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ORIGINAL PAPER

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Penile apoptosis in association with p53 under lack of testosterone

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Abstract It is known that testosterone deficiency induces apoptosis in the prostate and that p53 protein is involved in this apoptosis. Therefore, p53 protein may also be involved in apoptosis induction in a testosterone-deficient state in the penis. In this study, we investigated whether castration and chemical castration induce apoptosis at penile tissue in rats, and whether p53 protein is involved in this apoptosis. Male SD rats aged 8 weeks were divided into four groups: 1) the Control group; 2) the Castration group; 3) the Estrogen group, in which rats received βestradiol 17-(β-D-glucuronide) injection of 500 μg/body/day; and 4) the LH-RH group, in which rats received LH-RH analogue (leuprorelin acetate) injection of 2 mg/kg. The rats were sacrificed after treatment on days 1, 3, 5, 14, and 28 by cervical dislocation. Apoptotic cells and p53 protein-positive cells were observed on the 5th day after treatment and thereafter in all castration, estrogen, and LH-RH groups. These findings showed that both castration and chemical castration induced p53 protein in vascular endotherial cells in the corpus cavernosus during the process of losing testosterone. It was also suggested that in such states, apoptosis is induced in vascular endotherial cells in the corpus cavernosus.

Keywords Corpus cavernosus · Apoptosis · Testosterone · p53

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Introduction

The erection mechanism has recently been clarified by elucidating the construction and nervous system of the corpus cavernosus, and testosterone has been shown to act not only on the central nervous system but also directly on the erection in the local penile region. NO, which relaxes vascular smooth muscle in the corpus cavernosus, plays an important role in penile erection [2, 3, 21]. Testosterone is known to play a role in NO synthesis at nerve ending in the penis [14, 18], showing that testosterone plays an important role in local erectile action via NO [15, 16]. However, the direct action of testosterone on the corpus cavernosus has not been clarified. Androgen receptors exist in the penis, and a high concentration of testosterone exists in endotherial cells in the corpus cavernosus [5]. Some reports have suggested that testosterone acts not only on NO-mediated erection [17, 19, 22]. In castrated male dogs, the ratio of collagen increased in the corpus cavernosus [9]. It has been reported that apoptotic cells were observed in the corpus cavernosus on the 3rd and 5th days after castration [20]. Detection of apoptotic cells in the corpus cavernosus on diabetic rats has also been reported [1]. Therefore, apoptosis is induced in the corpus cavernosus in castrated and in diabetic state. In the prostate, abundant androgen receptors exist, and the development of this organ depends on male hormones [23]. Apoptosis is induced on the terms of testosterone deficiency [13], and p53 protein is involved in the apoptosis [6, 8]. Apoptotic bodies have also been reported in post-castrated penile specimens [20]. This report suggested that a remodeling process occurred in the penis of castrated rats involving apoptosis, especially in the erectile tissue. Therefore, we performed this study, hypothesizing that testosterone plays a role in maintaining the construction of the corpus cavernosus, and apoptosis involving p53 protein is induced in the corpus cavernosus in a testosterone-deficient state, which changes the construction of the corpus cavernosus.

Materials and methods

Animals

Male Sprague-Dawley rats (body weight 250–300 g, aged 8 weeks, Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were used for all the experiments. The rats were housed in our vivarium and allowed to acclimatize for 7 days. The housing environment was maintained at 21°C with a 12-h light: 12-h dark ratio. The rats were fed standard feed pellets and water. These rats were categorized into four groups as follows: 1) the Control group, which comprised 35 rats that underwent sham operation; 2) the Castration group, which comprised 35 rats that underwent bilateral orchiectomy; 3) the Estrogen group, in which 35 rats received βestradiol17-(β-D-glucuronide), sodium salt (Sigma Chemicals, St. Louis, MO, USA) injection of 500 μg/body/day; and 4) the LH-RH group, in which 35 rats received LH-RH agonist (leuprolelin acetate, Takeda, Osaka, Japan) injection of 2 mg/kg. The rats were sacrificed after treatment ondays 1, 3, 5, 14, and 28 by cervical dislocation. The penis was removed and isolated for histopathological evaluation and for immunohistochemical staining to detect apoptotic cells and cells that induced p53 protein.

