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Changes in Apoptosis-Related Genes (Bcl-2, Bax) in the Urethras of Old Female Rats Following Estrogen Replacement

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Following estrogen replacement in old female rats, changes in apoptosis-related genes (Bcl-2, Bax) in the urethra were investigated by immunohistochemical and RT-PCR analysis. A total of 40 old (16-month-old) female Wistar rats were divided into the following 5 groups: normal controls; rats replaced with low-dose estradiol (E_2) for 2 and 4 weeks, respectively; and rats replaced with high-dose E_2 for 2 and 4 weeks, respectively. After treatment, the urethras were removed, weighed and stained with hematoxylin and eosin, and then immunohistochemical stainings of Bcl-2 and Bax were performed. In addition, the expressions of the apoptosis-related genes *bcl*-2 and *bax* were investigated by PCR analysis, and then evaluated by computerized quantification. In the E_2-replaced rats, the immunostained urethras showed little immunoreactivity against the Bcl-2 protein, and in the control rats, no reactivity was seen. In contrast, the control rats showed immunoreactivity against the Bax protein, whereas the E_2-replaced rats showed little reactivity. Upon mRNA analysis, the expression of *bcl*-2 mRNA was uprisen in rats in the high-dose E_2 for 4 weeks group as compared with the controls, but the expression of *bax* mRNA was suppressed in the E_2-replaced rats as compared with the controls. As these results suggest, estrogen replacement up-regulated the expression of *bcl*-2 mRNA and down-regulated the expression of *bax* mRNA, which might suppress the apoptic action: these changes might alter lower urinary tract findings.

Key words: apoptosis; estrogen replacement; old rat; urethra

During menopause, the drop in serum levels of estrogen causes metabolic and atrophic alterations in many organs (Versi, 1990). Postmenopause urogenital atrophy disorder is characterized by dysuria, urinary urgency, pollakisuria and urinary incontinence (Suguita et al., 2000).

It is known that the urethra, like the vagina, is affected by estrogen, and that hypoestrogenism at menopause results in urogenital atrophy in all women, regardless of race, nationality or socioeconomic status. With progressing estrogen deficiency and advancing age, the urethral epithelium becomes thinner, and loses its vascularity, elasticity and regulation (Bachmann, 1998). In addition, estrogen depletion causes atrophic urethritis, and sometimes accounts for urinary incontinence (Elia and Bergman, 1993). Bhatia et al. (1989) reported that estrogen therapy could improve the proliferation and growth of the urethral mucosa, and that it could have a positive effect on urethral elasticity. In another study,

Abbreviations: E_2, estradiol; HE, hematoxylin and eosin; RT, reverse transcription; UIP, universal immuno-enzyme polymer

cytological examination revealed that estrogen treatment for 3 weeks induced proliferation of the atrophic urethral mucosa in postmenopausal women suffering from urinary incontinence (Ulmsten and Stormby, 1987).

Recent studies have indicated that estrogen regulates cell survival and cell death factors. In breast cancer cells (Teixeira et al., 1995; Kandouz et al., 1996; Huang et al., 1997), the expression of *bcl*-2 and other members of the *bcl*-2 family (*bax*, *bad* and *bcl*-x), by which estrogen regulates, has been shown to inhibit apoptosis. In brain cells (Dubal et al., 1999), estrogen prevented the injury-induced down regulation of *bcl*-2 expression.

In the present study, following estrogen replacement therapy, we investigated the changes in apoptosis-related genes (Bcl-2, Bax) on the urethras of old female rats.

Materials and Methods

Animals

The animal experiment was performed at Laboratory Animal Center of Tottori University, in accordance with the guidelines of the University. Estrogen replacement was performed according to Furuta et al. (2002). Female Wistar rats (Shimizu, Kyoto, Japan) were divided into 5 groups: each contained 8 animals. A silastic tube (inside diameter = 1.5 mm, outside diameter = 2.5 mm, length = 30 mm) (Kaneka Medix, Osaka, Japan) containing sesami oil (Kadoya, Tokyo, Japan) was subcutaneously planted into each rat at the age of 16 months. Ovariectomy was not performed in any groups. No additional treatment was performed in the control group (Group I). Plain sesami oil was replaced with the one containing 20 μg/mL of estradiol (E_2, Sigma, St. Louis, MO), and placed for 2 weeks (Group II) or 4 weeks (Group III), respectively. The higher dose of E_2 at 200 μg/mL was administered for 2 weeks (Group IV) or 4 weeks (Group V), respectively.

