Leydig Cells Undergoing Apoptosis in the Perinatal Rat Testis

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It has been suggested that fetal Leydig cells in rat testes decrease in number just before or soon after birth. However, recent studies have indicated that during the perinatal period, the total number of the Leydig cells does not significantly change. The present study examined apoptotic cell death in the rat testis from embryonic day 18 to postnatal day 49. Apoptotic cells were examined by immunohistochemistry with an antibody against single-stranded DNA (ssDNA), and *in situ* terminal deoxynucleotidyl transferase staining (*in situ* TdT). During the perinatal period, ssDNA-immunopositive cells and *in situ* TdT-positive cells were scattered in the interstitium but were barely present in the

seminiferous tubules. After postnatal day 7, the positive cells were abundant in the seminiferous tubules but scarce in the interstitium. Double-immunostaining for ssDNA and a steroidogenic enzyme, P450c17, demonstrated that parts of P450c17-positive Leydig cells were ssDNA-positive. Electron microscopic observations revealed that *in situ* TdT-positive cells had characteristic features of Leydig cells. These results suggest that fetal Leydig cells undergo apoptosis during the perinatal period, and that the number of Leydig cells is regulated by both the rate of cell proliferation and apoptotic cell death.

Key words: Rat testis, Apoptosis, Single-stranded DNA, *In situ* TdT, Electron microscopy

I. Introduction

Testosterone production in rat testes changes markedly in various developmental stages. During the final third stage of gestation, the production increases and reaches a maximum on the 19th embryonic day (E19); then, it decreases and reaches a minimum at about 14 days after birth. Thereafter, it increases again and attains adult levels by 60 days after birth [3, 7, 19]. These two phases of androgen-producing activity correlate with two distinct Leydig cell types, fetal and adult Leydig cells [6, 13, 15].

In the perinatal period of the rat, the fetal Leydig cells have been assumed to decrease in number just before or soon after birth, and accordingly, reduce testosterone production [13, 15, 17]. However, recent studies have indicated that the total number of fetal Leydig cells in testes

does not markedly change between the end of pregnancy and the beginning of the neonatal period, and that fetal Leydig cells persist even in adult testes [9, 11, 21].

To elucidate the fate of fetal Leydig cells, the present study examined apoptotic cell death in rat testes from E18 to 7 weeks after birth.

Apoptotic cell death in germ cells of the postnatal rat testes has previously been examined. The level of apoptotic DNA fragmentation is age-dependent and peaks in 16- to 28-day-old rats; apoptotic cells are preferentially found in spermatogenesis-stages I and XII–XIV; spermatocyte is the major cell type undergoing apoptosis [1]. Apoptosis in the fetal and neonatal rat testes, however, has not been extensively examined.

In situ 3' end-labeling of DNA fragmentation by terminal deoxynucleotidyl transferase (*in situ* TdT) is routinely used to detect apoptotic cells in various tissues including the testis [1, 4]. Recently, the antibody against single-stranded DNA (ssDNA) has been demonstrated to be a good marker of drug-induced apoptosis and programmed cell death during embryogenesis [14], and to recognize ssDNA with various base sequences [8]. Furthermore, electron microscopy is essential for confirming

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the morphological characteristics of apoptotic cells. Using these methods we detected apoptosis in the fetal and postnatal rat testes.

II. Materials and Methods

Experimental animals and tissue preparation

Fetal, neonatal and adult male Wistar rats (Japan SLC, Shizuoka, Japan) were used. The day when spermatozoa were found in the vaginal smear was designated as embryonic day zero (E0). The day of birth, usually E22, was designated as postnatal day zero (P0). The animals were maintained on a 12-hr light-dark cycle at 20°C and fed lab chow and water *ad libitum*.

On gestational days 18 and 21 (E18 and E21), pregnant rats were sacrificed by cervical dislocation and the fetuses were removed. The testes of the embryos were immediately placed in ice-cold fixative consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) and kept overnight at 4°C. The testes of male newborns on P1 were removed under anesthesia by intraperitoneal injection (i.p.) of pentobarbital sodium (25 mg/kg body weight) and immersed in the fixative overnight at 4°C. The male rats at later postnatal ages (P7, P21 and P49) were perfused through the left ventricle with warmed (37°C) heparinized 0.1 M PB (pH 7.4), followed by the fixative under anesthesia (pentobarbital sodium 50 mg/kg body weight, i.p.). The excised testes were immersed in the same fixative overnight at 4°C. After fixation, some of the tissues were dehydrated in ethanol and embedded in paraffin blocks, and others were soaked in 10%, 15% and 20% sucrose in 0.01 M phosphate-buffered saline (PBS), pH 7.4, and frozen in O.C.T. Compound (Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo, Japan).

