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Distribution and Change of Collagen Types I and III and Elastin in Developing Leg Muscle in Rat

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ABSTRACT

The distribution of collagen types I and III and elastin in the developing leg muscles were studied by immunohistochemistry in rat. From 0-day to 8-weeks old, the size of the gastrocnemius and plantaris muscles increased. The muscle connective tissue developed in the order of epimysium, perimysium and finally endomysium. The epimysium contained a considerable amount of collagen types I and III and some elastin in the neonates. These components in the epimysium remained almost unchanged in their distribution during development. The perimysium had little collagen type I and III or elastin at 0 day. Collagen type I and elastin slightly increased around 2 and 1 week, respectively, and returned to the previous levels. Collagen type III, however, increased and became abundant after 1 week. In the endomysium, the amounts of collagen type I and elastin were slight during postnatal growth, while collagen type III gradually increased after 2 weeks. The intramuscular tendons consistently showed intense reactivity for collagen type I and weak staining for elastin, whereas the staining for collagen type III decreased after 1 week and was finally restricted to the surface of intramuscular tendons. This study clearly demonstrated that the distribution of collagens, but not of elastin, significantly changed during development. The increase in collagen type III in the perimysium and endomysium, and its decrease in the intramuscular tendons probably reflect functional demands imposed on these connective tissues, i.e., shear forces in the former two and tensile loading in the latter.

Key words: *Collagen type I, Collagen type III, Elastin, Skeletal muscle*

Muscle connective tissue is histologically divided into three levels of organization: the epimysium (also referred to as the fascia), perimysium and endomysium. The epimysium surrounds the entire muscle, the perimysium bundles a group of muscle fibers into muscle fascicles, and the endomysium covers individual muscle fibers and fills out spaces among them. An intramuscular tendon may also be present in pennate muscles. These connective tissues have a diversity of roles, as follows: (1) make a framework and bind the muscle fibers together, maintaining their proper spatial alignment, (2) transmit forces, either actively produced by the muscle or passively imposed on the muscle, (3) act as a shock absorber, (4) provide considerable mobility for muscle to contract or extend by diminishing friction and allow smooth movements, and (5) serve as a pathway for nerves and blood vessels.

Collagen is one of the most important components in connective tissue. At least 19 different types of collagen have been reported[15]. Type I is the most abundant collagen in a variety of tissues. The major collagen types in skeletal muscle are types I and III[11]. Collagen has an extremely low compliance and is able to resist tensile force with minimal elongation of less than 10%[6]. On the other hand, elastic fibers can increase their length by 150%[6], and are considered to play a role in tissue mobility[10]. The mechanical properties of connective tissues, such as the ability to resist tension, compression, and extension, depend on the nature and proportions of the connective tissue components[6]. Small increases in the quantity of collagen in a muscle increase the stiffness of the tissue considerably[1]. Muscle connective tissue probably changes the amounts and types of collagen during muscle development due, in part, to

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the increase in the forces the muscle develops. However, the developmental process, which gives rise to differences in muscle connective tissue, and its functional significance have not been fully understood. Therefore, in the present study, the distribution of collagen types I and III and elastin were examined by immunohistochemistry during the development of the rat gastrocnemius and plantaris muscles.

MATERIALS AND METHODS

A total 18 Wistar rats of both sexes aged 0, 3 days, 1, 2, 4 and 8 weeks, 3 animals each, were used in this study. The animals were reared under conditions of 22–25˚C with a constant humidity of 55 ± 5% and a 12 hr light-dark cycle (light 8:00–20:00). Food and water were supplied ad libitum. Experimental procedures were approved by the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University.

Rats were sacrificed by overinhalation of diethyl ether. For 0- and 3-day-old rats, the hindlimbs were freed from the overlying skin and amputated at the thigh. Aqueous tragacanth gum jelly (6%) was placed on cork disks (about 20 mm in diameter and several mm thick) and the legs were supported by tragacanth gum on the cork disks so that the muscle could be cut either transversely or longitudinally. For 1-, 2-, 4- and 8-week-old rats, the gastrocnemius, soleus and plantaris muscles were removed en bloc and bones were excluded. These muscles had their deeper side stuck to balsa wood (1–2 mm thick) which had been freshly coated with 6% tragacanth gum. Each specimen on balsa wood was supported by tragacanth gum on a cork disk. Specimens and cork disk were then immersed and frozen together in isopentane cooled with liquid nitrogen. Cryosections (10 μm) of legs and muscles were cut in a cryostat and used for immunohistochemistry.