Serum testosterone level measurement

Before the rats were sacrificed, venous blood samples were obtained from the inferior vena cava and were assayed to determine the serum testosterone level with radio-immunoassay [7].

Histological procedure

The isolated penis were routinely processed for histological evaluation; the penile tissue was immediately embedded in paraffin and serial tissue sections were prepared. After thin sections (5 μm) were stained with haematoxylin and eosin (HE), the penile corpus cavernosus were observed.

In situ detection of apoptosis

To detect apoptotic cells in 5-μm thick sections of formalin-fixed and paraffin-embedded tissue, TdT-mediated dUTP-biotin nick end labeling (TUNEL) was used according to the procedures included in the Apop tag in situ apoptosis detection kit (Oncor, USA). Briefly, after routine deparaffinization and blocking of endogenous peroxidase with 0.3% hydrogen peroxide in methanol for 30 min at room temperature, sections were incubated with 100 μg/ml proteinase K (Sigma Chemicals, St. Louis, MO, USA) for 15 min at room temperature. After prehybridization treatment, the sections were exposed to TdT with digoxigenin-11-dUTP labelling for 30 min at room temperature, followed by exposure to 0.05% diaminobenzidine (DAB). Sections were counterstained with 0.5% methyl green solution. The penile sections were stained with an anti-FAS antibody and an anti-FAS-L antibody, and further stained using the TUNEL method. The anti-FAS antibody and an anti-FAS-L antibody were purchased from Sigma Chemicals (St. Louis, MO, USA), and the Biotin-21-dUTP3′-End Labeling Kit for the TUNEL procedure was purchased from CLONTECH (Palo Alto, CA, USA).

In situ detection of p53 protein

Five-μm sections of penile tissues were cut, air dried, washed three times in phosphate-buffered saline (PBS) and fixed in ethanol. The sections were stained by indirect immunofluorescence with a mouse monoclonal antibody against rat p53 protein (Development Studies Hybridoma Bank, Iowa City, USA), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappel Laboratories, Malvern, PA, USA), and also after tissue denaturation by incubation in acid urea (6 M urea/0.1 m glycine HCl buffer, pH 3.5) to disclose cryptic epitope.

Western blot analysis

For immunodetection, all procedures were performed in Tris-buffered saline (50 mM Tris-HCl, pH 7.5/0.15 M NaCl, 0.2% NaN3) containing 0.05% Tween 20. The membrane was blocked with 5% skim milk and subsequently incubated with a 1:2,000 dilution of rat anti-p53 antibody and 1:5,000 dilution of horse radish peroxidase (HRP)-conjugate of affinity-purified goat anti-rabbit IgG (Sigma Chemicals, St. Louis, MO, USA). The immunocomplex was detected on photographic film by H_2O_2/luminol chemiluminescence (Amersham, Little Chalfont, UK) and scanned. Significance of difference was analyzed by t-test.

Results

Serum testosterone level measurement

The testosterone level tended to decrease after the 5th day in the castration and estrogen groups compared with that in the control group, and the level further decreased significantly on the 14th and 28th days. In the LH-RH group, the testosterone level tended to decrease after the 14th day compared with that in the control group, as in the castration and estrogen groups, and it further decreased significantly on the 28th day (Table 1).

Histological procedure

On HE staining, no changes were observed over time in vascular endotherial cells, smooth muscle cells, or interstitial cells in the control group. In the castration and estrogen groups, no changes were observed until the 5th day. Interstitial collagen markedly increased after the 14th day compared with that in the control group, and further increased on the 28th day. In the LH-RH group, interstitial collagen increased after the 14th day compared with that in the control group, as in the castration and estrogen groups, but the increase was slight (Fig. 1).