Immunohistochemistry

The paraffin-embedded sections 6 μ m in thickness were cut, mounted on glass slides coated with chrome alum gelatin, and air-dried overnight at 37°C. The sections were deparaffinized and then rehydrated in water. The frozen sections 8 μ m in thickness were cut, mounted on gelatin coated slides and dried for about 1 hr. These sections were soaked in 0.2% Triton X-100 in 0.01 M PBS for 20 min. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide in distilled water for 20 min at room temperature, and endogenous avidin/biotin binding was blocked with avidin D, followed by biotin (Vectastain, Vector, USA). The paraffin sections were then incubated overnight with the antiserum against single-stranded DNA (ssDNA) [8, 14, 20] diluted 1 : 1,000 in 0.01 M PBS containing 1% normal goat serum (NGS) at 4°C. The frozen sections were incubated overnight with the antiserum against cytochrome P450 of 17 α -hydroxylase/C17,20-lyase (P450c17) (provided by Dr. Kominami, Faculty of Integrated Arts and Sciences, Hiroshima University, Hiroshima, Japan) [10, 16] diluted 1 : 4,000 in

0.01 M PBS containing 1% NGS at 4°C. Thereafter, the sections were incubated with biotinylated goat anti-rabbit IgG diluted 1 : 100 in 0.01 M PBS containing 1% NGS for 1 hr at 32°C, followed by streptavidin-peroxidase (Histofine, Nichirei, Japan) for 1 hr at 32°C. Immunoreactivity was visualized in 50 mM TRIS-HCl buffer, pH 7.6, containing 0.01% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide, and examined by light microscopy.

As the control, some sections were incubated with normal rabbit serum in place of the antiserum against ssDNA or P450c17.

Double immunolabeling

Some paraffin-embedded sections were double-immunostained for ssDNA and P450c17. The sections were incubated with antiserum against ssDNA (1 : 1,000) overnight at 4°C. Then, the sections were incubated with biotinylated goat anti-rabbit IgG (1 : 100) for 1 hr at 32°C, followed by streptavidin-peroxidase (Histofine, Nichirei, Japan) for 1 hr at 32°C. The immunoreaction was first visualized (brown color) in 50 mM TRIS-HCl buffer, pH 7.6, containing 0.01% DAB and 0.01% hydrogen peroxide. After washing with 0.01 M PBS, the sections were incubated overnight with antiserum against P450c17 (1 : 4,000) at 4°C, followed by biotinylated goat anti-rabbit IgG (1 : 100) for 1 hr at 32°C, and finally with streptavidin-peroxidase (Histofine, Nichirei, Japan) for 1 hr at 32°C. Immunoreactivity for P450c17 was visualized (purple color) in 50 mM TRIS-HCl buffer, pH 7.6, containing 0.01% 4-chloro-1-naphthol and 0.01% hydrogen peroxide.

In situ TdT reaction

We used an ApopTag Plus-peroxidase kit (Oncor, Gaithersburg, MD). In brief, this kit uses terminal deoxynucleotidyl transferase (TdT) to catalyze the template-independent addition of digoxigenin-dUTP and dATP to 3'-OH ends of fragmented DNA. The incorporated nucleotides form a random heteropolymer of digoxigenin-dUTP and dATP, and then an anti-digoxigenin antibody conjugated to peroxidase is added.

In practice, the paraffin-embedded sections were deparaffinized, rehydrated, and soaked in 0.2% Triton X-100 in 0.01 M PBS for 20 min. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide in 0.01 M PBS for 20 min at room temperature. The sections were then incubated with a mixture of digoxigenin-conjugated nucleotides and TdT at 37°C for 3 hr, and subsequently treated with anti-digoxigenin conjugated to peroxidase at 37°C for 30 min. Immunoreactivity was visualized in 50 mM TRIS-HCl buffer, pH 7.6, containing 0.01% DAB and 0.01% hydrogen peroxide. As the control, some sections were incubated without TdT.

Electron microscopy

Frozen sections were examined by ssDNA-immuno-

histochemistry and *in situ* TdT staining, without incubation with 0.2% Triton X-100. After the peroxidase reaction, the DAB chromogen was intensified with silver-gold [12]. After intensification, the sections were treated with 1% osmium tetroxide for 1 hr at room temperature, rinsed with 10% sucrose in distilled water, stained with a saturated solution of uranyl acetate for 30 min at room temperature, rinsed with 10% sucrose in distilled water, dehydrated in a graded series of ethanol, and embedded in Epon. Ultrathin sections were cut on a Reichert Ultracut E, mounted on copper grids, and examined using a Hitachi H-500 electron microscope.

