Morphogenesis of rat myotendinous junction

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Summary

Myotendinous junction (MTJ) is the highly specialized complex which connects the skeletal muscle to the tendon for transmitting the contractile force between the two tissues.
The purpose of this study was to investigate the MTJ development and rat EDL was chosen as a model. 1, 15, 30 day animals were considered and the junctions were analyzed by light and electron microscopy. The MTJ interface architecture increased during the development, extending the interaction between muscle and tendon. 1-day-old rats showed disorganized myofibril bundles, spread cytosol and incomplete rough endoplasmic reticulum, features partially improved in 15-day-old rats, and completely developed in 30-day-old animals. These findings indicate that muscle-tendon interface displays, during rat lifetime, numerically increased and longer tendon interdigitations, correlated with an improved organization of both tissues and with a progressive acquirement of full functionality.

KEY WORDS: morphogenesis, myotendinous junction, skeletal muscle, ultrastructure.

Introduction

In mammalian, the skeletal muscle transfers the contractile force to the tendon extracellular matrix (ECM) through a particular key structure: the myotendinous junction (MTJ). At ultrastructural level, the proximal extremity of the tendon forms interdigitations that penetrate into the muscle mass and thus increase the contact area between tissues[1].
There are significant differences in the architecture of fast and slow fibers. Many of the slow fibers are distributed along the entire length of a fascicle and they end sharply at MTJ level. In contrast, most fast fibers begin at the tendon but show a progressive decrease in cross-sectional area, to end far from MTJ. Thus, those fibers must transmit a large proportion of their force to the endomysium or adjacent muscle fibers[2].
In the MTJ, differences are revealed dependently on muscle fiber type. Red and white fibers differ in the angle of attachment to the tendon and in the general shape of MTJ. These differences are correlated to particular fiber orientation too. In addition, the finger-like extensions of the white fibers are smaller and more numerous than those of the red ones[3].
Both animal and human studies show that, during physical exercise, single muscle fascicles do not undergo the same changes in length as the whole muscle[4]. This difference is due to the influence of "elastic elements" and to the effect of the pennation angle[5,6]. Thus the role of MTJ is crucial not only for transmitting the contractile strength but even for its elastic capacity. In fact, although tendon and aponeurosis are passive structures, they act as "biological springs" that can be stretched elastically, storing and releasing energy during locomotion[6,7]. A part of this energy is returned during the concentric contraction, which involves an important contribution of tendinous tissues to the total shortening[8].
At the protein level, thin filaments that extend inside the terminal digit-like processes at the MTJ level are bundled into a sub-sarcolemmal dense plaque that provides a specialized site for adhesion and for force transmission across the cell membrane[9]. The molecular organization of these dense plaques is similar to the focal adhesion sites of cultured cells. In fact, both sites are enriched in the cytoskeletal proteins vinculin, talin, and α-actinin[10-14] and focal adhesions can provide physical coupling of actin filaments to β-integrins[15]. Furthermore, the extracellular domain of integrin heterodimers can bind ECM proteins, including collagens, fibronectin, vitronectin, and laminins[16], as well as each ECM components present at MTJ level[17].
A previous work demonstrated ultrastructual modifications of sternocleidomastoid MTJ among newborn (5 days old), adult group (4 months old) and old group (24 months old)[18]. In the present study, the morphological features of MTJ have been studied in rat EDL muscle. We analyzed three different time points of the

first month post-natal to follow the MTJ development. Furthermore, muscle and tendon ultrastructure close to the MTJ has been analyzed, for explaining the muscle-tendon interface changes.

Materials and Methods

9 albino Sprague-Dawley rats were used. Three 1-day-old animals (P1) were sacrificed. The other six rats were placed in cages and fed a standard diet without limitations. The room temperature was kept at 21 ± 1°C; 12 h of light was automatically alternated with 12 h of dark. At 15 days (P15), three animals were killed and at 30 days (P30) the remaining three rats were sacrificed.
Animal handling and mode of killing were conducted according to the European Community guidelines, to the Italian laws and to the Animal Experiment Committee of Urbino University. Rats were periodically examined by a veterinarian[19].

Light and electron microscopy

The rats were killed by an overdose of sodium thiopental. The EDL muscles were withdrawn from both the hind legs quickly, blotted dry and freed of connective tissue. Muscles, maintained under tension with pins, were immediately fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer for 3 h. The specimens were then minced into smaller (<1 mm^3) fragments and again fixed with glutaraldehyde for 1 h, postfixed with 1% OsO_4 in the same buffer for 1 h, dehydrated with alcohol, and embedded in araldite. Semithin sections were prepared, stained with 1% toluidine blue in distilled water at 60°C, and observed by light microscopy. The semithin sections were trimmed along the longitudinal plane of the muscle fibersin order to have an overview of myotendinous junction. For ultrastructural analysis nickel grids were used as supports. Thin sections, stained with uranyl acetate and lead citrate, were observed with Philips CM10 electron microscope[20].