In situ detection of apoptosis

Positive cells on TUNEL staining were detectable in the nuclei of penile tissues. No apoptotic cells were detected in the penile tissue in the control group. In contrast, in the castration and estrogen groups, apoptotic cells were observed in the penile skin and vascular endotherial cells in rats sacrificed after the 5th day after treatment, and the frequency of apoptotic cell-positivity did not change on the 14th or 28th day. In the LH-RH group, apoptotic cells were observed in the penile skin and vascular

Table 1 Serum testosterone level measurement

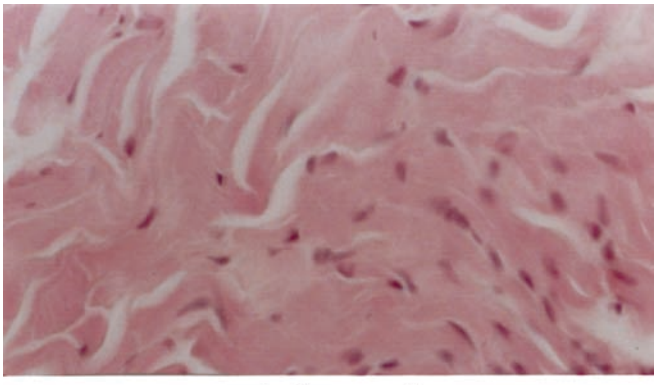
Group	Testosterone (ng/dl)				
	Day 1	Day 3	Day 5	Day 14	Day 28
Control	392 ± 61	390 ± 37	394 ± 39	387 ± 45	394 ± 53
Castration	345 ± 46	158 ± 40	97 ± 29*	42 ± 11*	17.9 ± 11**
Estrogen	310 ± 50	121 ± 42*	45 ± 37*	24 ± 10**	13.2 ± 9**
LH-RH	425 ± 62	364 ± 53	325 ± 50	156 ± 30	23.4 ± 9**

* and ** represent $p < 0.05$ and $p < 0.01$, respectively, which indicates that the values are significantly lower than those in the control group. The testosterone level tended to decrease after the 5th day in the castration and estrogen groups compared with that in the control group, and the level further decreased significantly on the 14th and 28th days. In the LH-RH group, the testosterone level tended to decrease after the 14th day compared with that in the control group, as in the castration and estrogen groups, and the level further decreased significantly on the 28th day

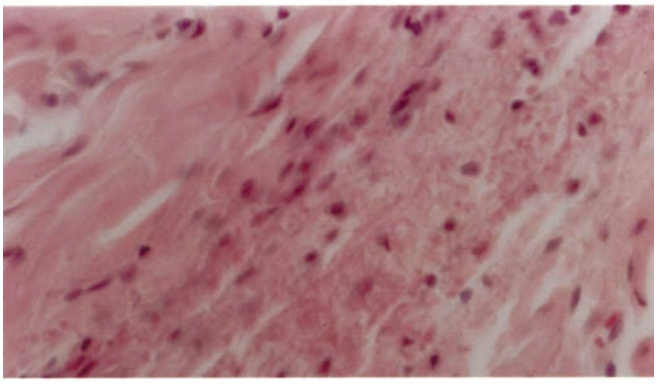
endotherial cells after the 14th day, and the frequency did not change on the 28th day (Fig. 2).

In situ detection of p53 protein

The expression of p53 protein was detected in the penile skin and vascular endotherial cells in the corpus cavernosus in rats sacrificed after the 5th day in the castration and estrogen groups. The frequency of p53 protein positivity did not change on the 14th and 28th days. In the LH-RH group, p53 protein was observed in the penile skin and vascular endotherial cells after the