III. Results

In the paraffin sections of fetal rat testes on E18 and E21, ssDNA-immunoreactive cells and *in situ* TdT-positive cells were abundant in the interstitium of the

testis, and scarce in the immature seminiferous tubules (Fig. 1a, b, d, e). On P1, ssDNA- and *in situ* TdT-positive cells were also scattered in the interstitium (Fig. 1g, h). However, on P7, ssDNA- or *in situ* TdT-positive cells were rarely localized in the interstitium and occasionally observed in the seminiferous tubules (Fig. 2a, b). On P21, many positive cells for ssDNA and *in situ* TdT were present in the seminiferous tubules with some developed structures (Fig. 2d, e). On P49, a few of the ssDNA- and *in situ* TdT-positive cells were observed in the mature seminiferous tubules (Fig. 2g, h).

Immunoreactive cells for P450c17, a steroidogenic enzyme and a marker of Leydig cells, were observed in the interstitium of the testis in the frozen sections. Many immunoreactive cells were observed in fetal and neonatal rat testes (Fig. 1). They decreased in number per section and showed cluster-formation on P7 (Fig. 2c). On P21 and P49, the immunopositive cells increased (Fig. 2f, i).

Fig. 1. Staining of ssDNA (a, d, g), *in situ* TdT (b, e, h), and P450c17 (c, f, i) in the rat testis on E18 (a–c), E21 (d–f) and P1 (g–i). ssDNA-immunopositive cells (left column: ssDNA, a, d, g) and *in situ* TdT-positive cells (middle column: TdT, b, e, h) are mainly localized in the interstitium of the testis, and scarce in the seminiferous tubules that show immature structure at these fetal and neonatal stages. Immunoreactivity for P450c17 (right column: c17, c, f, i) is localized in the cytoplasm of the interstitial cells in the testis. P450c17-positive cells in the sections on E21 or P1 seem to decrease in number as compared with those on E18. Positive cells are indicated with arrows. s, seminiferous tubules. Bar = 100 μ m.

Fig. 2. Staining of ssDNA (a, d, g), *in situ* TdT (b, e, h), and P450c17 (c, f, i) in the rat testis on P7 (a–c), P21 (d–f) and P49 (g–i). First row (a–c): on P7, ssDNA-immunoreactive cells (a) and *in situ* TdT-positive cells (b) are occasionally observed in the seminiferous tubules and rare in the interstitium; P450c17-immunopositive cells (c) mostly form clusters and are scattered in the interstitium. Second row (d–f): on P21, many positive cells for ssDNA (d) and *in situ* TdT (e) are localized in the seminiferous tubules that show a developing structure; P450c17-immunopositive cells (f) are increased in number in the interstitium. Third row (g–i): on P49, ssDNA- (g) or *in situ* TdT-positive (h) cells are occasionally localized in the seminiferous tubules with mature structure. P450c17-positive cells increase in number (i). Positive cells are indicated with arrows. s, seminiferous tubules. Bar = 100 μ m.

Fig. 3. Double immunostaining of ssDNA and P450c17 in the rat testis on P1 (a, b). Some of the P450c17-immunopositive cells (bluish purple) contain ssDNA-immunoreactivity (brown) in their cell nuclei (arrows). Some ssDNA-positive nuclei are spindle-shaped (arrowheads). s, seminiferous tubules. Bar = 30 μ m.

There was no reaction in the control sections incubated with normal rabbit serum or incubated without the TdT enzyme (data not shown).

The immunoreactivity of ssDNA was observed in the nuclei, and the P450c17 immunoreactivity was found in the cytoplasm (Fig. 3). The ssDNA-positive nuclei were round or spindle-shaped. Double immunostaining of ssDNA and P450c17 demonstrated that some of the P450c17-immunopositive Leydig cells contained ssDNA immunoreactivity in the nuclei (Fig. 3).

Although immunocytochemistry of ssDNA was not successful in the frozen sections, *in situ* TdT-positive cells

in the fetal and neonatal rat testes were observed by electron microscopy using the silver-gold intensification and pre-embedding method. Silver grains corresponding to the DAB chromogen were present in fragmented nuclei with condensed chromatin (Fig. 4). The grains were preferentially deposited on the surface of the nucleus. In Fig. 4a, lipid droplet-like small vesicles were observed in the shrunk cytoplasm surrounding the fragmented nucleus. Fig. 4b showed that *in situ* TdT-positive nuclei with condensed chromatin were phagocytosed by a cell with a bean-shaped nucleus.

