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Source: Biology of Reproduction, 85(1) : 105-112

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.110.089508

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BIOLOGY OF REPRODUCTION **85,** 105–112 (2011)
Published online before print 6 April 2011.
DOI 10.1095/biolreprod.110.089508

Distribution of Aromatase and Sex Steroid Receptors in the Baculum During the Rat Life Cycle: Effects of Estrogen During the Early Development of the Baculum[1]

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ABSTRACT

The baculum, also called os penis, plays an important role during copulation. However, the hormonal regulation of its development remains to be elucidated. To determine the direct involvement of sex steroids in the development of the baculum of rats, the distributions of androgen receptors (ARs), aromatase, and estrogen receptor alpha (ESR1) were observed immunohistochemically. On Postnatal Day 1, the rudiment of the baculum expressed ARs, aromatase, and ESR1. In the proximal segment of the baculum of neonatal rats, ARs were expressed in the parosteal layer but not in the periosteum or osteoblasts. Aromatase was expressed from the parosteal layer to the endosteum, particularly in the inner osteogenic layer. ESR1 was also abundantly expressed in almost all cells from the parosteal layer to the endosteum. ARs, aromatase, and ESR1 were all abundantly expressed during the neonatal period in the hyaline cartilage of the proximal segment and in fibrocartilage of the distal segment of the baculum. Expression in all the tissues was attenuated in an age-dependent manner and became quite weak at puberty. To determine the effect of estrogen on the growth of the baculum, the aromatase inhibitor 1,4,6-androstatrien-3,17-dione (ATD) was subcutaneously injected daily into pregnant rats from Days 19 to 23 of gestation and into pups on postnatal Days 1, 3, 5, 7, and 9. On Day 10, the length of the baculum in the ATD-treated rats was significantly shorter than that in the controls, although the body weight did not change. These findings suggest that not only androgen but also locally aromatized estrogen is involved in the early growth and development of the baculum.

estradiol, estradiol receptor, male reproductive tract, penis, steroid hormone receptors, steroid hormones

INTRODUCTION

The baculum, also called ''os penis,'' is a unique bone found in the penis of various mammalian species belonging to diverse orders, e.g., Insectivora, Chiroptera, Rodentia, Carnivora, and Primates (except in humans) [1]. In rats and mice, the baculum is present at the distal end of the corpus cavernosum and is composed of three portions [2–4]. The proximal portion is formed by the fusion of hyaline cartilage, and the central portion is made up of membranous bone. The distal segment is derived from fibrocartilage. The proximal and central portions fuse and begin to ossify within 5 days after birth to form the proximal segment, while the distal segment does not ossify until puberty [2, 4].

Regarding the functional relevance of the baculum, morphometric analysis has revealed that the baculum is subject to direct selection and therefore plays an adaptive role in copulation [5, 6]. An excellent previous study of *Hoxd13* mutant mice showed that the mice had normal corpora cavernosa but malformed bacula [7]. The mutant males could not father offspring, although they had normal germ cells and sexual behaviors. This indicates that successful impregnation requires specific bacular shape and/or movements during copulation.

Although the baculum plays an important role in force transfer during copulation, the hormonal regulation of its growth and development remains to be elucidated. Androgens play important roles in the growth and development of the baculum [2, 8, 9]. They are well known to act anabolically on osteoclasts and osteoblasts of general bones [10]. Neonatal castration or treatment of male mice with antiandrogens reduces the growth and calcification of the baculum [2]. Prepubertal treatment of castrated immature rats with testosterone propionate stimulates bone growth [11]. If genital tubercles are cultured beneath the renal capsule of castrated or androgen-treated rats, the baculum develops in an androgen-dependent manner [9]. In fact, androgen receptors (ARs) are expressed in the penile soft tissues of rats [12, 13]. However, the presence of ARs in the baculum has not been reported.

In contrast, the effect of estrogen on the ossification of the baculum is thought to be more complex. Endogenous estrogen in males is produced mainly in the testes and adrenal glands, although its plasma levels are much lower than those in females [14]. While exogenous estrogen treatment failed to affect the baculum of adult rats [2, 9], Goyal et al. [15–18] precisely clarified the teratogenic effects of pharmacologic doses of exogenous estrogen on the penile soft tissues of rats. Those studies rarely mentioned the baculum but found that neonatal exposure to diethylstilbestrol (DES) or estradiol valerate dose-dependently induced permanent penile aplasia [15, 16]. However, neonatal administration of tamoxifen, an estrogen receptor antagonist, also inhibited postnatal growth and differentiation of the mouse baculum [19, 20]. Taken together, these findings suggest that endogenous estrogen, besides androgen, also plays a direct and/or indirect role in the ossification of the baculum during the neonatal period.

Estrogen is produced by aromatase, a microsomal enzymatic complex. Aromatase irreversibly converts the aromatizable androgens such as testosterone to estrogens [21]. Because aromatase is expressed not only in the gonads but also in other tissues, some androgens are thought to act in a manner similar to that of estrogens converted locally [22, 23]. In rats, estrogen receptor alpha (ESR1) and aromatase are reportedly expressed

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Received: 2 November 2010.
First decision: 30 December 2010.
Accepted: 22 March 2011.
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eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

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in the region of neonatal penile soft tissues [23–25], but their precise distribution in the baculum remains unknown.