Immunohistochemistry

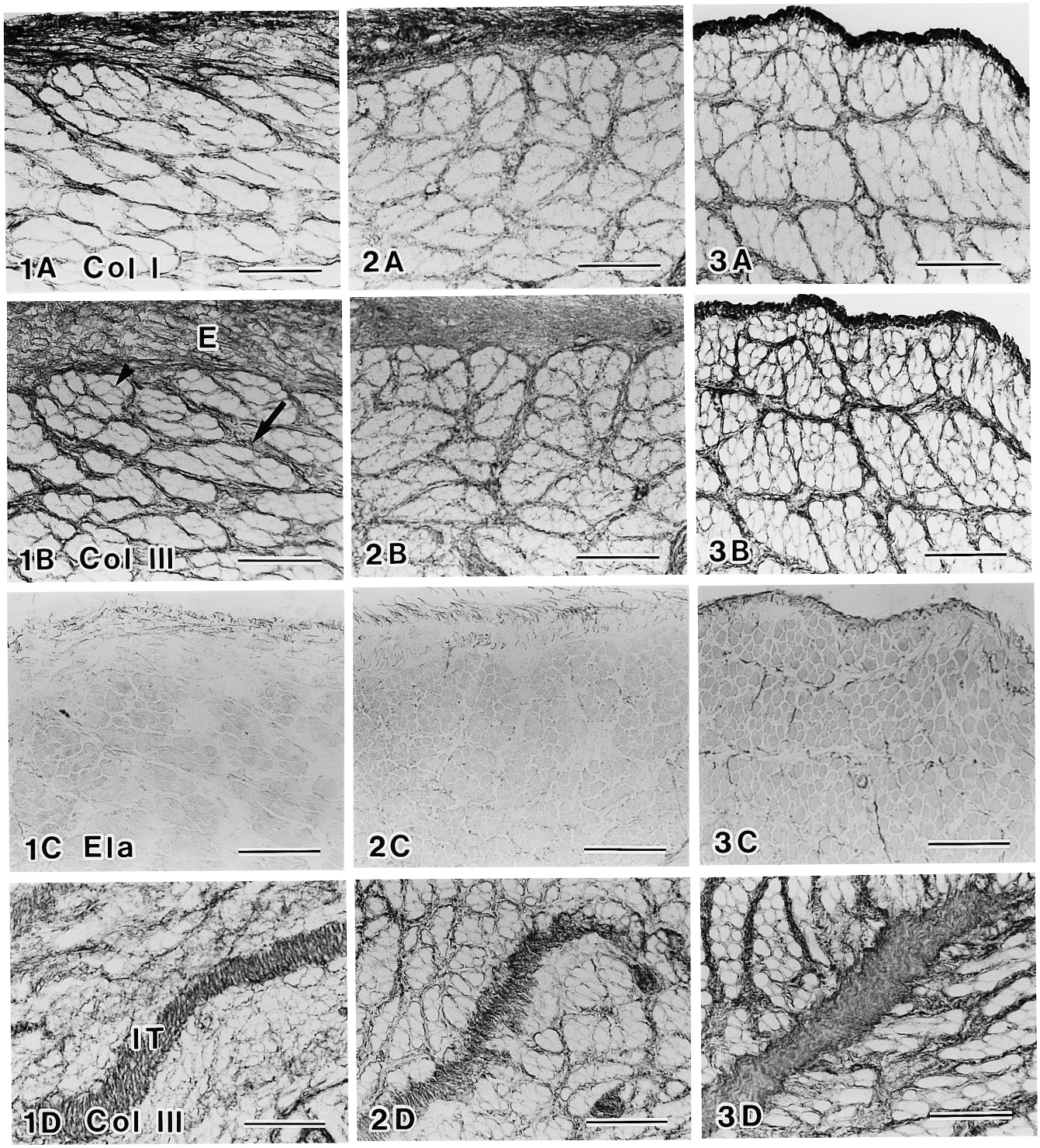
Sections were air-dried, fixed in acetone for 10 min and rehydrated in 0.01 M phosphate-buffered saline (PBS; pH 7.4) for 5 min. Endogenous peroxidase was inactivated by incubation of the sections in methanol containing 0.3% H_2O_2 for 20 min. Then, nonspecific binding sites were blocked by treating sections with PBS containing 1% normal horse serum (for collagen-type specific antibodies) or rabbit serum (for the anti-elastin antibody), depending on the second antibodies to be used. After blotting the blocking fluid, the sections were incubated with one of the following primary antibodies for 2 hr. Primary antibodies used were mouse monoclonal anti-rat collagen type I (1:4,000 dilution; C-2456, Sigma, St Louis, MO, USA), mouse monoclonal anti-rat collagen type III (1:8,000 dilution, C-7805, Sigma, St Louis, MO, USA) and goat polyclonal anti-rat elastin (1:10,000 dilution; RA75, Elastin Products Company, Inc., Owensville, MO, USA) antibodies. Collagen-type specific antibodies are guaranteed to show no cross reactivity against the other type of collagen examined in this study (as stated by the manufacturer). After washing with PBS three times (5 min each), sections were incubated with secondary antibodies, i.e., horse biotinylated anti-mouse IgG (1:250 dilution; BA-2001, Vector Laboratories, Burlingame, CA, USA) for anti-collagen antibodies, or rabbit biotinylated anti-goat IgG (1:30 dilution, Histofine, Nichirei, Tokyo, Japan) for the anti-elastin antibody for 1 hr. After washing with PBS twice for 5 min each time, sections were incubated with streptavidin-horseradish peroxidase for 30 min. Specimens were washed with PBS twice for 5 min each and immunoreactivity was visualized with 0.05% 3, 3'-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.2). The sections were then washed with PBS, dehydrated and mounted. Negative controls were incubated with mouse IgG (1:20 dilution, 2V-2001-10, Vector Laboratories, Burlingame, CA, USA) or goat IgG (1:20 dilution, 2V-3001-10, Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions instead of the primary antibodies. All procedures were conducted at room temperature.

