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Effects of castration on localization of TRPM-2/clusterin expression in the rat levator ani muscle

Mitsunobu ASATO[1)], Shoitsu SAWAMURA[2)], Hideyo ANDO[3)], Eiichi KAWAMOTO[4)], Kazuhiko SHIRAMA[1)], Jinzo YAMADA[1)]

[1)]Department of Anatomy (2), Tokyo Medical University, Shinjuku-ku, Tokyo 160-8402, Japan
[2)]Department of Health and Physical Education, Faculty of Education, Iwate University, Morioka, Iwate 020-0132, Japan
[3)]Department of Anatomy (1), Tokyo Medical University, Shinjuku-ku, Tokyo 160-8402, Japan
[4)]Animal Research Center, Tokyo Medical University, Shinjuku-ku, Tokyo 160-8402, Japan

Abstract

We sought to determine the actual localizations of testosterone-regressed prostatic message-2 gene (TRPM-2/clusterin) expression at the protein level in the levator ani (LA) muscles of both normal and castrated rats, using the Immuno-Gold TEM technique. In normal rat LA muscles, most gold particles were preferentially located in the I-band (73.7±16.3%), especially on the Z-line (53.1±10.2%). In castrated rats, the relative localization of TRPM-2/clusterin on the Z-line was significantly less than in normal controls (cont : 53.1±10.2% vs cast : 26.5± 4.1%, $p<0.05$). On the contrary, gold particles located outside the Z-line but within the I-band were significantly increased in castrated rats compared with controls (cont : 20.6±5.2% vs cast : 37.3±6.3%, $p<0.05$). This is the first report on the localization of TRPM-2/clusterin expression in the skeletal muscle. These results suggest that TRPM-2/clusterin appears to be one of the Z-proteins associated with the integrators between actin and other structural proteins such as α-actinin, titin, nebulin, spectrin and/or desmin within the Z-line of myofibrils. Therefore, it is possible that an androgen serves to maintain the function of TRPM-2/clusterin in the Z-line of LA muscles.

Introduction

The levator ani (LA) muscle is a skeletal muscle that loops around the rectum and attaches to the base of the penis in adult rodents. In adult females the LA muscle is absent[1)] or vestigial[2)]. This large sex difference emerges during perinatal life and is androgen-dependent[1,3)]. LA muscle size is permanently masculinized in females subjected to testosterone around the time of birth[2,3)]. The LA muscle of the male rat is exquisitely sensitive to androgens, much like the prostate or the seminal vesicles[2,4)]. In contrast to a typical skeletal muscle[5)], castration induces severe atrophy of the LA muscle resulting in important alterations in its contractile properties[6)].

Apoptosis has been reported to occur as a result of androgen withdrawal in androgen-sensitive organs such as the prostate and the seminal vesicles[7)]. The testosterone-regressed prostate message 2 (TRPM-2), also termed clusterin (TRPM-2/clusterin)[8,9)], is an anti-apoptotic gene which appears to be a general marker of apoptosis in hormone-dependent organs. In the prostate, TRPM-2/ clusterin was first considered to be an androgen-regressed gene with its protein product playing a role in regression of the gland after castration[10-12)]. Some studies have demonstrated that a close association

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Corresponding author : Dr. Mitsunobu Asato, Department of Anatomy, Tokyo Medical University, Shinjuku-ku, Tokyo 160-8402, Japan

exists between castration-induced apoptosis and the induction of TRPM-2/clusterin expression in the epithelium of the rat prostate[10–12]. In the LA muscle, Boissonneault[13] and Sawamura et al.[14] have shown that androgen withdrawal results in the overexpression of TRPM-2/clusterin, indicating that an anti-apoptotic response is triggered in response to androgen withdrawal. However, morphological aspects of TRPM-2/clusterin expression is not yet known in detail in the LA muscle as an androgen-dependent organs.

The present experiments were designed to determine the localizations of TRPM-2/clusterin expression, and to investigate the effect of androgen withdrawal on the expression of TRPM-2/clusterin in the LA muscle.

Materials and Methods

Specific pathogen-free Wistar rats were used. These animals (two females and one male) 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 0. Pups were sexed according to ano-genital 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 institutional guidelines of Tokyo Medical University.

Five animals at 11 weeks of age were gonadectomized under light ether anesthesia. All animals including intact controls (5 animals) were killed under heavy ether anesthesia at 7 days after gonadectomy, and the LA muscles were excised and weighed.