Results

The ultrastructural observations of EDL MTJs reveal interesting differences in the tissue organization during lifetime.
In P1 rats (Fig. 1), a cross section of EDL muscle displays numerous mitochondria, the progressive assembly of myofibril bundles and the presence of wide cytosolic areas. The rough endoplasmic reticulum appears incomplete and a lot of ribosomes are scattered in the intermyofibrillarspaces (A). In the tendon tissue near the MTJ, numerous fibroblasts display an extensive rough endoplasmic reticulum and are surrounded by collagen fibers, clearly observable in cross section (B). The muscle-tendon interface appears smooth and regular. In fact, the typical tendon interdigitations which penetrate into the muscle mass, are scarcely evident or frequently absent. Some junctions highlight discontinuous areas between tissues with an incomplete basal lamina (A-C). In this region, numerous pinocytotic vesicles are observable, which demonstrate an exchange phase between tissues (D). In longitudinal sections, the sarcomere disorganization and the abundant cytoplasm (E), containing incompletetubular structures (F), are highlighted. Furthermore, only few myofilament send up to the tendon where they appear to be well linked to the ECM (G).
The MTJs of P15 rats (Fig. 2) show interesting differences if compared to P1 one. Generally, the muscle-tendon interface appears more complex and compact. The tendon interdigitations are numerous and longer than in the newborn rats. Thus, there is a growing interpenetration between the tissues (A). The basement membrane is thickened and regular and the terminal myofilaments are connected to junctional electron-dense plaques. On the contrary, where myofilaments fail the attachment to the ECM, the thickness of basal lamina is reduced or gaps in the lamina are still present (B). Collagen fibers, surrounding the MTJ, and fibroblasts are present again in tendon (C), but these cells show a reduced RER compared to that of newborn rats (2C-1B).
In the muscle fiber, the disposition of myofibril bundles appears regular, showing aligned sarcomeres and mitochondria. A peripheral nucleus also appears, so indicating the progressive of differentiation of muscle (D). The sarcoplasm considerably decreases in the intermyofibrillar areas, showing the tubular system, and, occasionally triads (E-F).
In P30 rats (Fig. 3), numerous and deep interdigitations increase the contact surface between the tissues (A). Muscle tissue presents regularly arranged myofilament bundles and serial sarcomeres, perfectly aligned both near the MTJ and far from it (A-B). In longitudinal section, many triads, typical of adult muscle, are observed at this stage (B).
The tendon collagen fibers are frequently perpendicular to the sarcolemma, penetrating deeply into the MTJ finger-like processes (C). The terminal myofilaments extend from the last Z-line to the sarcolemma where they end into electron-dense areas (D)[21]. The fibroblasts are still present near to the MTJ but their RERs appear to be reduced in comparison to the cells of other stages (3D-2C-1B).

Discussion

Every muscle fiber sticks to the tendon by means of MTJ, so transmitting passive (elastic) and active (contractile) forces to the skeletal system.
In literature some authors analysed this anatomical region in animals throughout their lifespan. Ciena et al. (2012) have described its ultrastructural modifications at sternocleidomastoid muscle level, from newborn to aged rats, highlighting a high cellular activity in the muscle close to the junction, confirmed by the