RESULTS

In the present study, the gastrocnemius and plantaris muscles were selected for observation because the hamstrings were somewhat more advanced in development compared with other muscles. Young muscle had the least amount of connective tissue. As development proceeded, the muscle fibers and fascicles increased in size and the total connective tissue content increased. Based on the onset of collagen type III increase, it was considered that muscle connective tissue develops first in the epimysium, then in the perimysium and finally in the endomysium. Collagen types I and III and elastin were clearly stained by antibodies against them, while all negative control sections were unstained.

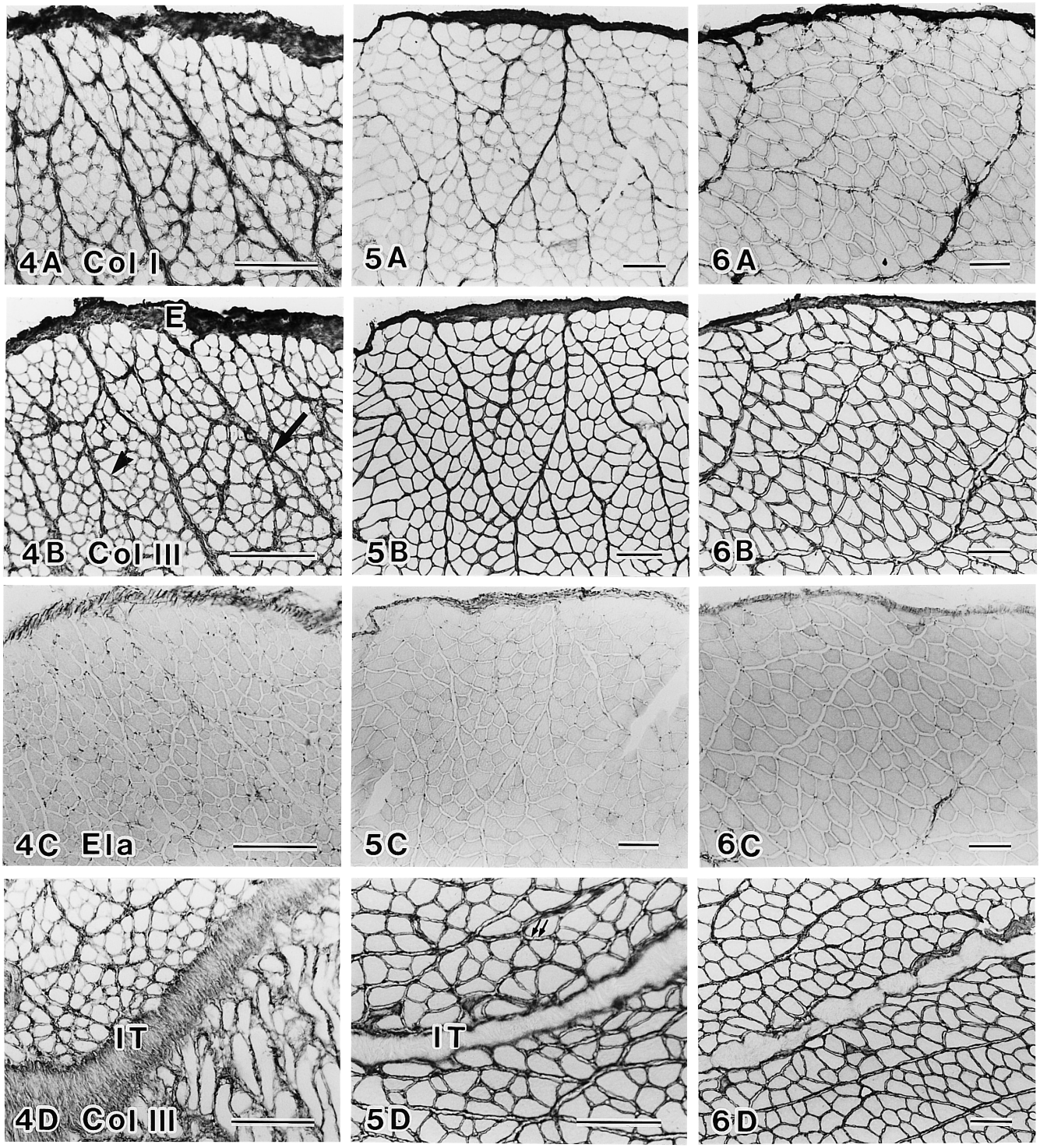
At 0 day old

The muscles were small and the connective tissue was generally scarce. In the epimysium, a considerable amount of collagen types I and III and some elastin were co-distributed (Fig. 1A, B, C). The perimysium was poorly developed and loosely assembled the muscle fascicles. Collagen types I and III loosely surrounded muscle fascicles without clear boundaries (Fig. 1A, B). Muscle fascicles were recognizable, but not well defined. The endomysium was almost unstained, containing lit-



Figs. 1, 2, 3. Transverse sections of the rat gastrocnemius muscle. Semiserial sections (panels A, B, C) are stained with antibodies against collagen type I (panels A), collagen type III (panels B) and elastin (panels C), respectively. Panels D show the intramuscular tendon stained for collagen type III. Col I, collagen type I. Col III, collagen type III. Ela, elastin. Scale bar = 100 μm.