Serum levels of estradiol

The serum E_2 concentrations were assessed by radioimmunoassay using a kit from Nihon DPC (Tokyo). In the control group, assay was performed on the 14th day after the operation, and in the E_2-replaced groups, 2 weeks (groups II and IV) or 4 weeks (groups III and V) after hormone replacement.

Histological examination of the rat urethra

After replacement therapy, the animals were all anesthetized by inhalation of diethyl ether and then weighed. In the control group (group I), this procedure was performed on the 14th day after the operation. The urethras were removed and weighed on a Mettler Basbal scale (Delta Range, Tokyo). After the procedure, all rats were sacrificed. Four urethras in each group were fixed in 10% formalin and embedded in paraffin, and the remaining 4 were frozen in liquid nitrogen. Each paraffin block was serially sectioned and routinely stained with hematoxylin and eosin (HE). For immunohistochemical staining of Bcl-2 and Bax, we used the monoclonal antibodies against Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) and Bax (Santa Cruz Biotechnology). The serial sections were also immunohistochemically stained with a Histfine Simple Stain Rat MAX PO (MULTI) kit (Nichirei, Tokyo) according to the manufacturer's instructions (UIP method).

Reverse transcription-PCR analysis

The remaining 4 urethras per group were frozen in liquid nitrogen. The total RNA was extracted from the dissected tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, treated with deoxyribonuclease [reverse transcription (RT) grade] (Nippon Gene, Tokyo) and then quantified spectrophotometrically. In this experiment, 5 μg of total RNA was reverse-transcribed in a 20 μL reaction volume. RT was carried out with Moloney murine leukemia

virus reverse transcriptase (Roche Molecular Systems, Branchburg, NJ), and PCR was performed using AmpliTag gold DNA polymerase (Roche Molecular Systems). The primers used for *bcl-2* were 5'-CACCCCTGGCATCTTCTCCTT-3' (sense) and 5'-AGCGTCTTCAGAGACAGCCAG-3' (antisense) (Genbank accession number U34964, 519 bp), and these for *bax* were 5'-CACCAGCTCTGAACAGAT CATGA-3' (sense) and 5'-TCAGCCCATCTTCTT CCAGATGGT-3' (antisense) (Genbank accession number RRU 49729, 540 bp) (Ananth et al., 2001). The PCR cycles consisted of denaturation at 94˚C for 30 s, annealing at 68˚C for 30 s and extension at 72˚C for 1 min for 26 cycles (*bcl-2*); and denaturation at 94˚C for 30 s, annealing 62˚C for 30 s and extension at 72˚C for 1 min for 26 cycles (*bax*). As a control, a 285-bp DNA of rat β-actin was also amplified using 5'-TCATGAAGT GTGACGTTG ACATCCGT-3' (sense) and 5'-CCTAGAAGC ATTTGCGGTGCAGGATG-3' (antisense) primers (Ananth et al., 2001). The temperature cycling conditions were as follows: 10 min at 94˚C 18 cycles of (94˚C for 30 s, 65˚C for 30 s, 72˚C for 30 s) and a final extension at 72˚C for 30 s. The PCR product was size separated by electrophoresis on a 2% cyber green-containing agarose gel, and photographed. For quantification, photographs showing the PCR products were scanned and analyzed using Densitograph version 4.0 software (ATTO, Tokyo). On each sample, signal strength was normalized by that of β-actin.

Statistical analysis

All data was analyzed by Student's *t*-test. Statistical significance was defined as $P < 0.05$.

Results

Serum levels of estradiol

The results of serum E_2 levels are recorded in Table 1. The serum E_2 levels in the replaced groups were significantly higher than those in the control group. Compared in terms of the E_2 dose, the serum E_2 levels were about 10 times higher in the high-dose groups (groups IV and V) than in the low-dose groups (groups II and III) (Table 1).

Body and urethral weight

There were no significant differences among groups in body weight. However, urethral weight was significantly heavier in the E_2-replaced groups than in the control group, although there were no significant differences among the E_2-replaced groups in urethral weight (Table 1).

Table 1. Body weight, urethral weight and serum E_2 levels

	Body weight (g)	Urethral weight (mg)	Serum E_2 levels (pg/mL)
Group I	353 ± 37.0	23.0 ± 4.6	7.3 ± 2.5
Group II	358 ± 28.0	30.7 ± 4.8	25.0 ± 4.8
Group III	339 ± 24.3	30.4 ± 6.8	29.4 ± 10.7
Group IV	332 ± 34.4	29.8 ± 2.8	258.1 ± 55.0
Group V	340 ± 23.2	33.7 ± 6.4	213.6 ± 67.3

Data are expressed as mean ± SD.
Statitical analysis by Student's *t*-test.
E_2, estradiol.
* Significant differences; $P < 0.05$.
** Significant differences; $P < 0.01$.