In this study, the distributions of ARs, aromatase, and ESR1 in the three portions of the baculum were observed immunohistochemically throughout the life cycle of rats. To determine the effect of local estrogen on the growth of the baculum, the effect of perinatal treatment with aromatase inhibitor was examined.

MATERIALS AND METHODS

Animals

Wistar-Imamichi rats were used for all experiments, as histological data for the baculum are available for this strain [2, 7]. For experimental purposes, rats were maintained in a closed colony at a temperature of 23 ± 2°C, with lights on from 0500 h to 1900 h (14L:10D). They were always allowed free access to food and water. Each litter contained eight pups, and weaning occurred at the age of 3 weeks. The day of birth was considered Postnatal Day 1. We used 32 rats in the histological and immunohistochemical analyses and seven mothers with their 29 pups in the aromatase inhibition test. All experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals, School of Veterinary Medicine and Animal Sciences, Kitasato University, Japan.

Radiological Analysis

The rats were killed by decapitation under ether anesthesia on Days 1, 5, 10, 30, 60, 90, and 180. Their blood and penises were harvested. The bacula were visualized with a soft X-ray apparatus (Softex, Kanagawa, Japan). The lengths of the bacula were analyzed by interpretation of the radiographs.

Measurement of Plasma Steroid Hormone Levels

Blood samples were immediately put into heparinized tubes and centrifuged at 4°C for 10 min at 800 × *g*. Harvested plasma was stored at 20°C until assayed. Plasma testosterone levels were measured by the time-resolved fluoroimmunoassay method [26]. The europium-labeled rabbit antibody against bovine serum albumin (BSA) conjugated with testosterone was kindly donated by Dr. Y. Hasegawa, Kitasato University, Aomori, Japan [26]. For the assay, 70 μl of the plasma was diluted in 430 μl of water, and 3 ml of diethyl ether was added to extract testosterone from the plasma. The organic phase was transferred to another glass tube and volatilized using a dry thermo unit. The volatilized organic phase was reconstituted with 350 μl of the assay buffer (140 mM NaCl, 0.5% BSA, 0.05% gamma globulin, 0.00078% diethylenetriaminepentaacetic acid, 0.05% sodium azide, 0.01% Tween 40, and 50 mM Tris-HCl, pH 7.8). Wells of polystyrene microtiter strips were coated with BSA-testosterone conjugate (Steraloids, Newport, RI). After all the wells of the microtiter strips were washed, 100 μl/well of serially diluted standard (0.001–10 ng/ml) and reconstituted samples was added. Thereafter, the europium-labeled antibody against the BSA-testosterone conjugate was added to each well and incubated for 4 h. After all the wells were rewashed, 100 μl/well of enhancement solution was added, and the fluorescence intensity of each well was measured with a time-resolved fluorometer (Multilabel Counter model 1420 ARVO; Wallac Oy, Turku, Finland). The range of linearity was 0.001–10 ng/ml. The intra- and interassay coefficients of variance (CVs) were 9.0% and 9.9%, respectively.

In addition, plasma estradiol concentrations were also measured using similar assay protocols. Steroids were extracted from 1-ml plasma samples on Days 5–120. The plasma estradiol levels in the rats treated with aromatase inhibitor or control reagent (see *Effect of Aromatase Inhibition* below) were also determined. The europium-labeled rabbit antibody against BSA conjugated with estradiol was also kindly donated by Dr. Y. Hasegawa [26]. The range of linearity was 1–1000 pg/ml. The intra-assay CV was 5.6%.

Histological and Immunohistochemical Analyses

Penile tissues were fixed in 10% neutral formalin immediately after radiology, decalcified in formalin with 10% ethylenediaminetetraacetic acid for 2–4 weeks after fixation, and then embedded in paraffin. Blocks were cut into 2-μm-thick sagittal sections and dehydrated through the use of an ethanol series and xylene. Some sections were stained with hematoxylin and eosin for histological examination, and others were used for immunohistochemistry. The sections were incubated at 80°C overnight to retrieve antigens and incubated in 0.3% hydrogen peroxide for 20 min to quench endogenous peroxidase. To block nonspecific antibody binding, the sections were incubated in 10% normal goat serum (Nichirei, Tokyo, Japan) for 30 min. The rabbit polyclonal antibodies against ARs (catalog no. sc-816; Santa Cruz Biotechnology, Santa Cruz, CA), ESR1 (catalog no. ab37438; Abcam, Cambridge, U.K.), aromatase (catalog no. 3599-100; BioVision, Mountain View, CA), and ESR2 (catalog no. PA1-310B; Affinity BioReagents, Golden, CO; and catalog no. ab3577, Abcam) were diluted at a ratio of 1:1000 in 10 mM PBS and incubated with the sections in a humidified chamber at 4°C for 14 h. Immunohistochemical staining was carried out by the labeled streptavidin biotinylated antibody method by using a Histofine Simple Stain MAX PO kit (catalog no. 424141; Nichirei). Immunoreactions were visualized with 3,3′-diaminobenzidine. Counterstaining was performed with Mayer hematoxylin solution (Merck, Whitehouse Station, NJ). Negative controls, in which the primary antibodies were replaced with nonimmunized sera, did not show nonspecific staining. The observed sites are presented in Figure 1, which shows toluidine blue staining of the adult penis.