Dissected LA muscles were fixed with 4% formaldehyde in 0.1 M phosphate buffer. Samples were rinsed in buffer, dehydrated through a series of graded ethanols and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan), which was polymerized at 60°C for 3 days. Ultrasections, 85–100 nm, were cut using a diamond knife on a Reihert-Jung ultramicrotome, and mounted on formvar-coated nickel grids. Sections were rinsed in 0.01% phosphate buffered saline (PBS). After treatment with 10% H_2O_2 for etching, sections were treated with 1% bovine serum albumin (BSA) in 0.01 M PBS to block nonspecific binding, and incubated with primary anti-human TRPM-2/clusterin rabbit antibody diluted 1 : 50 with 1% BSA/PBS overnight at 4°C. After incubation, the sections were rinsed with PBS and incubated for 2 hrs at room temperature with anti-rabbit IgG conjugated with 10 nm colloidal gold particles (BB International, Cardiff, UK) diluted 1 : 100 in BSA/PBS. Grids were washed with PBS and then with distilled water. After the final washing, grids containing tissues were stained with uranyl acetate and lead. Tissues were then examined and photographed using a JEM-1200EXII transmission electron microscope.

To investigate the localization of gold particles in the LA muscle, five sections in each specimen dissected at the center of LA muscles were used. For quantification, pictures were printed at magnification of 45,000–75,000. Then 175 sarcomeres in control animals and 185 sarcomeres in castrated animals were selected at random on 2–3 photographs in each section. The dispersed gold particles in the sarcomeres were counted manually. To study the distribution of gold particles in the LA muscles, sarcomeres were divided the according to following criteria. A. Myofilament ① within the I-band (a: on the Z-line, b: out of the Z-line) ② within the A-band. B. Sarcoplasm (out of the myofilaments). The ratio of each criterion to total gold particles in the myofibrils was calculated.

Values were expressed as mean±S.E.M. Statistical significance between the means was assessed with Student's *t*-test (two-tailed test). A value of $p<0.05$ was regarded as indicating a statistically significant difference.

Results

Organ weights

The effects of castration on the wet weights of LA muscles were studied. The weights at 7 days following castration were significantly decreased when compared with those of intact controls (cast: 203±12 mg, $n=5$ vs cont: 243±15 mg, $n=5$, $p<0.05$).

Immuno-Gold TEM examination

As shown in Figs. 1 and 2, gold particles were predominantly dispersed around the I-band, especially on the Z-line in normal animals. In castrated animals, the particles also preferentially located in the I-band, but some particles shifted to out of the Z-line (Figs. 3 and 4).

The result of quantitative study on the localization of TRPM-2/clusterin expression in the sarcomeres of LA muscles is presented in Table 1. Most gold particles were located in the myofilaments of sarcomeres in LA muscles from both control (88.0%) and castrated rats (84.9%). The other gold particles were scattered in the mitochondria and around the interstitial connective tissue (sarcoplasm) of the myofibrils. In LA muscles from castrated rats, gold-particles located on the I-band slightly reduced as compared with that in intact controls. The percentage of gold particles located on the Z-line significantly reduced in castrated rats when compared with intact control rats. On the contrary, gold particles expressed out of the Z-line but within the I-band significantly increased in castrated rats compared with that in controls. Although no significant difference between the two was observed, the ratio of gold particles located on the A-band tended to increase in castrated