Histological changes in the rat urethras

There were no significant differences between the control and E_2-replaced rats in the HE stains of the urethra. Figures 1a and b show representative light microscopic photographs of the urethras immunostained by the antibody against Bcl-2: Fig. 1a for the control rats, and Fig. 1b for the group V rats replaced with high-dose E_2 for 4 weeks. Bcl-2 was not detected clearly in the urethra of the control rats. However, in the mucosal region of the urethra of the E_2-replaced rats, weak immunoreactivity against Bcl-2 was detected.

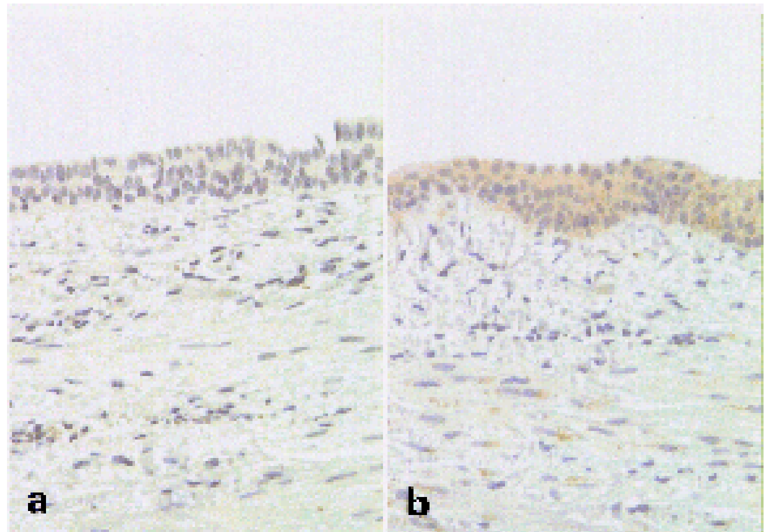

Fig. 1. Immunohistochemical stains of the rat urethras by an antibody against Bcl-2 protein.
a: A control rat (× 200).
b: A group-V rat replaced with high-dose E_2 for 4 weeks (× 200).

Figures 2a and b show urethras similarly stained by the antibody against Bax in the control rats and the group V rats. Bax was detected strongly in the mucosal region of the urethra of the control rats, but in the urethra of the E_2-replaced rats, weak immunoreactivity against Bax was detected.

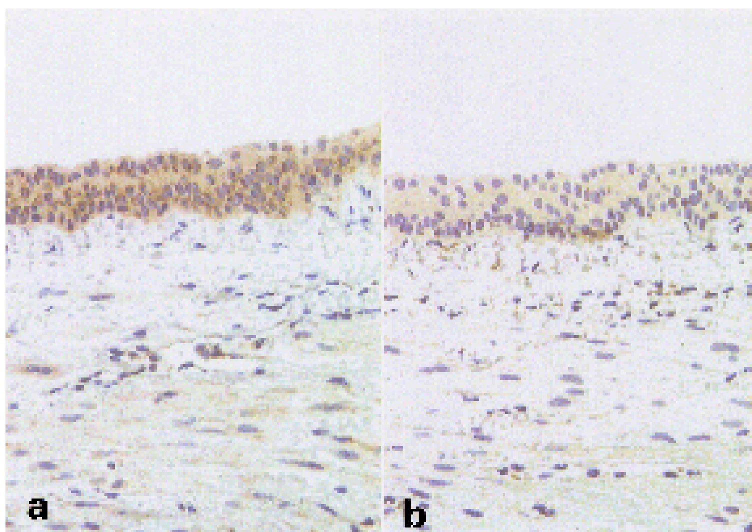

Fig. 2. Immunohistochemical stains of the rat urethras by an antibody against Bax protein.
a: A control rat (× 200).
b: A group-V rat replaced with high-dose E_2 for 4 weeks (× 200).

RT-PCR analysis of expression of bcl-2 and bax mRNA

The expressions of *bcl*-2 mRNA were significantly increased in group V compared with the control group. In the other E_2-replaced groups, there were no significant differences in the expressions of *bcl*-2 mRNA compared with the control group. However, the expressions of *bax* mRNA were significantly decreased in groups III and V compared with the control group. In groups II and IV, the expressions of *bax* mRNA showed no significant differences compared with the control group. The *bax*/*bcl*-2 ratio was significantly lower in groups III and V than in the control group (Fig. 3).

