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Effects of neonatally administered androgen on the development of the levator ani muscle in female rats

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Abstract

The present experiments were designed to determine the influence of neonatally (on day 1 after birth) administered testosterone propionate (TP) on the sensitivity to re-administered TP and on development of the female rat levator ani (LA) muscle.

A single injection of TP on day 57 or 147 after birth resulted in the re-growth of the LA muscle in neonatally TP-treated (androgenized) female rats, but not in oil-treated female rats. A single re-administration of TP on day 57 or 147 significantly increased the weights of LA muscles in androgenized female rats compared with in oil-treated females. In neonatally oil-treated female rats, TP injection on day 57 of age had no effects on the development of LA muscles. From histological examinations, in females treated with TP at the day of birth, abortive regeneration was seen to accompany muscle destruction. Only a thin fibrous strand was seen around the site of the LA muscle. Occasionally fragments of muscle fibers with rudimentary cross-striations were also found in the strand. In androgenized females subjected again to TP on day 57 of age, regeneration of the LA muscle was completed ; cross-striation of myofibrils was clearer and myofilaments displayed normal alignment, as in fully-developed skeletal muscle. Although the origin of regenerated muscle cells after androgen re-administration is not known, present results indicate that exposure of neonatal female rats to androgen modifies the sensitivity of the LA muscle to re-administered androgen in mature animals.

Introduction

The levator ani (LA) muscle of the rat provides a valuable model for studying the effects of androgens on muscle structure and function. Previous studies have demonstrated that this particular skeletal muscle possesses high affinity androgen receptors that are found in greater quantities than other skeletal muscles[1)-3)]. Following castration of adult male rats the LA muscle undergoes an atrophic response whereas androgen administration results in hypertrophy of this muscle[4)]. In adult female rats the LA muscle is absent[5)] or vestigial[6)]. This large sex difference emerges during postnatal life and is androgen-dependent[5)7)].

On the other hand, when female rats were given androgen injections during the first 10 days after birth, permanent effects on the hypothalamo-pituitary axis were produced[8)]. When this hormone was given before the hypothalamus was completely developed, it interfered with the maturation of the anterior-preoptic hypothalamic area ; in adulthood, this area governs the cyclic release of the ovulating hormone[9)]. Thus, adult female rats treated neonatally with androgens do not ovulate and develop persistent estrus. It is believed that androgen's effects are dependent on 2 factors ; dosage, and age at treatment time[10)]. If testosterone propionate (TP)

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is given in low concentrations or at an advanced age, treated animals ovulate during their postpubertal period but eventually become anovulatory[11]. Therefore, it is possible that neonatal androgen injection may exert permanent effects on the sensitivity to androgen when animals are fully matured.

In the present study we have investigated the influences of neonatally administered TP on the development and regeneration of the LA muscle in female rats.

Materials and Methods

Specific pathogen-free Wistar rats were used. One male and one female animal were kept per filter-top cage in a room with controlled temperature (24±2°C) and light (14 h of light, 10 h of darkness). Food and water were available *ad libitum*. Animals were observed daily for delivery of pups, and the day of birth was designated day 1. Pups were sexed according to anogenital distance, and female pups were removed. Animals were weaned on day 21 and subsequently housed two or three per cage. Animal care and all experiments were carried out according to the institution guidelines of Tokyo Medical University.

Normal animals in both sexes were killed under heavy ether anesthesia on day 1 (the day of birth), 4 or 7 of ages, dissected the area of under-abdominal tissues and fixed in neutralized 10% formalin solution. Female animals received a single subcutaneous injection of TP (Fluka Chemie, Buchs, Switzerland, 20 μg in 0.02 ml of sesame oil) on the day of birth (androgenized). Androgenized females were killed under heavy ether anesthesia, dissected under abdominal tissues, and fixed in formalin solution. Both oil-injected and androgenized females were treated with TP (1 mg in 0.05 ml of sesame oil) on 57 and 147 or 52, 54 and 57 days of age. TP-treated females including oil-treated females were killed under heavy ether anesthesia on 60 and 150 days of age, and the LA muscles were excised and weighed. Tissues of these animals were divided into two sets for light and electron microscopical examinations. For microscopical examination, the tissue around the rectum in 1-, 4- and 7-day-old females was cut transversely, fixed in neutralized 10% formalin solution, embedded in paraffin and sectioned serially in the plane transverse to the rectum. The sections were stained with hematoxylin and eosin.