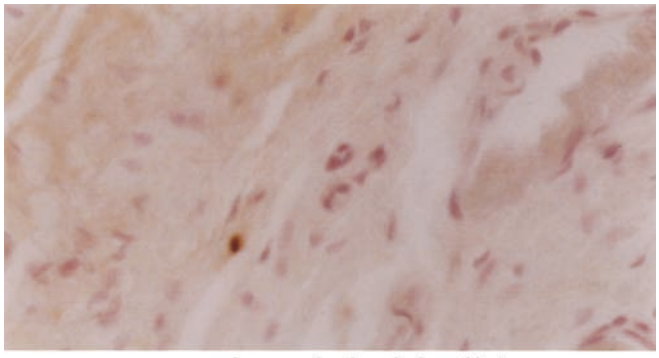


control group A

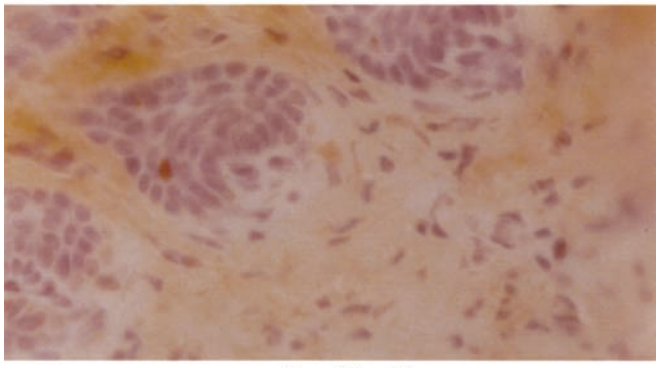


castration group B

Fig. 1A, B Section at day 28 from Control group (**A**) and Castration group (**B**). All sections, haematoxylin and eosin (HE). There were no time-course changes in the vascular endotherial cells, smooth muscles cells, or interstitial cells in the control group. In contrast, in the castration and estrogen groups, no changes were observed until the 5th day, but interstitial collagen markedly increased after the 14th day compared with that in the control group, and collagen further increased on the 28th day. **A, B** ×400



vascular endotherial cell A



penile skin B

Fig. 2A, B Detection of apoptotic cells by TUNEL staining. A tissue section on the 28th day. TUNEL-positive cells detected in the LH-RH group. No TUNEL-positive cells were observed, nor were there apoptotic cells in the penile tissue in the control group. In contrast, in the castration and estrogen groups, apoptotic cells were observed in penile skin and vascular endotherial cells in rats killed after the 5th day post-treatment, and the frequency of apoptotic cell positivity did not change on the 14th or 28th day. In the LH-RH group, apoptotic cells were observed in the penile vascular endotherial cells (**A**) and skin (**B**) after the 14th day, and the frequency of apoptotic cell-positivity did not change on the 28th day. **A, B** ×400

14th day and the frequency did not change on the 28th day (Fig. 3).

Western blotting

The band at lane of p53 protein was detected in the tissue from rats by Western blotting after the 5th day, and p53 protein was not detectable in the control group. In the castration and estrogen and LH-RH groups, the expression of p53 protein was detected (Fig. 4).

Discussion

During the development and maturation of most organisms, the removal of unwanted cells (programmed cell death) is important; the cells are destroyed by apoptotic pathway [10, 11]. Apoptosis also plays an important role in the development of several diseases. When apoptosis is hindered, precancerous cells are not eliminated, viral infections are enhanced, and autoimmune reactions are not suppressed. Conversely, when apoptosis is promoted, neurodegenerative disorders such as Alzheimer's disease and graft rejection may develop [4]. Apoptosis may cause some reduction of the erectile tissue within the corpora. It has been shown by Klein et al. [12] that the cavernosal neurotomy of the rat induces the loss of rat erectile cells by apoptosis and may account for postoperative impotence or the perceived reduction in penile mass after radical prostate surgery. There is a report that there is a very high number of apoptotic bodies seen in post castration penile specimens, particularly in the corpora cavernosa and corpus spongiosum [20]. The findings described above suggest that testosterone plays a role in the penile tissue construction, and apoptosis is induced in the penile tissue in a testosterone-deficient state, which may change the tissue construction. Testosterone plays an important role in the development and tissue construction of the prostate. When testosterone is deficient, apoptosis is induced, and p53 protein is involved in the apoptosis. In the penis, which is affected by testosterone similarly to the prostate, apoptosis may also be induced in a testosterone-deficient state, and p53 protein may be involved in the apoptosis. In this study, the greatest decrease in the serum testosterone level was observed in rats in the castration and estrogen groups sacrificed on the 14th and 28th days.

Collagen increased in the corpus cavernosus in rats in the castration and estrogen groups sacrificed on the 28th day compared with that in the control group. The induction of apoptotic cells in the corpus cavernosus was observed in rats in the castration and estrogen groups sacrificed after the 5th day. On immunostaining, p53 protein was detected in the corpus cavernosus in rats sacrificed after the 5th day in both the castration and estrogen groups.