Effect of Aromatase Inhibition

An aromatase inhibitor, 1,4,6-androstatrien-3,17-dione (ATD), was purchased from Steraloids. When deciding the ATD dose in this experiment, we referred to a previous report [27]. Pregnant rats were subcutaneously injected daily with 0.5 mg of ATD in 30 μl of sesame oil from Days 19 to 23 of gestation. Control pregnant rats were injected with the vehicle only. The litter size was adjusted to eight pups. The male pups were subcutaneously injected with 0.5 mg of ATD in 50 μl of sesame oil on Postnatal Days 1, 3, 5, 7, and 9. Control rats were injected with the vehicle only. The animals were killed on Postnatal Day 10. Their penises were harvested, and the bacula were visualized with a soft X-ray apparatus. The lengths of the bacula were analyzed by interpreting the radiographs.

Statistical Analysis

To determine the differences in body weight and the lengths of the bacula between control and ATD-treated rats, the effect of ATD treatment was evaluated by a two-tailed Student *t*-test with a significance threshold of $P < 0.05$.

RESULTS

As shown in Figure 2, the calcified bacula of male rats are visible on radiographs. The proximal segment of the baculum appeared on day 5. Measurement of the longitudinal lengths of the bacula on the radiographs showed that the proximal segment elongated dramatically from birth to Day 40. Only a few changes were noted in the rats between 60 and 180 days (approximately 4–5 mm). In contrast, the distal segment could not be observed on the radiographs until Day 40. It lengthened greatly from Days 40 to 60 and then developed gradually until Day 180 (approximately 2–3 mm). Plasma testosterone levels were determined using the time-resolved fluoroimmunoassay method. We detected temporarily high secretion from Days 1 to 5 (with the peak level on Day 1). The testosterone levels in immature rats from Days 10 to 40 remained low. After puberty, on Days 60, 120, and 180, testosterone was secreted at a constant level of 2 to 3 ng/ml. Plasma estradiol concentrations on Days 5–120 were <1 pg/ml, which was the minimum standard level of measurement in our immunoassay (data not shown). The estrogen levels on Day 1 were not measured, as the harvested plasma volume was not large enough.

The penile tissues were used for immunohistochemistry after radiological analysis. We observed the immunolocalization of ARs, aromatase, and ESR1 in the baculum during the life cycle of normal Wistar-Imamichi rats. In Day 1 penile sections, the ossified baculum was not observed, but the rudiment was seen on the dorsal side of the urethra (Fig. 3). AR- and ESR1-immunopositive signals were detected in the mesenchymal cells in the rudiment and other tissues. For aromatase, weak but diffuse immunopositive signals were detected. After Day 1, three sites of the baculum were

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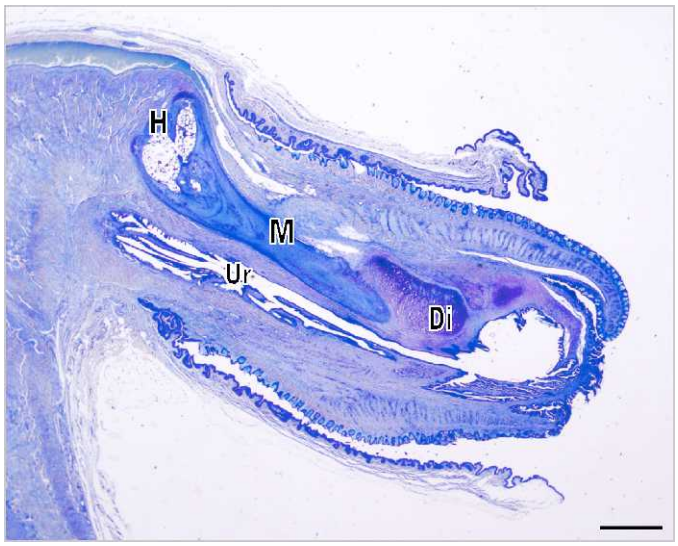
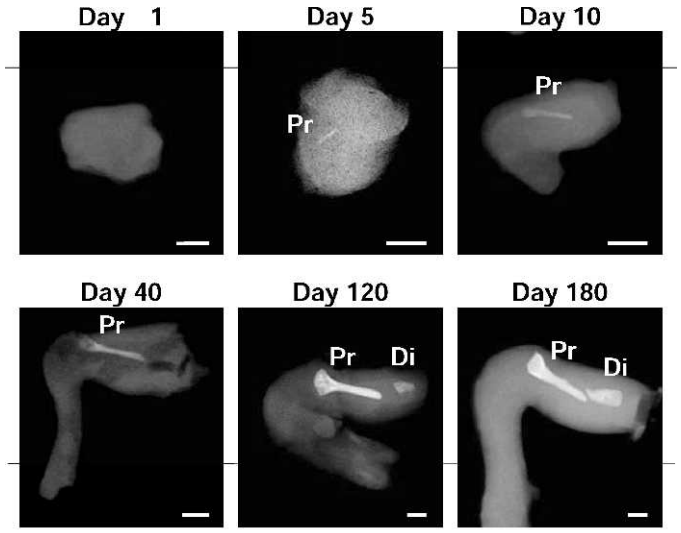