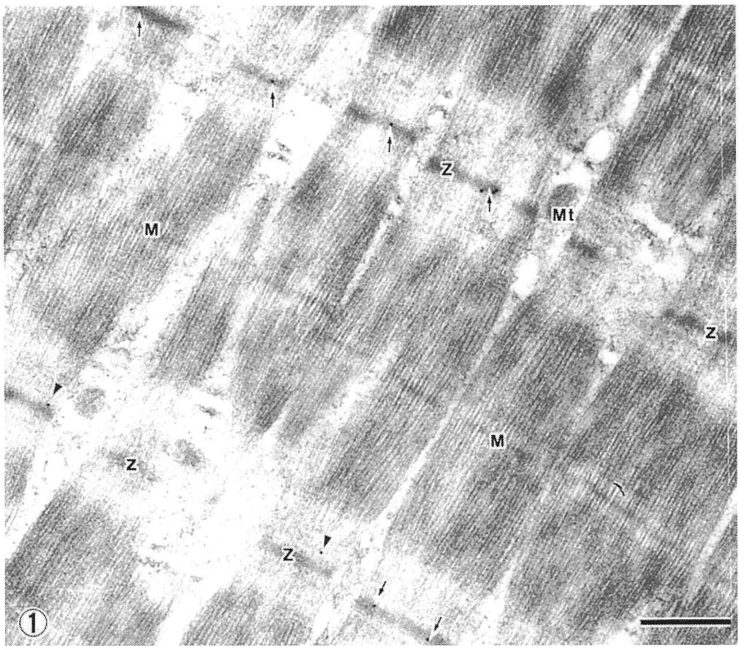

Fig. 1 Immuno-gold transmission electron micrograph showing TRPM-2/clusterin in the LA muscle of intact control rat. Immuno-gold particles were preferentially located in the I-band, especially on the Z-line (Z) (arrows) of the myofibrils. Some particles were dispersed out of the Z-line (arrowheads), but within the I-band. M : M-line of the A-band. Mt : mitochondrion. Bar : 0.5 μm

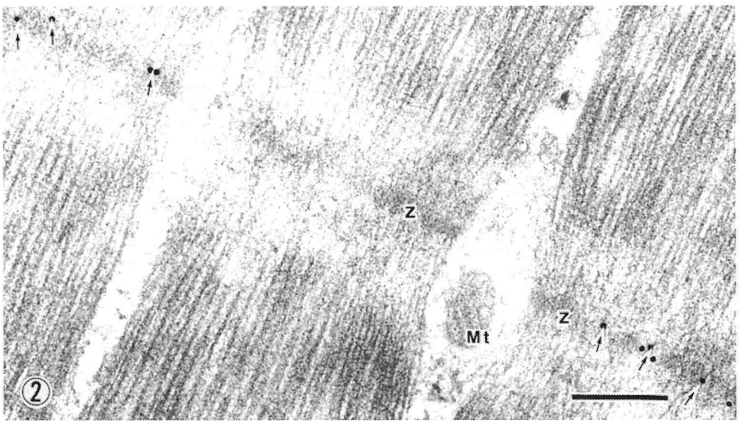

Fig. 2 Higher magnification of LA muscle in the intact control rat. All immuno-gold particles were located on the Z-line (Z). Mt : mitochondrion. Bar : 0.2 μm

Table 1 Effects of castration on the localization of TRPM-2/clusterin expression in the rat LA muscle.

Immuno-gold particles on the myofibrils of LA muscles were counted manually on electron-photomicrographs. Data represent the mean of percentages (%) in each experimental group containing 5 rats (total sarcomeres : control = 175 ; castration = 185) ± S.E.M *$p < 0.05$ compared with control group

	Control (Total particles : 2,153)	Castration (Total particles : 2,294)
Myofilaments	88.0 ± 19.2%	84.9 ± 20.1%
I-band	73.7 ± 16.6%	63.8 ± 17.4%
On the Z-line	53.1 ± 10.2%	26.5 ± 4.1%*
Out of the Z-line	20.6 ± 5.2%	37.3 ± 6.4%*
A-band	14.3 ± 4.8%	21.1 ± 5.8%
Sarcoplasm	12.0 ± 4.3%	15.1 ± 3.9%