Fig. 4. Electron micrograph of *in situ* TdT-positive cells in the rat testis on P1. **a:** Silver grains, which correspond to the location of the DAB endproducts, are present in the fragmented nucleus with condensed chromatin (arrows). Small lipid droplets (arrowheads) are observed in the shrunk cytoplasm surrounding the fragmented nucleus. **b:** Silver grains are also observed in the nuclei with condensed chromatin (arrows). Some of these *in situ* TdT-positive nuclei are phagocytosed by a cell with a bean-shaped nucleus (N). Bars = 2 μ m (**a**), 3 μ m (**b**).

IV. Discussion

This study has demonstrated that ssDNA-immunoreactive and *in situ* TdT-positive cells are preferentially localized in the interstitium of the testes of fetal and neonatal rats. Parts of the ssDNA-immunopositive cells also possessed P450c17-immunoreactivity in the cytoplasm. These results suggest that some fetal Leydig cells undergo apoptotic cell death.

Fetal Leydig cells of rats were believed to decrease in number just before or soon after birth [13, 15, 17]. However, recent studies have indicated that the total number of fetal Leydig cells does not change markedly in the perinatal period, even though the number of Leydig cells per unit volume decreases [9, 11, 21]. The present study has demonstrated apoptosis of fetal Leydig cells, and suggests that fetal Leydig cells keep a balance between cell proliferation and cell death in the perinatal period, and that consequently the total number of the Leydig cells does not change.

In the adolescent and adult rat testes, cells in the interstitium rarely expressed ssDNA- or *in situ* TdT-reactivity (Fig. 2). This difference in frequency of apoptosis may be of a different character which lies between fetal and adult Leydig cells. Apoptotic cell death of germ cells in the postnatal rat testis has already been elucidated [1].

There were some ssDNA-positive and P450c17-negative cells in the interstitium of fetal and neonatal rat testes (Fig. 3). Parts of these cells had round nuclei and may be Leydig cells with shrunk cytoplasm undergoing apoptosis. Other cells have demonstrated spindle-shaped nuclei and may be fibroblasts or Leydig cells with shrunk nucleus and cytoplasm. We could not determine the characteristics of these cells in this study. Further electron microscopic study is needed to elucidate the characteristics of ssDNA-positive and P450c17-negative cells.

The antibody against single-stranded DNA has been recently demonstrated to be a marker of drug-induced apoptosis and programmed cell death during embryogenesis [14]. It is suggested that degradation of the supercoiling organization of DNA to single strands is one of the DNA degradation steps during apoptosis and programmed cell death as well as of oligonucleosomal fragmentation [14]. The present study has demonstrated that ssDNA-immunopositive cells have nearly identical localization and number as *in situ* TdT-positive cells at all stages of development. These results suggest that ssDNA-immunohistochemistry detects apoptotic nuclei as effectively as *in situ* TdT-staining.

Apoptotic cell death is usually confirmed by electron microscopic detection of nuclear chromatin condensation and cytoplasmic shrinkage [18], because *in situ* TdT-positive cells are occasionally necrotic cells such as those in the liver of CCl₄-injected rat [5]. This study has confirmed the apoptotic characteristics of the cells in the interstitium of perinatal rat testes using the *in situ* TdT and the silver-gold intensification method [12]. The intensification

method facilitated the detection of *in situ* TdT-positive cells as shown in Fig. 4. These positive cells may be apoptotic bodies, which are shrunk apoptosis-cells splitting into small membrane-bounded bodies [18]. Because lipid droplet-like vesicles are found in these apoptotic bodies (Fig. 4a), the bodies may be derived from fetal Leydig cells. Apoptotic bodies are usually phagocytosed by macrophages and neighboring cells. The phagocytosis is shown in Fig. 4b, and the phagocyte may be a macrophage because of its characteristic bean-shaped nucleus. Recently, Collins *et al.* [2] has demonstrated that *in situ* nick-translation signals are localized around apoptotic nuclear fragments, and that they have a circular or ring pattern. The silver grains in Fig. 4 are also located in the periphery of the nucleus with condensed chromatin. DNA fragmentation of the apoptotic nucleus may preferentially occur in the periphery of the nucleus.

In summary, this study has shown that apoptotic cells are localized in the interstitium of perinatal rat testis. These results suggest that fetal Leydig cells undergo apoptosis, and that the number of fetal Leydig cells is regulated by the balance between cell proliferation and apoptotic cell death.

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VI. References

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