FIG. 1. A representative overview image of an adult rat penis stained with toluidine blue. M, membranous bone site of the proximal segment of the baculum; H, hyaline cartilage site of the proximal segment of the baculum; Di, distal segment of the baculum; Ur, urethra. Bar = 1 mm. M, H, and Di are roughly the sites shown in Figures 4, 5, and 6, respectively.

observed in the sections: membranous bone site of the proximal segment, hyaline cartilage site of the proximal segment, and distal segment (Fig. 1). Figure 4 shows the membranous bone site of the proximal segment of the baculum. ARs were most strongly expressed in the parosteal layer on Day 5, following which the expression attenuated in an age-dependent manner. ARs were not expressed in the periosteum or osteoblasts. Aromatase was expressed in the cytoplasm of the parosteal layer, periosteum, osteoblasts, and endosteum on Day 5. After Day 5, aromatase expression was observed particularly in the inner osteogenic layer; it declined in the other tissues and was scarce on Day 40. ESR1 was expressed in almost all the cells at this site until Day 10, but the expression weakened in an age-dependent manner. Multinucleate osteoclast-like cells were not found in any section throughout the life cycle of the rats in this experiment. The hyaline cartilage site of the proximal segment of the baculum is shown in Figure 5. Chondrocytes in the hyaline cartilage expressed ARs, aromatase, and ESR1 on Days 5–10. These immunoreactive intensities were very weak or not observed after Day 40. Immunolocalization in the distal segment of the baculum was also observed during the life cycle of rats (Fig. 6). AR-, aromatase-, and ESR1-immunopositive cells were observed in the fibrocartilage of the distal segment on Days 5–10. These immunoresponses were also very weak or not observed after Day 40. The immunopositive signals and their negative controls are shown in Supplemental Figure S1 (available online at www.biolreprod.org). A summary of the immunolocalization is provided in Table 1. The expression of ESR2 was also examined, but its signal was too weak to provide the data for this study. The reasons for the extremely weak expression of ESR2 would be the presence of some artificial factors and not the absence of ESR2, because in a previous study, ESR2 was reportedly expressed at least in the rudiment in the postnatal period [24].

The effects of perinatal treatment with aromatase inhibitor are shown in Figure 7. The body weights and lengths of the bacula were observed in 10-day-old rats treated with ATD or the vehicle as control. In the ATD-treated rats, the length of the proximal segment of the baculum was significantly reduced



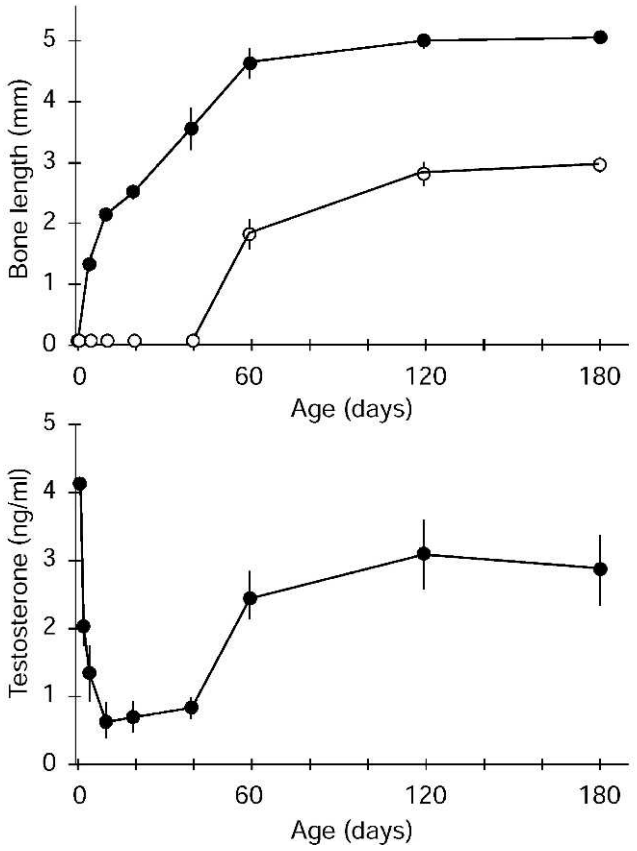


FIG. 2. Radiographs show the bacula (**A**), change in the length of the baculum (**B**), and plasma testosterone levels (**C**) in normal male rats. Radiographs show the representative bacula of rats at ages 1, 5, 10, 40, 120, and 180 days. Bars = 1 mm. The proximal segment (Pr) clearly ossified from Day 5, and the distal segment (Di) was observed from Day 60. The lengths of the bacula were measured on the radiographs. Closed and open circles represent the longitudinal dimensions of the proximal and distal segments, respectively.

(ATD treatment, 1.76 ± 0.048 mm; control, 2.1 ± 0.039 mm), although body weights did not differ significantly between the two groups. The estradiol concentrations were lower than the minimum assay limit (1 pg/ml) in not only the samples from the ATD-treated pups but also those from the control pups.