For electron microscopy, the small pieces of LA muscles were fixed in phosphate-buffered 2.5% glutaraldehyde (pH 7.3) for 4-5 h and then postfixed in 1% osmic acid (phosphate-buffered to pH 7.3) for 1-2 h. Tissue fragments were dehydrated in graded concentra-

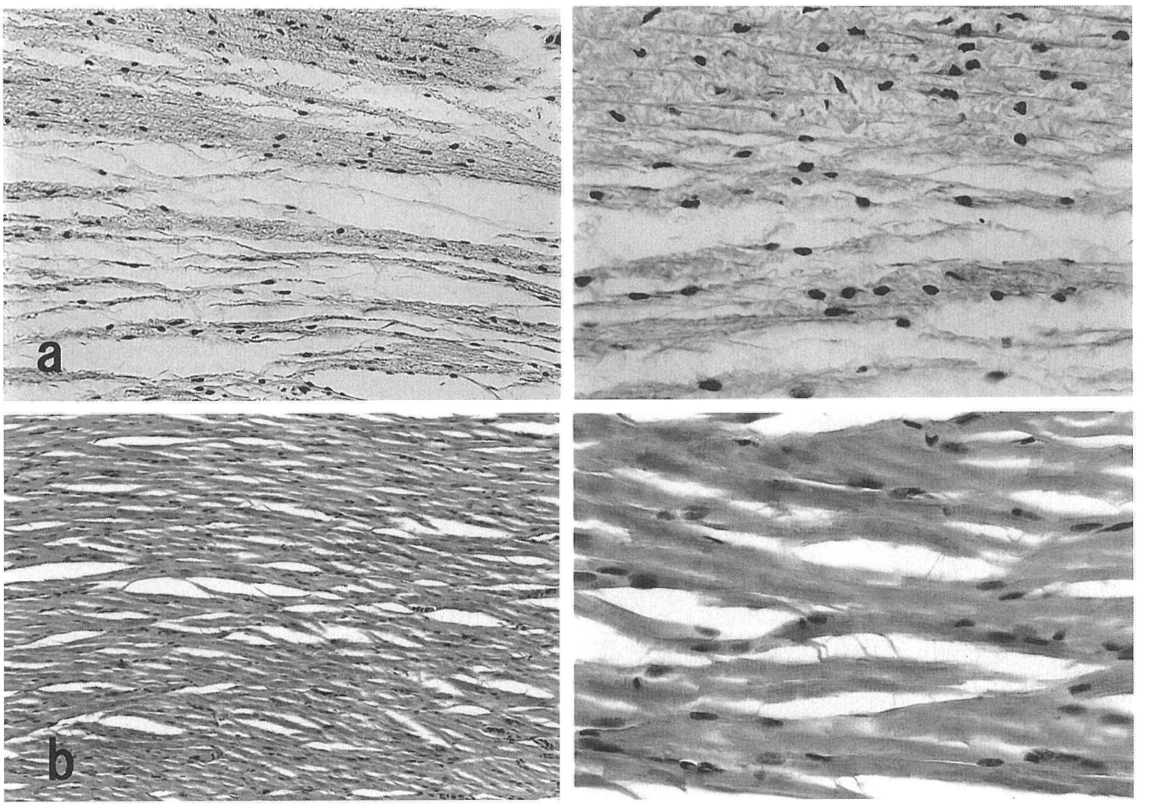

Fig. 1 Light microscopic photographs of longitudinal-section of muscle fibers in the LA muscle of androgenized 60-day-old female rats without (a) and with (b) TP re-administration on day 57. Note the transformation to fibrous strands and muscular destruction (a), and well-developed muscle fibers (b). Left : ×100, Right : ×400 (original magnification). H & E.

tions of ethanol and embedded in Quetol 812. Survey semi-thin sections were cut and stained with toluidine blue. Localized areas of the muscle were selected and thin sections were cut on a Reichert ultratome. Grids with thin sections were stained with uranyl acetate followed by lead. Sections were screened on a JEMXI1200 transmission electron microscope.

Statistical analysis

Statistical analysis of difference between groups was performed by one-factor ANAVA.

Results

(Permanent effect of neonatal TP injection on the regeneration of LA muscles)