1. At 0 day old. The epimysium (E) contains a considerable amount of collagen types I and III and some elastin, whereas the perimysium (arrow) and endomysium (arrowhead) are poorly developed. The intramuscular tendon (IT) shows intense staining for collagen type III.
2. At 3 days old. The perimysium is not well organized and the endomysium shows little collagen type I and III or elastin.
3. At 1 week old. The perimysium contains relatively abundant collagen type III. The endomysium has a small amount of collagen type III and a little collagen type I and elastin.



Figs. 4, 5, 6. Transverse sections of the rat gastrocnemius muscle. Semiserial sections (panels A, B, C) are stained with antibodies against collagen type I (panels A), collagen type III (panels B) and elastin (panels C), respectively. Panels D show the intramuscular tendon stained for collagen type III. Col I, collagen type I. Col III, collagen type III. Ela, elastin. Scale bar = 100 μm.

4. At 2 weeks old. The epimysium (E) contains abundant collagen types I and III and small amount of elastin. The perimysium (arrow) is well defined and contains abundant collagen types I and III. In the endomysium (arrowhead), type III collagen is increased. Intramuscular tendon (IT) shows diminished staining for collagen type III inside it (panel D).

5. At 4 weeks old. Muscle fibers are significantly increased in size. The endomysium shows intense staining for collagen type III and an unstained gap (small arrows in panel D) is observable between muscle fibers. In the intramuscular tendon (IT), collagen type III is restricted to the surface.