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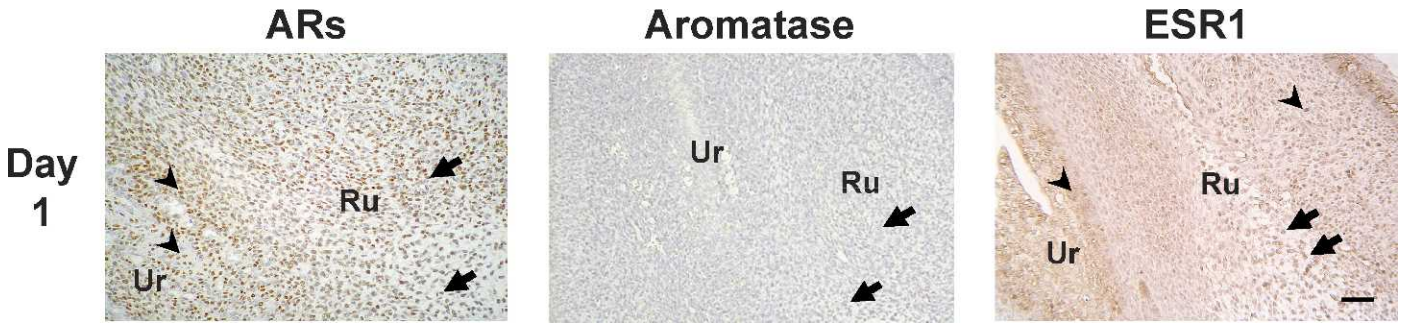


FIG. 3. Immunolocalization of ARs, aromatase, and ESR1 are shown in the rudiment (Ru) of the baculum on Day 1. In Day 1 penile sections, the baculum was not observed, but the rudiment was seen on the dorsal side of the urethra (Ur). Immunopositive signals were detected in the mesenchymal cells in the rudiment (arrows) and other tissues (arrowheads). Bar = 25 μm.

DISCUSSION

The histology of the baculum has been reported in detail in mice and rats [2–4], but no morphological study determining the period for which the baculum grows longitudinally during the life cycle of rats has been conducted. In this study, we demonstrated the normal development of the baculum. The proximal segment was clearly visualized by soft X-ray on Day 5. It elongated dramatically in the postnatal-to-juvenile period. The slope of the growth curve decreased with age, and only a few changes were detected between 60- and 180-day-old rats. These results closely agreed with those of a previous report with mice [2]. The distal segment did not clearly calcify until Day 40. It lengthened greatly in the pubertal period from Days 40 to 60 and then gradually slowed with age. The distal segment in mice reportedly calcifies in the juvenile period [2], indicating that the timing of calcification of the distal segment of the baculum is not very different between mice and rats. On Day 180, the length of the proximal segment finally reached 4–5 mm, and that of the distal segment reached 2–3 mm. Both of these lengths were similar to those reported in mice [2]. In some previous reports, the penile length in a normal rat is reported to be almost 40 mm and that in mouse to be almost 16–20 mm [15–17]. Although the penile length in a rat is more than 2-fold that in a mouse, the length of a rat baculum is not very different from that of a normal mouse baculum.

The development of the baculum is thought to be mainly due to androgens [2, 9, 11]. In this study, the plasma testosterone levels harvested from rats were also determined. While alteration of testosterone levels during the life cycle of rats was similar to that indicated in previous reports [28, 29], it did not correlate with the growth curve of the baculum. In other words, the period of maximal growth rate of the baculum was observed with rather low plasma testosterone levels. Furthermore, the plasma estrogen concentrations were also measured, but they were less than the minimum measurement limit throughout the life cycle of control and ATD-treated rats. Thus, we hypothesized that some testosterone could be locally aromatized in the baculum if estrogen