In 60-day-old female rats subjected to oil at the day

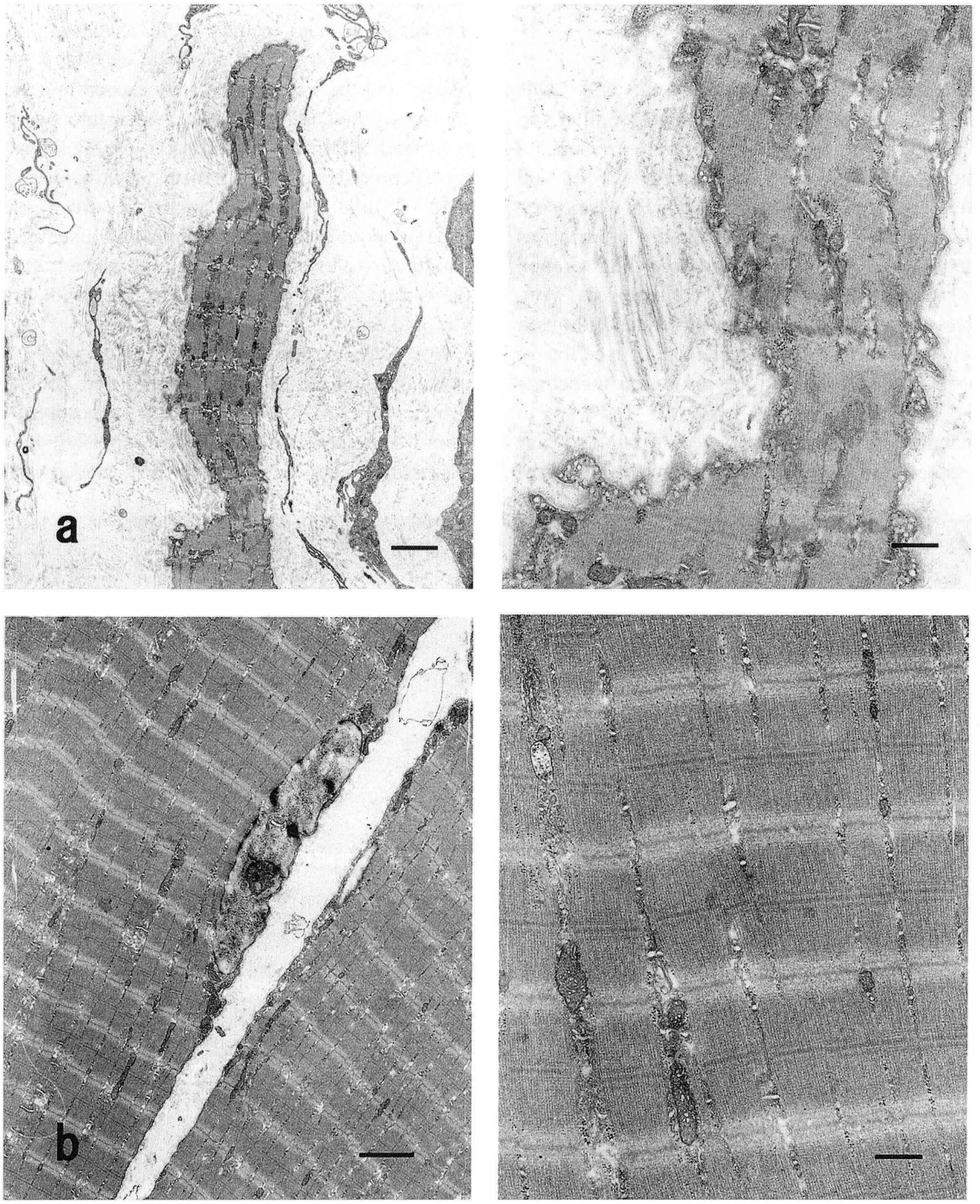

Fig. 2 Electron micrographs of the longitudinal section in the LA muscle of androgenized 60-day-old female rats without (a) and with (b) TP re-administration on day 57. Note the fragment of muscle fiber with myofibrillar disorganization (a) and the normal alignment of myofilaments in the muscle fibers (b). a and b : Bar=2 μm. right : higher magnification, Bar=0.5 μm.

of birth and TP at the attainment of puberty, LA muscles did not develop and the weights, therefore, could not be measured as noted in non-treated females. In females treated with TP at the day of birth, abortive regeneration clearly accompanied muscle destruction, when the females matured. Only a thin fibrous strand was seen around the site of LA muscle (Fig. 1a). Occasionally fragments of muscle fibers with rudimentary cross-striations were found in the strand (Fig. 2a). In neonatally TP-injected female rats subjected again to TP in the postpubertal period, development of the LA muscle was complete; myofibrils showed clear cross-striation as observed in normal adult males (Figs. 1b, 2b). A single injection of TP on day 57 or 147 of age significantly increased in the weights of LA muscles in neonatally TP-treated females compared with that in oil-treated females (Fig. 3). Triple injections of TP on days 51, 54 and 57 of age further increased the weights of LA muscles (Fig. 3).