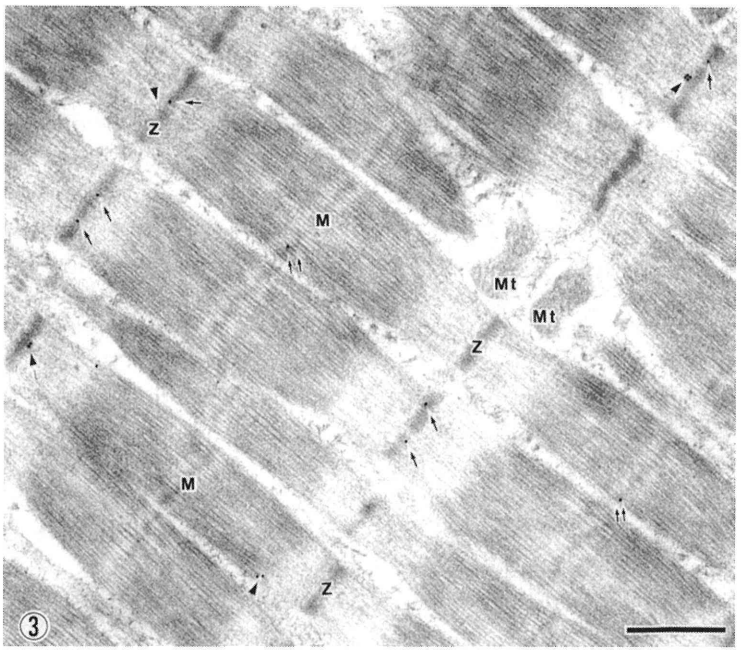

Fig. 3 Immuno-gold transmission electron photograph showing TRPM-2/clusterin in the LA muscle of a castrated rat 7 days after gonadectomy. Immuno-gold particles were dispersed on the Z-line (arrows), out of the Z-line but within the I-band (arrowheads) and M-line (M) of the A-band (double arrows). Mt : mitochondria. Bar : 0.5 μm

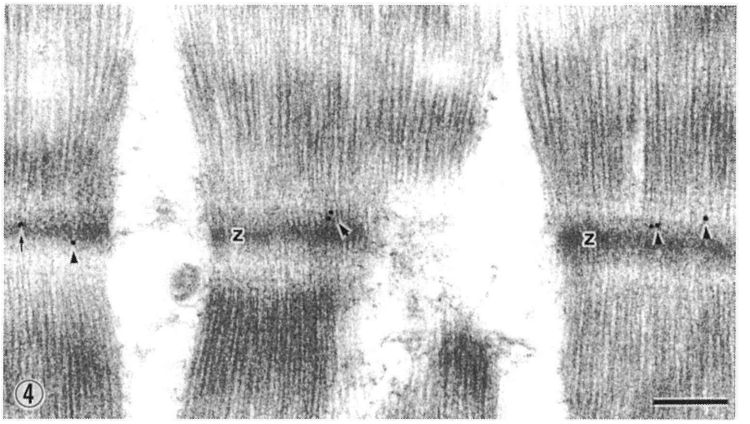

Fig. 4 Higher magnification of the LA muscle in a castrated rat 7 days after operation. Most immuno-gold particles were dispersed out of the Z-line (Z) (arrowheads), but within the I-band. Only one particle was located on the Z-line (arrow). Mt : mitochondrion. Bar : 0.2 μm

rats as compared with that in control rats.

Discussion

Our results showed that TRPM-2/clusterin was predominantly expressed around the I-band, especially on the Z-line in rat LA muscles. This is apparently the first report to demonstrate the localization of TRPM-2/clusterin expression in the skeletal muscle. Moreover, we demonstrated that androgen withdrawal caused an aberration of TRPM-2/clusterin expression sites. In the prostate, it has been postulated that transcriptional activation of the TRPM-2/clusterin gene is an important mediator of castration-induced prostatic involution[11,15]. Recently, Sawamura et al.[14] have shown the morphological aspects of TRPM-2/clusterin expression in castrated rat prostate. Small granules were formed around the Golgi complexes in the glandular epithelium and subsequently fused to form larger granules (secretory granules), often with irregular outline. These results indicated that the mechanism of TRPM-2/clusterin synthesis appears to be the same as that of other glandular products such as citric acid in the prostate. Since the site of TRPM-2/clusterin expression is the boundary of sarcomeres in the LA muscle, it is not clear whether TRPM-2/clusterin is associated with the apoptotic process in the skeletal muscle as indicated by other hormone-dependent organs such as the prostate. Recently, Boissonneault[13] has shown that androgen withdrawal results in the overexpression of TRPM-2/clusterin in rat LA muscles, indicating that an anti-apoptotic response is

triggered in response to androgen withdrawal. Actually Sawamura et al.[14] confirmed overexpression TRPM-2/clusterin in castrated rat LA muscles, using Western blot analysis. In spite of the above results, the present results showed a possibility that TRPM-2/clusterin is not only regarded as an anti-apoptotic protein but also a structural protein in the Z-line of skeletal muscle, because TRPM-2/clusterin was also detected in LA muscle from intact control rat and in vastus lateralis muscle which is a characteristic fast-twitch skeletal muscle[16].

In normal differentiated muscle fibers, the repeating structural unit, to which all the morphological events of the contractile cycle are referred, is the sarcomere, which is defined at the segment between two successive Z-lines. Sarcomeres are highly organized structures composed of actin (thin) and myosin (thick) filaments that slide past each other during contraction. Of particular importance is the organization of the actin filaments in muscle fibers, where a stable anchorage of actin filaments at the Z-line of the sarcomeres is required for the transmission of mechanical strain along the length of the serially arranged sarcomeres, ultimately along the length of the muscle.