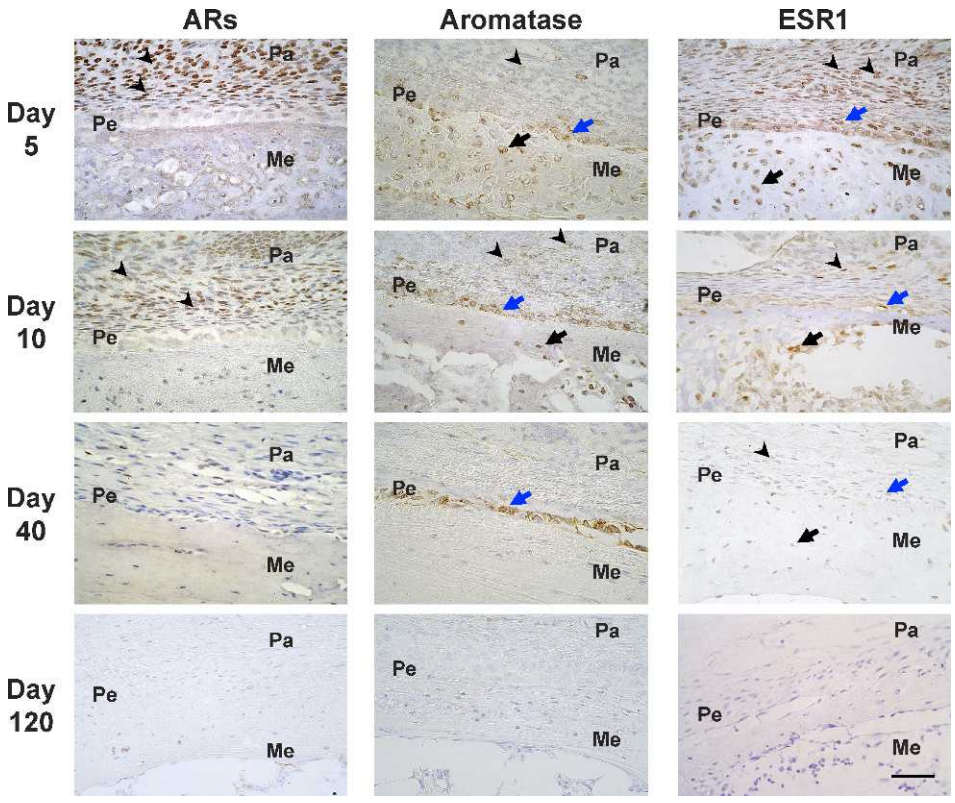


FIG. 4. Immunolocalization of ARs, aromatase, and ESR1 are shown at the membranous bone site of the proximal segment of the baculum during the life cycle of rats. This site is shown as M (membranous bone site of the proximal segment of the baculum) in Figure 1. Longitudinal sections are shown here, with the proximal segment of the baculum to the left and the distal segment to the right. Immunopositive cells were variously observed: osteoblasts in the membranous bone (Me; black arrows), inner osteogenic layer cells in the periosteum (Pe; blue arrows), and cells in the parosteal layer (Pa; black arrowheads). Bar = 25 μm. The distributions changed with age. See details in Table 1.

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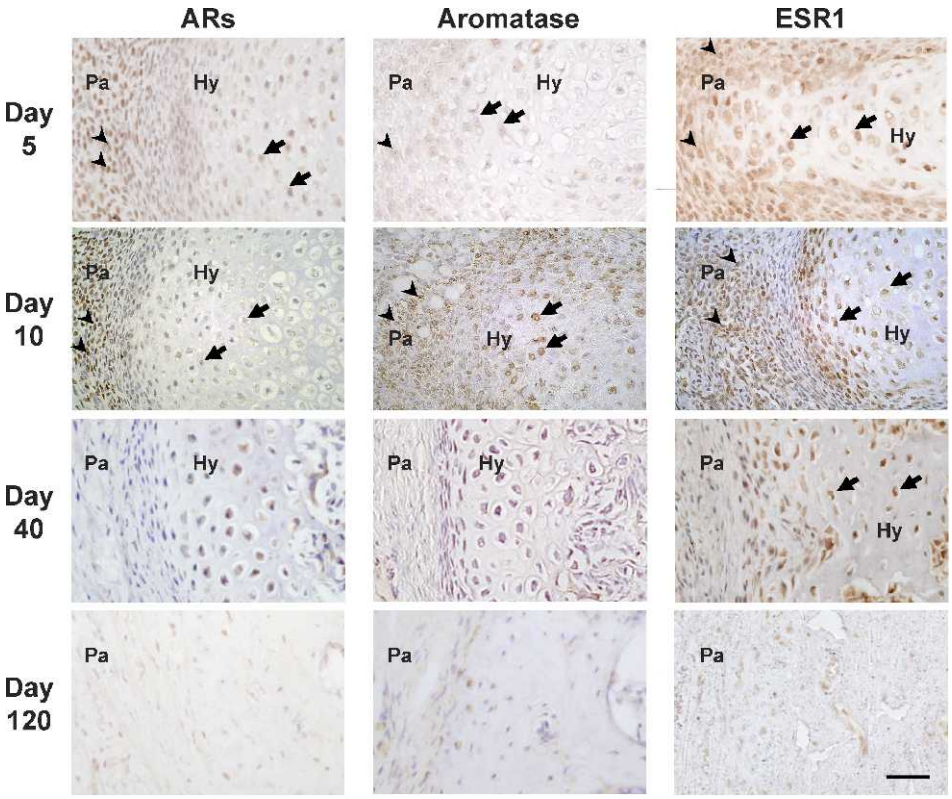


FIG. 5. Immunolocalization of ARs, aromatase, and ESR1 at the hyaline cartilage site of the proximal segment of the baculum during the life cycle of rats. This site is shown as panel H in Figure 1. Immunopositive cells were variously observed: chondrocytes in the hyaline cartilage (Hy; black arrows) and cells in the parosteal layer (Pa; black arrowheads). Bar = 25 μm. The distributions changed with age. See details in Table 1.

is involved in baculum development. To determine the involvement of androgens, estrogen, and local aromatization in the development of the baculum, the distributions of ARs, aromatase, and ESR1 were investigated throughout the life cycle. On Day 1, the receptors and enzyme were extensively expressed in the rudiment. These findings indicate that androgen and locally aromatized estrogen played a direct role in the early development of the baculum.