On Western blotting of p53 protein in the tissue from rats sacrificed after the 5th day, p53 protein was not detected in the control group, but was detected in the castration and estrogen groups. Our study suggests that a remodeling process occurs in the penis of castrated and chemically castrated rats involving apoptosis, especially in the erectile tissue. p53 protein was suggested to be

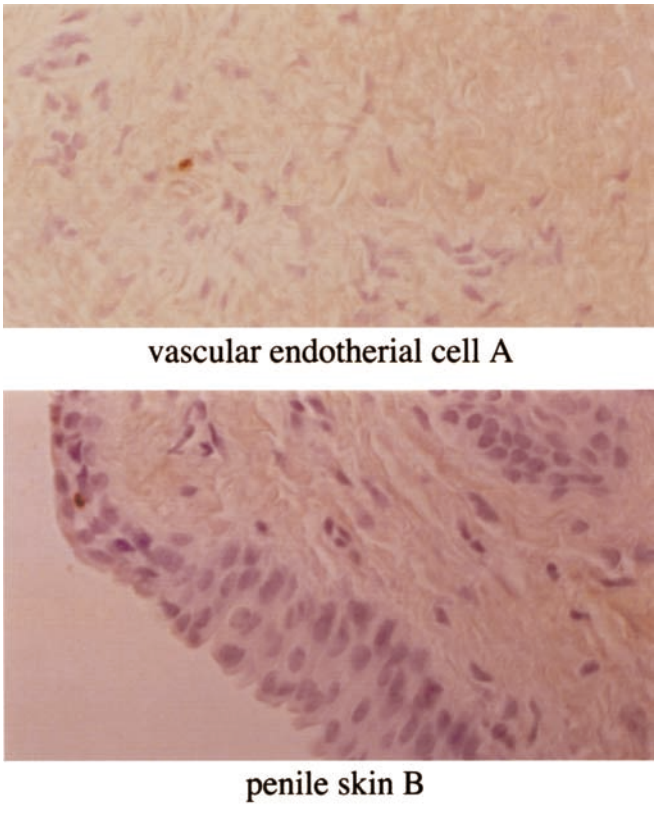


Fig. 3A, B Detection of p53 protein expression by immunohistochemistry. A tissue section on the 5th day. Occurrence of p53 protein-positive cells in Castration group. In the control group, no p53-positive cells were observed. p53 protein was detected in the penile skin and vascular endothelial cells in the corpus cavernosus from rats killed after the 5th day post-treatment in the castration and estrogen groups. The frequency of p53 protein positivity did not change on the 14th and 28th days. In the LH-RH group, p53 protein was observed in the penile skin and vascular endothelial cells after the 14th day, and the frequency of p53 protein positivity did not change on the 28th day. **A** p53-positive cells detected in the vascular endothelial cells in the corpus cavernosus from rats killed after the 5th day post-treatment in the castration group. **B** p53-positive cells detected in the penile skin in the corpus cavernosus from rats sacrificed after the 5th day after treatment in the castration group. **A, B** ×400

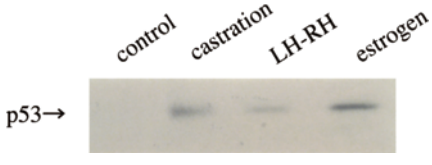


Fig. 4 Western blotting of p53 protein in the tissue from rats killed on the 14th day. p53 protein was not detectable in the control group. In the castration, estrogen, and LH-RH groups, p53 protein was detected

involved in the apoptosis. The above findings showed that p53 protein was induced in vascular endotherial cells and the penile skin in the corpus cavernosus during the process of losing testosterone after castration and chemical castration. It was also shown that in such states, apoptosis was induced in vascular endotherial cells and the penile skin in the corpus cavernosus and interstitial collagen increased, which may change the penile tissue construction. It is also considered that castration and chemical castration irreversibly change the corpus cavernosus tissue. Further research is needed to identify the role of apoptosis in the pathophysiology of erectile dysfunction caused by castration and chemical castration.

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