The details of the interrelation of filaments of successive sarcomeres at the Z-line are still a subject of debate, but certain points seem to be adequately established. Z-lines aid to maintain the sarcomere in register in the myofibril. They serve as an anchoring plane of the actin filaments; they link titin and actin filaments from opposing sarcomere halves in a lattice connected by α-actinin and contain desmin. Titin (connectin), the most abundant and largest muscle cytoskeletal protein, occurs in actin filaments that run parallel to the myosin filaments of the sarcomere. Nebulin, another cytoskeletal protein, appears to run parallel to, and in close association with, actin filaments. Desmin, a small cytoskeletal protein occurs within and between Z-lines of adjacent sarcomeres to maintain lateral association between sarcomeres[17–19].

Studies employing protein interaction analysis demonstrate that two types of titin interactions are involved in the assembly of α-actinin into the Z-line. Titin interacts by means of a single binding site with the two central spectrin-like repeats of the outermost pair of α-actinin molecules. The function of spectrin that occurs within the central Z-line is the interaction of titin with multiple α-actinin molecules by means of their C-terminal domains. This activity permits the assembly of a complex of titin, actin, and α-actinin with constraint of the path of titin in the Z-line[20]. In immunogold TEM studies, we clearly demonstrated that gold particles preferentially localized at the boundary of sarcomeres (=Z-line) of the myofibrils in intact control LA muscle, and that the particles shifted from the Z-line to other parts of the I-band in the muscle of castrated rat. From these results, it semms very likely that TRPM-2/clusterin appears to be one of the Z-proteins associated with the integrators between actin and other Z-line filaments such as α-actinin, titin, nebulin, spectrin, or desmin in the Z-line. Moreover, these results suggest the possibility that an androgen serves to maintain the function of TRPM-2/clusterin in the Z-line of myofibrils. However, further studies will be needed to determine whether TRPM-2/clusterin acts as a structural protein in an androgen-sensitive skeletal muscle.

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ラット肛門挙筋における TRPM-2/clusterin 発現部位とそれに対する去勢の影響

安里満信[1)] 澤村省逸[2)] 安藤英世[3)]
川本英一[4)] 白間一彦[1)] 山田仁三[1)]

[1)]東京医科大学解剖学第二講座
[2)]岩手大学教育学部保健体育講座
[3)]東京医科大学解剖学第一講座
[4)]東京医科大学動物実験センター

【要旨】 Testosterone-regressed prostatic message-2 (TRPM-2) は、前立腺等の Androgen 依存性器官に去勢により発現する Anti-apoptotic 遺伝子である。今回の報告は、Androgen 依存性骨格筋である肛門挙筋における TRPM-2/clusterin 発現 (蛋白レベル) 部位を金コロイド免疫電顕法で検討すると共に、その局在に対する去勢の影響も併せて検索したものである。

正常ラット肛門挙筋では、殆どの金コロイド粒子は筋原線維の筋フィラメント内 (88.0±19.2%) に散在し、その多くは I−帯 (73.7±16.6%) とくに Z−線上 (53.1±10.2%) に限局した。去勢後 7 日目のラット肛門挙筋では、筋フィラメント内 (84.9±20.1%) および I−帯内 (63.8±17.4%) 金粒子の相対数には正常動物と比較して有意な差はないものの、I−帯内の Z−線上 (26.5±4.1%) の金粒子は有意に減少していた。今回の結果は、TRPM-2/clusterin が正常肛門挙筋にも発現し、特異的に筋節間に局在することを意味するものであった。また、この発現部位は去勢により Z−線上からずれることを示していた。以上の結果から、同じ Androgen 依存性器官であっても前立腺等とは異なり、肛門挙筋における TRPM-2/clusterin は Anti-apoptotic な蛋白というよりはむしろ、Z−線上における actin と α-actinin や titin 等の他の構造蛋白との統合に関与する Z−蛋白の一つであると考えられる。従って、Androgen は肛門挙筋の Z−線における TRPM-2/clusterin の機能を調節している可能性が示唆される。

〈**Key words**〉 TRPM-2/clusterin、肛門挙筋、Z−線、去勢、ラット