After Day 5, in the proximal segment of the baculum, membranous bone differentiated and rapidly elongated in the central area, which consisted of periosteal cells and osteoblasts. In the proximal area of this segment, hyaline cartilage and

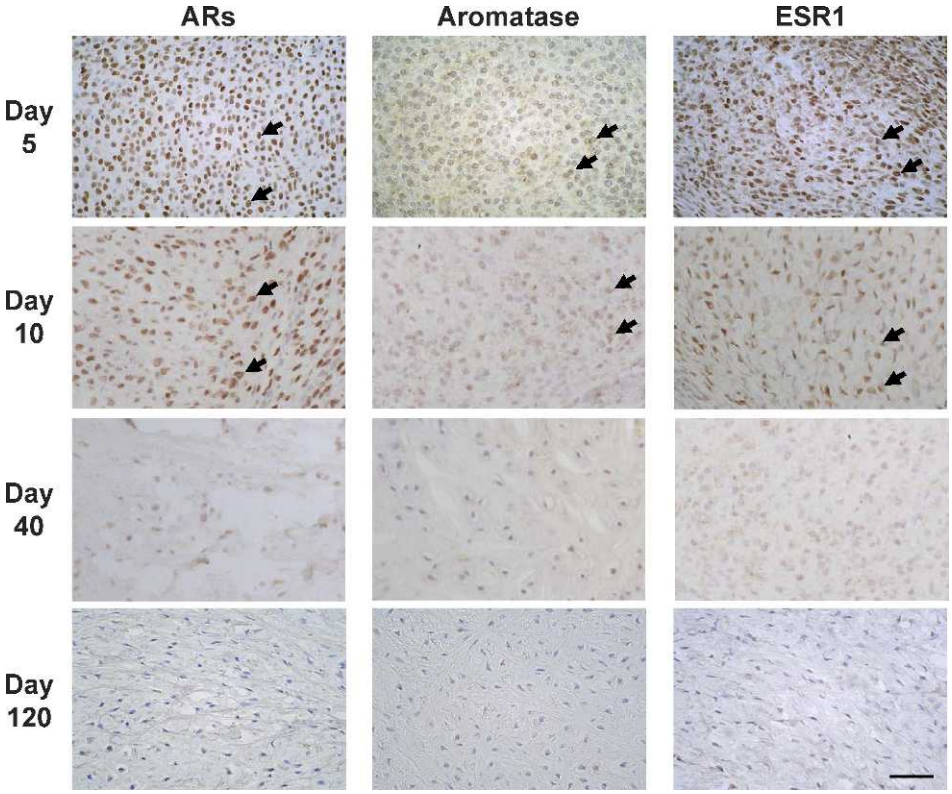


FIG. 6. Immunolocalization of ARs, aromatase, and ESR1 in the distal segment of the baculum during the life cycle of rats. The distal (Di) segment is shown in Figure 1. Immunopositive signals were detected in the chondrocytes in the distal segment (black arrows). Bar = 25 μm. The distributions changed with age. See details in Table 1.

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TABLE 1. Distribution of aromatase and sex steroid receptors in the baculum during the life cycle of rats.[a]

	Paraosteum			Periosteum			Osteoblasts			Chondrocytes			Distal segment		
Day	ARs	Arom	ESR1	ARs	Arom	ESR1	ARs	Arom	ESR1	ARs	Arom	ESR1	ARs	Arom	ESR1
5	+	±	+	−	+	+	−	+	+	±	+	+	+	+	+
10	+	±	+	−	+	+	−	+	+	±	+	+	+	+	+
40	±	−	±	−	+	±	−	−	±	±	±	+	−	−	−
120	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] ARs, androgen receptors; Arom, aromatase; ESR1, estrogen receptor alpha; –, not expressed or very weakly expressed; ±, slightly expressed; +, strongly expressed.

chondrogenic ossification occurred. Androgens mediate stimulatory effects on general periosteal bone formation and chondrogenesis in male mice and rats [30, 31]. In the femoral and tibial bones of rats, ARs are expressed in the periosteal layers and proliferative chondrocytes [32, 33]. In the present study, however, ARs were not expressed or were only weakly expressed in the periosteum and osteoblasts or the inner osteogenic layer in the central area of the baculum, although it was localized in the parosteal layer, chondrocytes, and other penile tissues other than the baculum. These data imply that chondrogenesis could be directly caused by androgens in the hyaline cartilage of the proximal area, whereas membranous ossification could be affected indirectly in the central area of the proximal segment.

In the penile tissues other than the baculum, localization of aromatase and ESR1 has been precisely reported. Aromatase is abundantly expressed in the primordial corpus cavernosum and corpus spongiosum of neonatal rat penis but is downregulated in adulthood [23]. ESR1 is localized in the mesenchyme and subepithelial stroma in the perinatal period and is also expressed in the corpus cavernosum and corpus spongiosum in the postnatal period [24]. In adult rats, ESR1 is concentrated in the urethral epithelia and vascular and neuronal structures [24]. In this study, we determined the localization of aromatase and ESR1 in the bacula of rats throughout their life cycle. In the proximal segment of the baculum, aromatase was extensively expressed on Day 5. After Day 5, its expression appeared specifically in the inner osteogenic layer of the periosteum and in chondrocytes but declined in the other tissues and disappeared on Day 40. ESR1 was strongly expressed in almost all the cells in the proximal segment until Day 10, but the expression weakened with age and disappeared on Day 40. The expression patterns of aromatase and ESR1 were closely correlated with the timing of the elongation of the proximal segment. This indicates that androgen involvement in the development of the proximal segment of the baculum depends specifically on the local aromatization of androgens into estrogen.