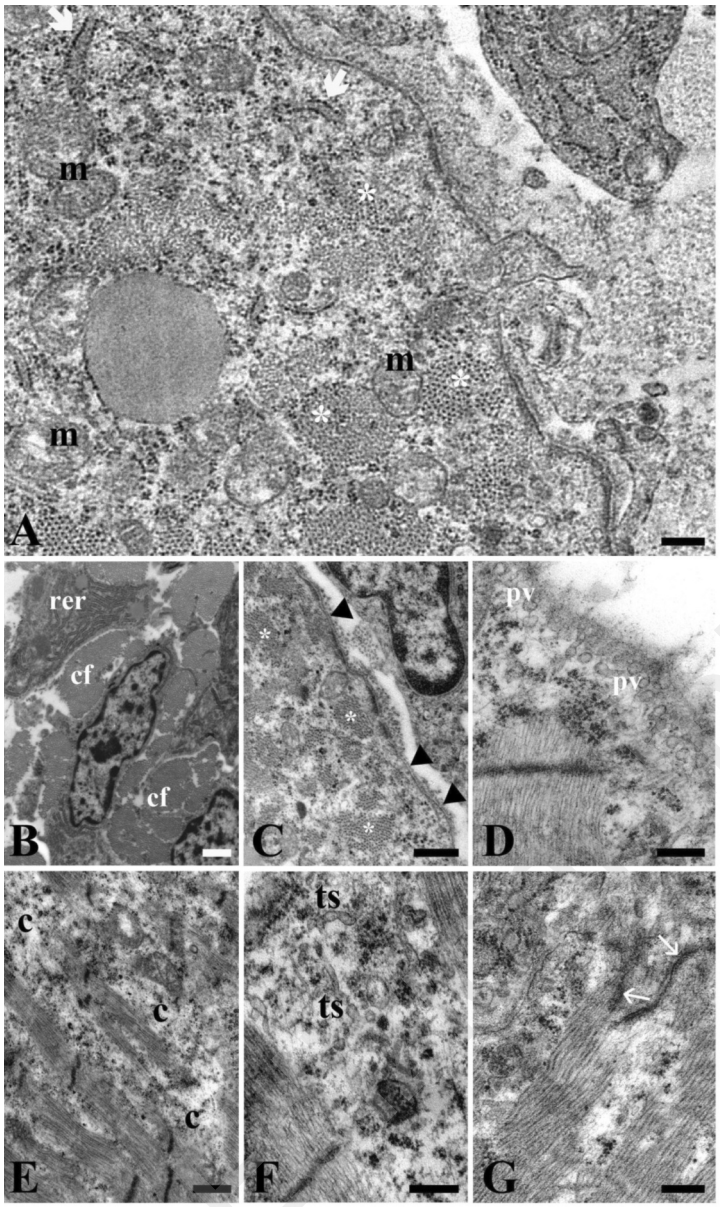

Figure 1. TEM observations of P1 rats. Muscle cells with mitochondria (m), incomplete rough endoplasmic reticulum (→) and a lot of ribosomes among the cross section of myofibrillar areas (*) are shown (**A**). Collagen fibers (cf) and active fibroblasts, which display an extensive rough endoplasmic reticulum (rer), are present in the tendon tissue (**B**). Smooth MTJ folds with a discontinuous basal lamina (▶) are visible and numerous pinocytotic vesicles are observable at this level (**C-D**). Longitudinal sections highlight sarcomere disorganization and wide cytosolic areas (c) (**E**), showing an incomplete tubular system (ts) (**F**). The few myofilaments, ending on the basal lamina, appear well linked to the tendon ECM (→)(**G**). Bars A, D, F, G: 0.25 μm; B:1 μm; C,E:0.5 μm.

presence of numerous mitochondria and capillaries[18]. Moreover, Charvet et al. (2012) showed that MTJ reveals not only important morphological changes but also molecular modifications during life time[22].

In this work, the transmission electron microscopy has been used to analyze MTJ ultrastructure during the development, also investigating the tissue modifications close to the muscle-tendon interface. Our data clearly display, how MTJ formation is a gradual organization process which involves both tissues. In fact, the muscle structure features appear very different in the considered three time points (P1, P15, P30).

The tissue disorganization of the newborn rats is characterized by a low number of myofilaments, spread cytosol among these and by an interrupted contiguity between terminal myofilaments and tendon matrix. Muscle tissue structure changes in the P15 animals, where myofibrils appear in closer contact each other. However, these rats display an altered sarcoplasm organization, still highlighting an incomplete development.

On the contrary, in P30 rats the muscle organization appears regularly assembled, as shown by the correct disposition of sarcomeres and mitochondria, the small cytoplasmic areas within myofilaments and the close contiguity between the terminal myofilaments and the tendon ECM.

Moreover, in the tendon tissue of the newborn rats, the presence of numerous fibroblast with abundant RER, which indicates an intense production of extracellular matrix, decreases in a progressive way, respectively, in P15 and P30 animals, where they appear also with a reduced RER.