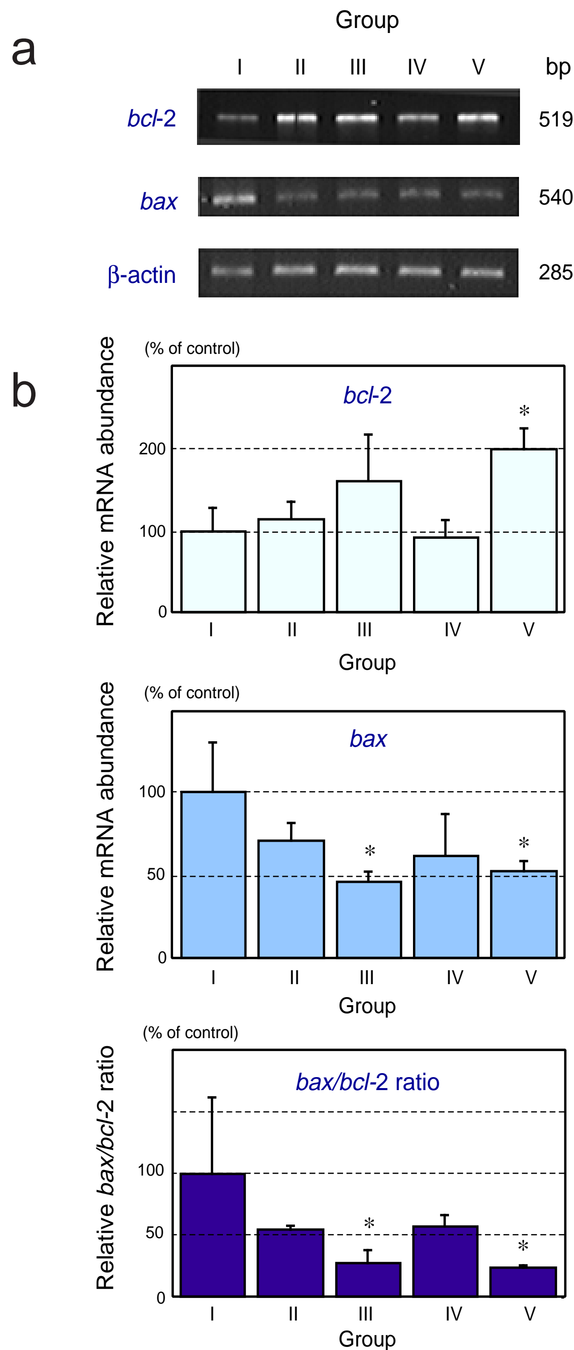

Fig. 3.

a: Semiquantitative RT-PCR analysis of mRNA expression of *bcl*-2, *bax* and β-actin in rat urethras. RT, reverse transcription.

b: Relative densities of *bcl*-2 and *bax* mRNA signals, and relative *bax*/*bcl*-2 ratio. The signals are normalized with corresponding β-actin signals. Each bar represent the mean ± SEM. Statistical significances were examined in comparison to the control level expressed as 100%; significant difference; $P < 0.05$.

Discussion

Rats are fertile until approximately 15 months of age, and thereafter their estrous cycle stops. Several studies indicated that the E_2 levels are less than 10 pg/mL in ovariectomized female rats (Shulman et al., 1987; Albert et al., 1991). Other studies have indicated that serum E_2 levels of 15 pg/mL are slightly below the mean level of E_2 throughout the estrous cycle (9–27 pg/mL), or about 1/3 of the maximum value E_2 (35–52 pg/mL) reached during the estrous cycle (Dupon and Kim, 1973; Overpeck et al., 1978). In the present study, the E_2 levels were lower than 10 pg/mL in the control group. In the E_2-replaced groups, the mean serum E_2 levels reached over 20 pg/mL in the low-dose groups, and 200 pg/mL in the high-dose groups. Thus, this method of estrogen replacement was adequate for studying the effect of estrogen on the rat urethras.