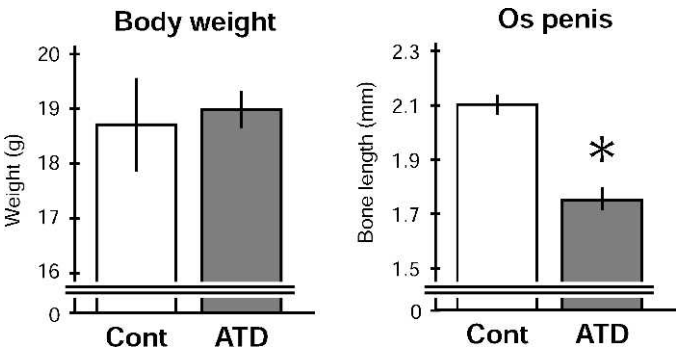


FIG. 7. Body weight and length of the bacula of 10-day-old rats treated with the aromatase inhibitor ATD are shown. White and gray columns indicate averages of the values obtained for the control (Cont) and ATD-treated (ATD) groups, respectively, and vertical bars represent the SEM. Asterisk indicates the significant difference between the ATD-treated and control groups ($P < 0.05$). In the ATD-treated rats, the length of the baculum was reduced, although the body weight did not change.

Estrogen involvement in bone ossification is controversial. In male rats, estrogen is not thought to stimulate periosteal expansion. Treatment with an aromatase inhibitor or an ER antagonist does not exert significant effects on appendicular skeletal growth [34, 35]. In a previous study, bone mineral density in femurs did not change in male ESR1-conditional knockout mice [36]. However, some studies have reported that estrogen is involved in ossification. Estradiol administration stimulates periosteal bone formation in rats [33]. Estrogen prevents bone loss via ESR1 and induction of the Fas ligand in osteoclasts [37]. Osteopenia develops in human male patients genetically deficient in ESR1 [38] or aromatase activity [39]. In this study, the aromatase inhibitor ATD was administered from Day 19 of gestation to 10 days after birth. The longitudinal length of the proximal segment of the baculum was significantly reduced, although the body weights were not significantly different between the ATD- and vehicle-treated rats. This result demonstrates the importance of local aromatization in the development of the proximal segment of the baculum. However, this does not mean that only estrogen stimulates the development of the baculum. Inhibition of aromatase action not only reduces estrogen but also increases testosterone metabolites, including 5α-adrostanediol, a ligand for ESR2. Thus, it is not definitively concluded that all the effects elicited by ATD were due to reduction in estrogen synthesis. To clarify this issue completely, additional experiments are essential in the future, involving treatment with aromatase inhibitor plus estradiol. On the other hand, treatment with an estrogen analog, DES, causes serious abnormalities in the developing penis via ESR1 [15, 16]. Alterations in the baculum were not elaborated in these reports, but the representative radiographs showed that the bacula of the DES-treated rats appeared smaller than those of the controls. Indeed, estrogens exert a biphasic effect on general bone growth: low levels of estrogens exert an apoptotic effect on osteoclasts and an antiapoptotic effect on osteoblasts, but higher concentrations inhibit linear growth and promote growth plate closure [10]. Because ESR1 expression was highly age-dependent, an appropriate level of estrogen production in the neonatal period could be important in the normal development of the baculum. However, evidence exists that androgen action is an important factor in the growth of the baculum [2, 9]. Indeed, estrogen and testosterone might use different cellular pathways to regulate osteoclastogenesis and bone resorption, as reported in a study using human osteoclast precursors [40]. Taken together, maintaining an adequate balance of local estrogen and androgen levels may be necessary for the normal growth of the baculum.

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In the distal segment, immature fibrocartilage was observed on Days 5–40; thereafter, it was replaced with nonlamellar bone by endochondral ossification. These histological observations closely agreed with those in a previous report on this area in rats [41]. The slope of the growth curve of the distal segment after puberty closely correlated with that of plasma testosterone levels, superficially indicating that the distal segment could be stimulated by androgen after puberty. In this study, AR-, aromatase-, and ESR1-immunopositive cells were observed in the fibrocartilage of the distal segment on Day 5. However, these immunoresponses weakened in an age-dependent manner. This suggests that AR and ESR1 levels in the distal segment after puberty would be lower than those in the juvenile period. Appropriate receptors can be detected by using more sensitive means in future studies. According to a study by Murakami et al. [42], once the fibrocartilage is calcified, endochondral ossification can take place without androgens. Thus, besides androgen, local estrogen could also be involved in the early developmental process of the fibrocartilage of the distal segment.

In conclusion, our results provide evidence that ARs are expressed in the parosteal layer, while aromatase and ESR1 are expressed in the periosteum, endosteum, and various tissues in the bacula of neonatal rats. Moreover, we demonstrated that treatment with an aromatase inhibitor reduced the growth of the baculum. These findings suggest that not only androgen but locally aromatized estrogen also promotes the development of the baculum of rats during the neonatal period.

ACKNOWLEDGMENT

We thank Dr. Yoshihisa Hasegawa (Kitasato University) for providing the testosterone antibody and assay materials.

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