6. At 8 weeks old. The perimysium and endomysium were stained intensely for collagen type III and slightly for collagen type I and elastin.

tle collagen type I and III or elastin (Fig. 1C). Muscle fibers were small and of various sizes. The intramuscular tendons were composed of thick collagen fibers containing collagen types I and III (Fig. 1D) and little elastin.

At 3 days old

Collagen types I and III and a little elastin were co-localized in the epimysium (Fig. 2A, B, C). Collagen type III in the perimysium, with regionally varying thickness, was not well organized and surrounded oval- or irregular-shaped muscle fascicles (Fig. 2B). In the perimysium, some collagen type I and little elastin were present. The endomysium showed little collagen type I and III or elastin. The intramuscular tendons contained relatively abundant collagen types I and III (Fig. 2D).

At 1 week old

Muscles and their connective tissue were slightly developed. The epimysium was thick and contained abundant collagen types I and III and some elastin (Fig. 3A, B, C). Muscle fascicles were separated by a thick perimysium (Fig. 3B). The perimysium contained abundant collagen type III, but only some collagen type I and a little elastin. The endomysium had a small amount of collagen types I and III. In the endomysium, a little elastin was recognizable (Fig. 3C). Intramuscular tendons were intensely stained for collagen type I throughout the tendon. Immunostaining for collagen type III was somewhat reduced inside the intramuscular tendon (Fig. 3D).

At 2 weeks old

Muscles and connective tissue were relatively well developed. The epimysium contained abundant collagen types I and III and a small amount of elastin (Fig. 4A, B, C). The perimysium was almost regular in thickness and separated muscle fascicles distinctly. The endomysium was thin and surrounded most muscle fibers. In the endomysium, collagen type III increased its staining intensity (Fig. 4B), but collagen type I and elastin increased only a little (Fig. 4A, C). The immediate periphery of each muscle fiber was intensely outlined by collagen type III, whereas weakly or

Table 1. Collagen type I immunostaining

Age	Epimysium	Perimysium	Endomysium	Intramuscular tendons
0 day	++	+	–	+++
3 days	++	+	±	+++
1 week	++/+++	+	±/+	+++
2 weeks	++	++	±	+++
4 weeks	++	+	–/±	+++
8 weeks	++	+	–/±	+++

–, negative; ±, faintly positive; +, weakly positive; ++, moderately positive; +++, strongly positive.

Table 2. Collagen type III immunostaining

Age	Epimysium	Perimysium	Endomysium	Intramuscular tendons
0 day	++	+	–/±	+
3 days	++	+	±	++
1 week	++	++	±	±
2 weeks	++/+++	++	+	–/±
4 weeks	++/+++	++	++	–/±
8 weeks	++/+++	++	++	–/±

–, negative; ±, faintly positive; +, weakly positive; ++, moderately positive; +++, strongly positive.

Table 3. Elastin immunostaining

Age	Epimysium	Perimysium	Endomysium	Intramuscular tendons
0 day	+	±	±	+
3 days	+	±	±	+
1 week	+	+	±	+
2 weeks	+	±	±	+
4 weeks	+	±	±	+
8 weeks	+	±	±	±

–, negative; ±, faintly positive; +, weakly positive; ++, moderately positive; +++, strongly positive.

unstained gaps were occasionally recognized between neighboring muscle fibers. Intramuscular tendons demonstrated a strong reactivity for collagen type I, while the staining was diminished for collagen type III inside it (Fig. 4D).

At 4 weeks old

Muscles and connective tissue were well developed at this stage. The epimysium contained abundant collagen types I and III and a small amount of elastin (Fig. 5A, B, C). The perimysium had usually a constant thickness and muscle fascicles were clearly defined (Fig. 5B). The perimysium was stained intensely for collagen type III and to a lesser extent for collagen type I and a little elastin. The endomysium showed intense staining for collagen type III, but collagen type I and elastin were hardly stained (Fig. 5A, C). Collagen type III was positive at the surface of muscle fibers and an unstained gap was often observable between neighboring muscle fibers (Fig. 5D). Intramuscular tendons demonstrated intense staining for collagen type I throughout the tendon, whereas collagen type III was located on the surface of the intramuscular tendon with the inside being virtually negative (Fig. 5D).