(Normal- and TP-induced development of the LA muscle)

Normal postnatal development of LA muscle in male rats showed progressive proliferation and differentiation of the muscle fibers (Fig. 4). At the day of birth (1 day old), the muscle composed of myotubes, was slender and showed ventral thickening. We observed longitudinal myofibrils and cross-striation. The 4-day-old muscle increased in size, cross-striation of the myofibrils was clearer and myotubes changed into muscle fibers with characteristic shifting of the nuclei to the periphery. In some parts of the muscle, typical myotubes were still found. On day 7 after birth, most of the myotubes had differentiated into muscle fibers; cross-striation was clearly recognizable and surrounding fibrous tissue had transformed into adipose tissue.

The LA muscle of females developed similarly to that of males until the day of birth. On the day of birth, the LA muscle showed regressive changes. Thin myotubes of the LA muscle contained very few myofibrils, and these showed no cross-striation. This involution was rapid and thereafter the LA muscle disappeared around the rectum (Fig. 5).

A single injection of TP on the day of birth resulted in the development of LA muscle in female rats. On day 4 after birth, the muscle of the female developed similarly to that of 4-day-old male rats (Fig. 6a). The proximal parts of the muscle were thick and raphe could easily be recognized. The myotubes differentiated into muscle fibers with clearer cross-striation. At 7 days of age the muscle showed abortive regeneration accompanying muscle destruction (Fig. 6bc). The muscle loop surrounding the rectum was outlined by a fibrous strand, this arrangement still suggesting the original shape of the muscle (Fig. 6b). A few myotubes containing myofibrils were still present. In some females, the muscle loop was interrupted and from the ends of the remaining myotubes regenerating strands of cytoplasm with rows of nuclei and occasional stumps of myofibrils could be seen (Fig. 6c).

Discussion

The present experiments demonstrated that a single injection of TP at the day of birth resulted in the regeneration of LA muscle following re-administration

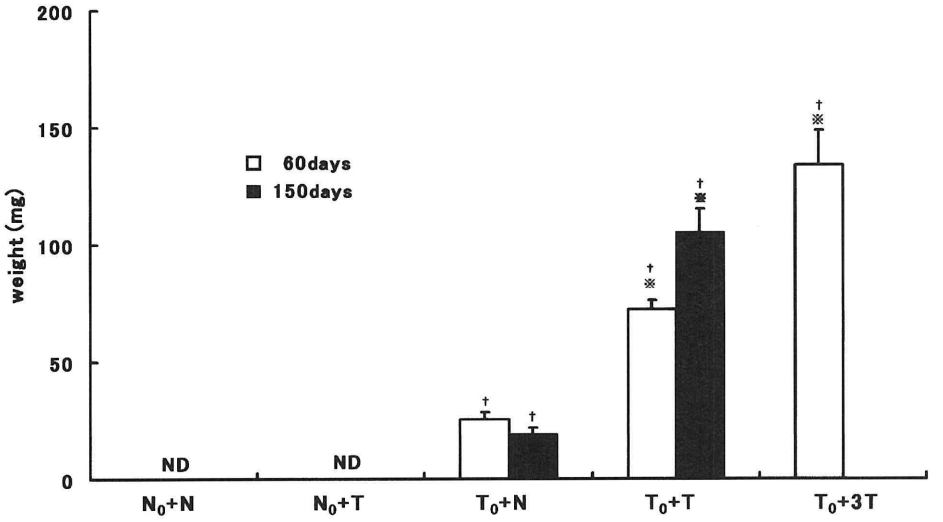

Fig. 3 LA muscle weights in neonatally oil- (N_0) and TP- (T_0) treated female rats subjected again to oil (N) and TP (T) on day 57 or 147 after the first injection. 3T represents TP injections on days 51, 54 and 57 (three times) after birth. Outlined and solid bars represent the neonatally androgenized female rats subjected to TP re-administration on 57 and 147 days of age, respectively. ND: not distinctive. Values are means±S.E.M. of 5-10 animals. +$P<0.01$ compared with neonatally oil-treated animals subjected to TP on day 57 of age. *$P<0.01$ compared with neonatally TP-injected animals subjected to oil on day 57 of age (Anova).

of TP in adult female rats, though TP injection could not develop the LA muscle of adult females without neonatal TP-injection. These results indicated that neonatal injection of TP induces a permanent effect on muscle sensitivity to re-administered TP during the postpubertal period. On the other hand, when female rats were given androgen injections during early postnatal life, permanent effects on the hypothalamo-pituitary axis were produced[8]. When this hormone was given before the hypothalamus was completely developed, it interfered with the maturation of the anterior-preoptic hypothalamic area; In adulthood, this area governs the cyclic release of the ovulating hormone[9]. Thus, adult female rats treated neonatally with androgens do not ovulate and develop persistent estrus. Therefore, it seems very likely that neonatal application of androgen produces permanent effects on the differentiation of the central nervous system and the muscular susceptibility to re-administered androgen. In androgenized females without the re-administration of androgen, the site of the muscle was outlined only by fibrous strands, although their arrangement still suggested the original shape of the muscle. A few destroyed muscle fibers remained until the puberty. Although androgen re-administration resulted in the regeneration of LA muscle, the origin of regenerated muscle fibers is not known. During muscle regeneration, up-regulation in the expression of growth factors is essential to nurture the proliferation of muscle precursor cells, their differentiation and fusion into myoblasts and their

