytochemical data suggest two speculated mechanisms as follows. First, the Fas-expressing cells had potentiality for apoptosis, but they did not easily apoptose, because of relatively less amount of Fas-ligand in extracellular matrix. Second, if the Fas in secretory vesicles is almost soluble Fas, which is competitive against the membrane Fas for binding to Fas-ligand, the Fas-positive cells could survive in a manner of consuming the Fas-ligand. In other words, the soluble Fas-positive epithelial cells mean a surviving cell population. The present immunocytochemical study clearly indicates that Fas was produced in secretory forms, probably soluble Fas, in prostate epithelial cells of castrated rats.

Then it raised other important questions. The first question is whether soluble Fas in the vesicles plays a role as a receptor for the cell death signal. As mentioned above, Fas in the prostate epithelial cells can be produced in both types, such as a cell-surface protein and a soluble one. In the two different forms, the Fas can not only mediate the apoptotic signal upon binding with Fas-ligand, but also block the apoptosis through competitive binding with Fas-ligand, as discussed before (Papoff et al., 1996). In humans, the soluble Fas is reported to be elevated in serum of the patients with malignancies, indicating that it is synthesized and released from the tumor cells to protect themselves from

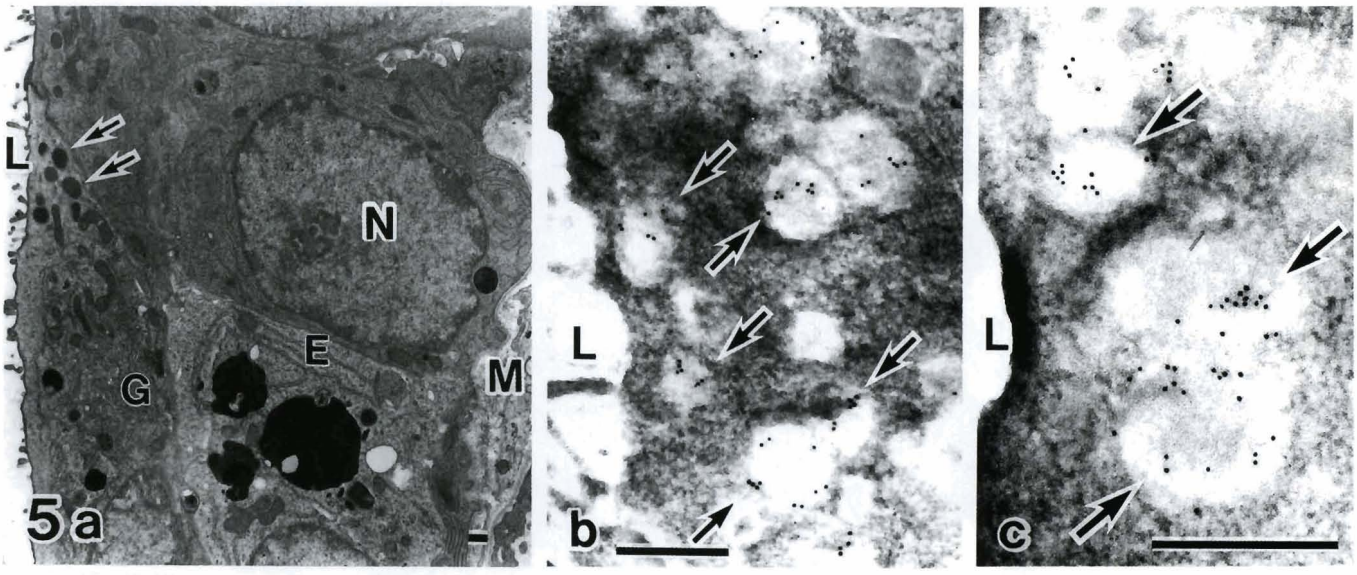

Fig. 5. Electron micrographs (a) and Fas immunogold labeling (b, c) of prostate epithelial cells on day 4 after castration. **a.** Many fewer cell organelles are seen in almost all epithelial cells. Arrows indicate secretory vesicles. Apoptotic bodies are also seen in an epithelial cell (right bottom). N: nucleus; G: Golgi apparatus; E: endoplasmic reticulum; L: acinar lumen; M: interacinar matrix. x 4,000. Bar: 0.5 μm. **b and c.** Some secretory vesicles are still labeled with immunogold particles for Fas (arrows). L: acinar lumen. b, x 30,000; c, x 50,000. Bar: 0.5 μm.

immune reactions (Lee et al., 1998). It was already reported that the soluble Fas also existed in rats (Kobayashi and Koike, 1996; Kimura et al., 1994). When the Fas-immunopositive secretory vesicles in the epithelial cells as seen in the present study contained lots of soluble Fas as described before, neutralization of Fas-ligand with the soluble Fas would inhibit triggering their apoptosis. So the soluble Fas-expressing cells may survive under the apoptotic conditions in comparison with Fas-negative cells. The second question is where Fas-ligand can be produced in the rat prostate. In this study, we could not yet detect Fas-ligand immunopositive cells in rat prostate tissues. It is speculated that relatively low amounts of Fas-ligand were consumed in the epithelium of castrated rat prostates for surviving under the condition of androgen ablation.

The last question is whether Fas-positive epithelial cells could apoptose immediately after the expression of Fas in their cytoplasm. As mentioned above, Fas-expressing epithelial cells were found to be more than a half of the whole prostate epithelium on day 2 after castration, as shown in Figure 1b. If all of the Fas-immunopositive cells immediately induce their apoptotic changes, they must disappear later. However, the rate of apoptosis in the epithelial cells was reported to be only about 10% at a peak under the similar experimental condition, as reported before (Kubo et al., 1998). Other investigators also reported that the prostate epithelial cells contained extremely high levels of Bcl-2 to survive after the castration, as compared with those in intact normal rats (McDonnell et al., 1992; Perlman et al., 1999). Therefore, some residual epithelial cells may survive in the apoptotic microenvironment, because of protective functions of Bcl-2, though other Fas-positive cells may express low levels of Bcl-2, resulting in apoptosis. It will be necessary to perform other biochemical, immunological or genetic confirmation of both soluble Fas and membrane Fas, Bcl-2 and Fas-ligand in the prostate tissues after castration, including their immunohistochemical examination.

Acknowledgements. We thank Drs. Nobuo Terada and Yasuhisa Fujii, and also Miss. Yasuko Kato, Department of Anatomy, Yamanashi Medical University, for their technical assistance during our present experiment.

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Accepted December 19, 2000