Previous studies showed that estrogen depletion resulted in increased body weight (Eika et al., 1990). In the present study, there was no significant difference in body weight was observed among groups. However, the mean urethral weight of the E_2-replaced rats was significantly higher than that of the control rats. These results suggest that estrogen replacement after menopause increases urethra weight in rats. No previous studies have shown changes in female rats' urethral weight after estrogen replacement. However, a few studies indicated that estrogen replacement might raise bladder weight as the collagen content of the bladder increases and the bladder epithelium thickens (Suguita et al., 2000; Eika et al., 1990). On the other hand, several studies have demonstrated the presence of estrogen receptors in the human lower urinary tract. This provides evidence for the direct actions of estrogens on the bladder and urethra (Batra and Iosif, 1987; Versi, 1990). Estrogen therapy could improve the proliferation and growth of the urethral mucosa (Bhatia et al., 1989), and cause an increase in urethral collagen content and urethral blood flow (Batra et al., 1986). These

changes in E_2-replaced rats might increase the urethral weight as the bladder weight. However, the present study did not show significant differences in the HE staining of urethras between the control and E_2-replaced rats. The differences in urethral weight between the control and E_2-replaced rats was about 5 to 10 mg. It might be difficult to detect differences in urethral mucosa or collagen content by HE staining.

In the present immunohistochemical staining, immunoreactivity against Bcl-2 was not detected clearly in the urethras of the control rats, but slightly detected in the mucosal region of the urethras of the E_2-replaced rats. However, immunoreactivity against Bax was detected more strongly in the mucosal region of the urethras of the control rats than in the E_2-replaced rats.

In the RT-PCR analysis, the expressions of *bcl*-2 mRNA were significantly increased in group V (high-dose E_2 replaced for 4 weeks) compared with the control group. In the other E_2-replaced groups, the mean expressions of *bcl*-2 mRNA were higher than in the control group. However, the expressions of *bax* mRNA were significantly decreased in groups III (low-dose E_2 replaced for 4 weeks) and V (high-dose E_2 replaced for 4 weeks) compared with the control group. In the other E_2-replaced groups, the mean expressions of *bax* mRNA were lower than in the control group. The *bax*/*bcl*-2 ratio was significantly lower in groups III and V than in the control group. The administration period for 2 weeks might have been too short to cause a difference in gene expression. RT-PCR analysis suggested that estrogen replacement up-regulates the *bcl*-2 gene expression and down-regulates the *bax* gene expression. Considering the histological results, these changes occured mainly in the mucous membrane of the rat urethra. Thus, it seems that epithelium cells were estrogen dependent rather than submucosal cells.

It is known that Bcl-2 inhibits most types of cell death, implying a common mechanism of lethality. Bcl-2 is localized to intracellular sites of oxygen-free radical generation, including mitochondria, endoplasmic reticula and nuclear membranes. The mechanism by which Bcl-2 exerts its antiapoptotic effects is not fully resolved, although it has been speculated that Bcl-2 acts as either a regulator of an antioxidant pathway that prevents oxidative damage, such as lipid peroxidation caused by the generation of free radicals (Tsujimoto and Croce, 1986; Hockenbery et al., 1993), or as a regulator of intracellular Ca^{2+} compartmentalization (Kane et al., 1993). The antiapoptotic activity of Bcl-2 correlates with its intracellular ratio to another recently described protein called Bax. Bcl-2 heterodimerizes with Bax. High levels of Bax have been shown to favor apoptosis in cells subjected to growth factor deprivation, whereas high levels of Bcl-2 prolong cell survival under the same conditions (Bafy et al., 1993). In other studies, Bcl-2, a cell survival factor, has been identified as an estrogen responsive gene in reproductive tissues. Estrogen may directly up-regulate this survival factor through receptor-mediated interactions with regions of the *bcl*-2 promotor, which contains several putative estrogen-responsive sites, or by an indirect pathway. Estrogen depletion results in a marked decrease in *bcl*-2 expression, and in the absence of estrogen, the *bax*/*bcl*-2 ratio is increased. In this type of situation, estrogen-dependent tissues undergo apoptosis (Teixeira et al., 1995).

In the present study, considering the changes in apoptosis related genes (Bcl-2, Bax), estrogen replacement might prevent apoptosis mainly in the urethral mucosa of old female rats. However, it is difficult to demonstrate the reason why the immunoreactivity was observed mainly in the mucosa. Further studies need to be performed to assess the relation between the expression of apoptosis-related genes on the female urethra and estrogen replacement.

It appears from our study that in the mucosa of the rat urethra, estrogen replacement up-regulates the *bcl*-2 gene expression and down-regulates the *bax* gene expression. These results indicate that estrogen replacement may prevent apoptosis in the urethras of old female rats.

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