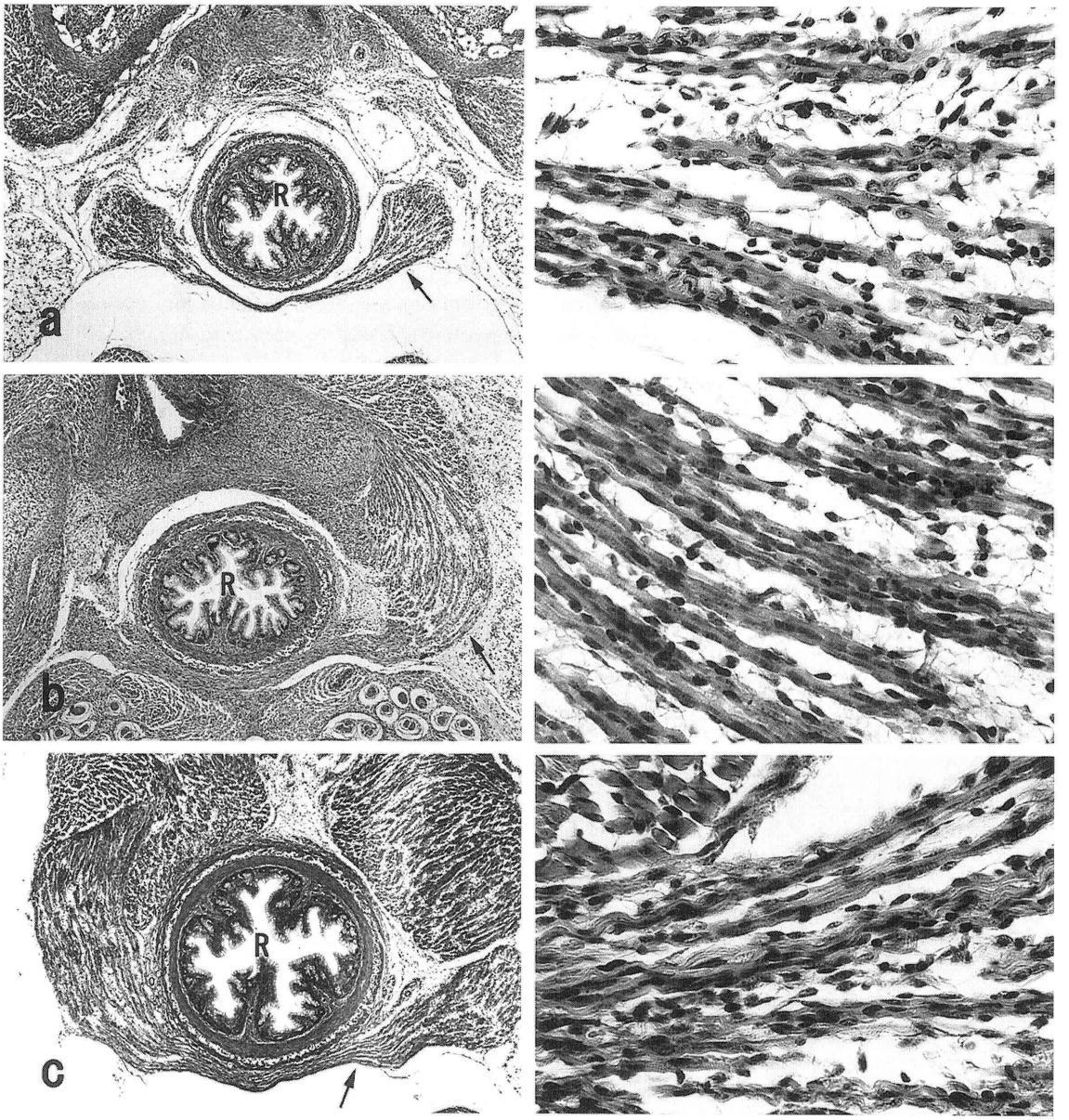

Fig. 4 Light microscopic photographs of the LA muscle in male rats. Note the progressive growth and differentiation of the LA muscle. a: 1-day-old male rat (day of birth), b: 4-day-old male rat, c: 7-day-old male rat. Arrow: LA muscle, R: rectum. Left: ×40, Right: ×400 (original magnification). H & E.

development into mature muscle fibers[12)13)]. An increased expression of insulin-like growth factor (IGF)-1 was associated with the regeneration[14)] and hypertrophy[15)] of muscle fibers and is produced by muscle, independent of growth hormone[14)]. In the present study, the effect of TP on regeneration of the LA muscle may be associated with the over-expression of IGF.