At 8 weeks old

Muscles and connective tissue were well developed in this almost adult stage. The epimysium contained abundant collagen types I and III, as well as fragmentary elastin (Fig. 6A, B, C). The perimysium was thin and extended among muscle fascicles. The perimysium and endomysium contained abundant collagen type III and a little collagen type I and elastin. Collagen type III in the endomysium was largely restricted to the surface of muscle fibers and there was a thin unstained gap between muscle fibers (Fig. 6B). Intramuscular tendons were intensely positive for collagen type I, but collagen type III was restricted to the surface (Fig. 6D).

The semiquantitative interpretations of the reactivity of collagen types I and III and elastin were evaluated subjectively and graded one of 5 grades on a scale – to +++, as presented in Tables 1, 2, and 3.

DISCUSSION

Muscle connective tissue has been examined using antibodies against collagen types I and III in bovine muscle[7] and chick muscle[3]. These studies revealed that the epimysium mainly contains collagen type I, and the perimysium and endomysium predominantly contain collagen type III. Bailey and Sims[2] analyzed collagen contents in bovine muscle by electrophoresis. Light and Champion[13] demonstrated biochemically that the percentages of collagen type III / collagen types (I + III) were 16.4%, 28.0% and 62.3% in the epimysium, perimysium and endomysium, respectively, in the bovine pectoralis profundis muscle. Järvinen et al[8] reported that the epimysium contains mainly collagen type I, with some collagen type III, the perimysium collagen types I and III, and the endomysium collagen type I with little type III in normal rats. Although some discrepancies exist, most studies suggest a tendency towards the percentage, but not content, of collagen type I being the highest in the epimysium and the lowest in the endomysium. This tendency is in good agreement with our observations. In addition, the present study clearly demonstrated that such a regional difference in collagen types among muscle connective tissue becomes evident during growth, and that the muscle connective tissue develops from the epimysium to the perimysium and finally the endomysium. The increase and change in collagen probably relates to the locomotive activity of rats, since rats wean and take food by themselves by 3 weeks after birth.

It is well known that collagen type I is very strong against tensile stress. The increase in collagen type I during muscle growth in the epimysium and tendons is reasonable because strong tensile forces are imposed on these structures. Collagen type I in the epimysium seems to prevent overstretching and over-contraction of the muscle, and enhances muscle mobility by reducing friction between overlying skin and the muscle, probably in a similar way to the subcutaneous connective tissue[10]. Elastin seems to allow sliding among collagen layers when shear force is imposed, restoring the position of collagen layers after the removal of the force[10]. However, the elastin content in muscle is generally low. The elastin content in bovine muscle is less than 0.2%[4] or usually below 1%[17] of dry weight, while the collagen content accounts for 1–10%[17]. Due to the scarcity and good extendability of elastin, elastic fibers (which contain elastin) seem unlikely to play a major role in active force transmission.

Collagen type III is a major component in the endomysium[3,7,13]. Collagen type I confers tensile strength and rigidity, whereas collagen type III confers compliance to tissue[11]. In addition, a variety of extracellular substances, such as proteoglycans[14], fibronectin and thrombospondin-1[9] have been identified in the extracellular matrix of the perimysium and endomysium. These substances are not visualized in this study and may be present in the unstained gaps. It is speculated that tension is efficiently transmitted between adjacent muscle fibers through the endomysium by a shear mechanism[16,20,21], rather than by in-plane tension[16,18]. Trotter[20] considered that the trans-laminar shear properties are physiologically relevant for the endomysium, and that the mechanical properties of the muscle connective tissue compo-

nent must make an important contribution to the overall mechanical properties of the muscle. The fibrils in the endomysium are much more aligned circumferentially to the muscle fiber axis at short sarcomere lengths than at rest sarcomere lengths[16]. Taking these things together, it is likely that collagen type III and other components, in the perimysium and endomysium, probably play important roles in the transmission of forces by a shear, rather than a tensile mechanism. Another possible function of collagen type III is the regulation of collagen fibril diameter. It was clearly demonstrated in vitro that the ratio of collagen type III to collagen type I is inversely proportional to the diameter of collagen fibrils[12,17,19], and Birk and Mayne[5] have shown that the increase in collagen fibril diameter is associated with a decrease in collagen type III in developing tendons of the chick embryo. It is also demonstrated that the endomysium contains thin collagen fibrils in human, rat and dog lingual muscles[22]. Further analysis of the components in the perimysium and endomysium is necessary and their functions need to be clarified.

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