Androgen activity in androgen-dependent muscles is known to be mediated through androgen receptors (AR). Antonio et al.[16)] demonstrated that androgen administration caused an increase in AR levels. Although it is not yet known whether AR is expressed in neonatal female LA muscle, our experiments indicate that muscle hypersensitivity to androgen may persist until puberty. Since the present experiments do not elucidate the exact role of AR and its mechanisms of action in the muscle response to TP, we needed to certify the expression of AR in female LA muscles during the perinatal life.

The involution of the LA muscle is an example of "apoptosis" and lead to complete sexual dimorphism. No doubt the lack of male hormones is the definitive factor for involution. It is well known that the differentiation and development of the genital tract at birth was not completed[17)]. It was speculated that the gonads of male fetuses are primarily the source of an active influence on sexual differentiation, lack of the gonads would lead to a loss of accessory sexual organs, such as involution of the LA muscle in the rat[18)19)]. The involution which appears to be closely associated

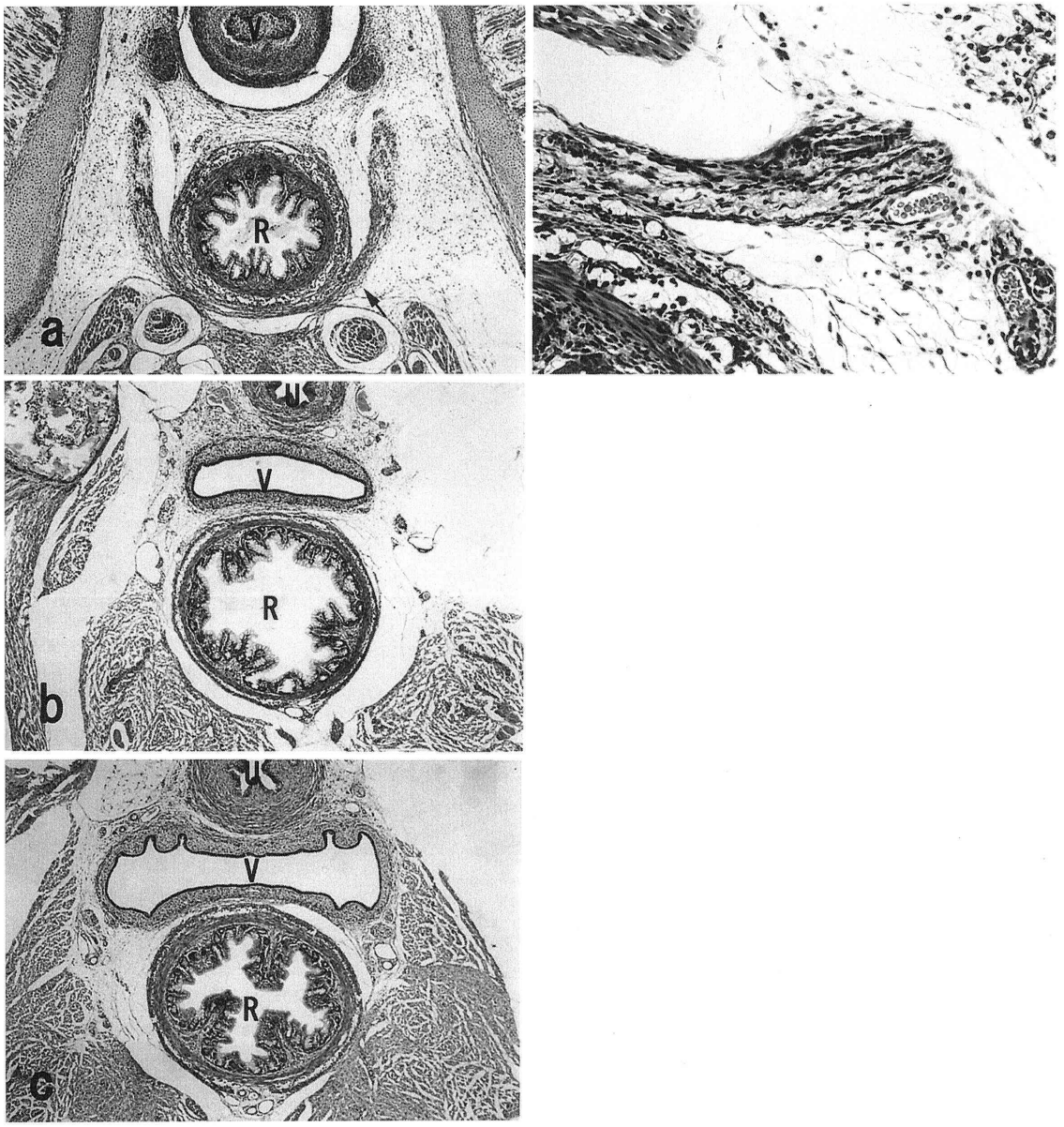

Fig. 5 Light microscopic photographs of the LA muscle in female rats. Note the abortive regeneration of the LA muscle. a : 1-day-old female rats, b : 4-day-old female rats, c : 7-day-old female rats. Arrow : LA muscle, R : rectum, V : vagina, U : urethra. Left : ×40, Right : ×400 (original magnification). H & E.

with changes in the interplay of hormones could be induced by other mechanisms (neural etc). It is of interest that androgen injection maintains the muscle even when denervation is performed perinatally[20]. This finding suggests an absolute dependence of this muscle at hormonal level, at least in the early stages of development. Despite numerous studies dealing with hormonal sensitivity, relatively little is known about the development of the female rat LA muscle. In the present experiments, the development of the female LA muscle appears to be the same as that of male until the day of birth, and thereafter regresses rapidly within a few days after birth, while the muscle of the male develops progressively. This suggests that studies on the mechanisms involved in the development of the LA muscle during the gestational and perinatal periods are needed.

Moreover, apoptosis has been reported to occur as a result of androgen withdrawal in androgen-responsive organs such as the prostate and the seminal vesicles[21]. Of particular interest is over-expression of the testosterone-regressed prostate message 2 (TRPM-2), also referred to as clusterin. TRPM-2/clusterin is an anti-apoptotic gene which appears as a general marker of apoptosis in hormone-dependent organs[22-24]. Sawamura et al.[26] showed that androgen withdrawal results in the over-expression of TRPM-2/clusterin in rat LA muscle as well as the prostate, indicating that an anti-apoptotic response is a trigger in response to an-

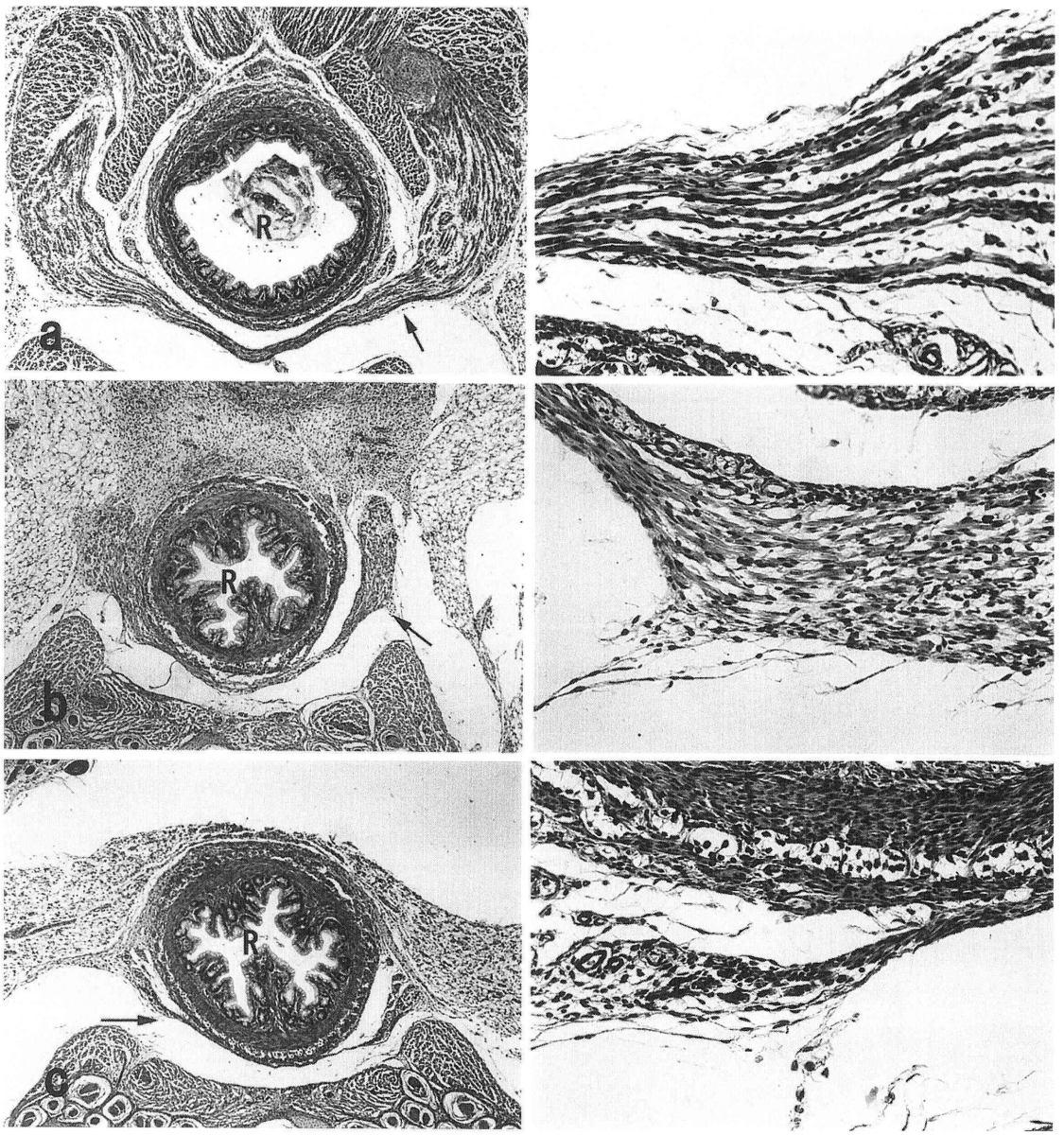

Fig. 6 Light microscopic photographs of the LA muscle in female rats treated with TP on the day of birth. Note the development and then abortive regeneration of the LA muscle. a : 4-day-old (3 days after TP injection) female rats, b) c) : 7-day-old female rats. Arrow : LA muscle, R : rectum. Left : ×40, Right : ×400 (original magnification). H & E.

drogen withdrawal. Based on those results, Asato et al.[27] clearly demonstrated the actual localization of TRPM-2/clusterin expression at the protein level in the LA muscle both in normal and castrated rat, using the immuno-Gold TEM technique. They also showed that TRPM-2/clusterin preferentially localized at the boundary of sarcomeres (Z-lines) of the myofibrils in intact control LA muscle, and that the TRPM-2/clusterin shifted from the Z-line to other parts of the I-band in the muscle of castrated rats. These results suggested that TRPM-2/clusterin appears to be one of the Z-proteins associated with binding(s) between actin and other Z-line filaments. Therefore, these results indicated the possibility that an androgen serves to maintain the function of TRPM-2/clusterin in the Z-line of myofibrils. However, it is not yet known whether TRPM-2/clusterin acts as an anti-apoptotic protein or a structural protein in the LA muscle.

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雌ラット肛門挙筋の発達に対する出生直後のアンドロジェン投与の効果

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【要旨】 今回の研究は、雌ラットにおける肛門挙筋の発達に対する出生直後のアンドロジェン投与の影響を検討するために計画されたものである。動物はすべて Wistar 系ラットを用いた。アンドロジェンとしては Testosterone propionate (TP) を使用した。

出生直後に TP を一回投与し、性成熟後に再度 TP を投与すると、肛門挙筋は発達し、筋線維の筋フィラメント配列も正常であった。出生日に TP を投与し、性成熟後にゴマ油を与えた動物では、肛門挙筋の輪郭は認められたが、筋線維は線維の網工に置き換わっていた。また、網工の中にはしばしば退化した筋線維も観察された。他方、出生直後にゴマ油を投与した動物では、性成熟後の TP 投与による筋の発達はみられなかった。性成熟後の TP 投与によって再生する筋線維の由来は明らかではないが、今回の結果は、周産期の TP 投与が性成熟後の雌肛門挙筋の再生に重要な意味をもつことを示すものであった。

正常雄動物の肛門挙筋は出生直後より加齢と共に分化・発達し、性成熟後では顕著であった。一方、性成熟後の雌では肛門挙筋は確認できないが、出生時には退化構造を示し、生後 4 日目以降は消失した。しかし、出生日に TP を投与すると、投与 3 日目 (4 日齢) には発達し、雄のものと同等構造にあったが、7 日齢以降は退化した。このことは、肛門挙筋の発達が胎生期ではアンドロジェン非依存性であり、出生後ではアンドロジェン依存性であることを意味する。今後、周産期のアンドロジェン投与が性成熟後の雌肛門挙筋の再生を誘導する機構について更に検討する必要がある。

〈キーワード〉 肛門挙筋、アンドロジェン、発達、再生、雌ラット