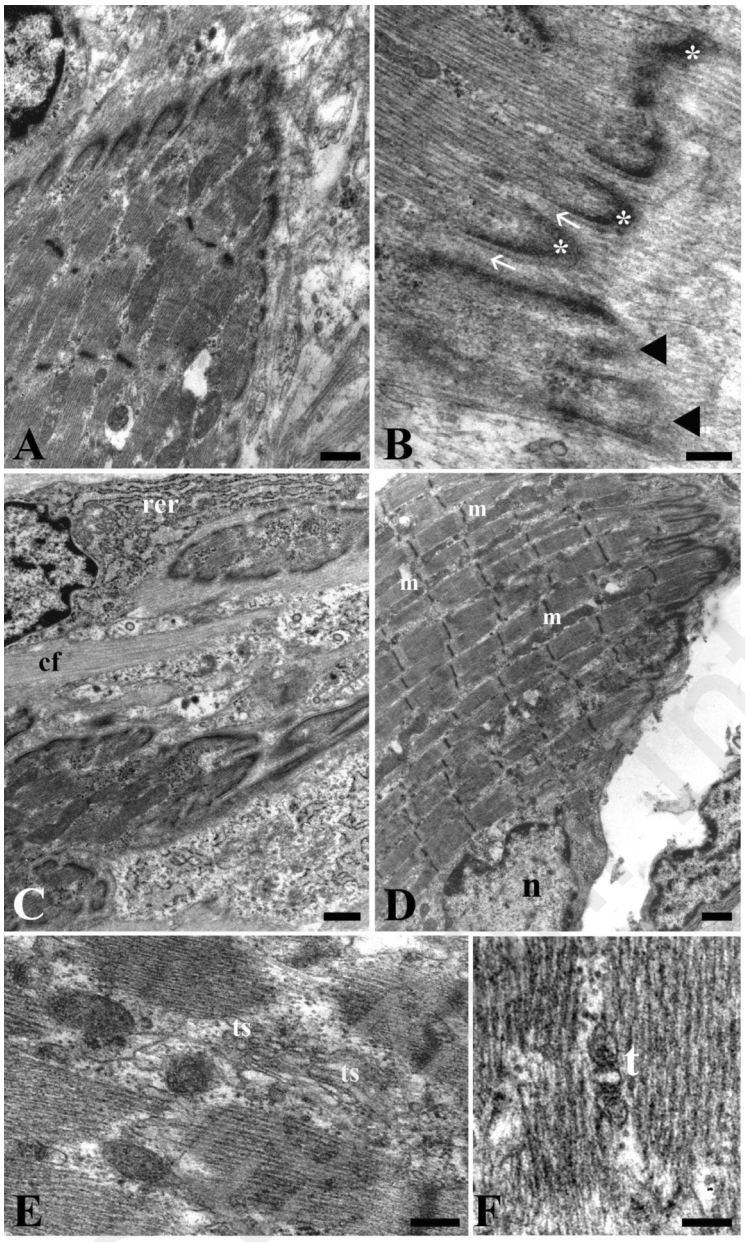

Figure 2. TEM analysis of P15 rats. The MTJs display an interdigitate profile (**A**), where numerous finger-like processes (→) are present (**B**). Muscle basement membrane is thickened and the myofilaments extend to junctional electron-dense plaques (*). In some areas, the close contiguity between muscle and tendon still seems to be absent (▶) (**B**). Collagen fibers (cf) and a fibroblast, with its rough endoplasmic reticulum (rer), are visible near MTJs (**C**). In **D**, the muscle shows a regular arrangement of sarcomeres and mitochondria (m) and a peripheral nucleus (n). In sarcoplasm, an organized tubular system (ts) and some triads (t) are present (**E**-**F**).
Bars A, C: 0.5 μm; B, E: 0.25 μm; D: 1 μm; F: 125 nm.

Furthermore, collagen fibrils seem to acquire a new orientation and organization with age. In particular, the extracellular matrix forms the tendon processes typical of adult MTJ.

Taken together, our observations demonstrate that muscle-tendon interface displays interesting structural changes during life time. At the beginning, MTJ appears interdigitation free, with a smooth and regular interface. At junctional level, the muscle basal lamina appears not always continuous but displays some gaps, where there is not a contiguity between the terminal myofilaments and the ECM. The development of both tissues involves the contact surface between muscle and tendon: in fact, the MTJs of P15 and P30 reveal, respectively, an increasing number of interdigitations which penetrate deeper into the muscle mass. During life time, the growing contact surface between muscle and tendon, could be explained as an adaptation to tension increase generated by tissue development. In fact, when analyzing the images of P30 MTJ compared to the newborn one, a growing number of myofilaments, taking contact with the tendon, is observable.

Our data are in agreement with the results of previous studies on MTJ development of muscles with different role and structure, showing similar modifications in different species too[18,22]. Differently, we demonstrated that the MTJ may reveal ultrastructural changes in the shorter time (e.g. after two weeks), which are correlated with morphologic modi-

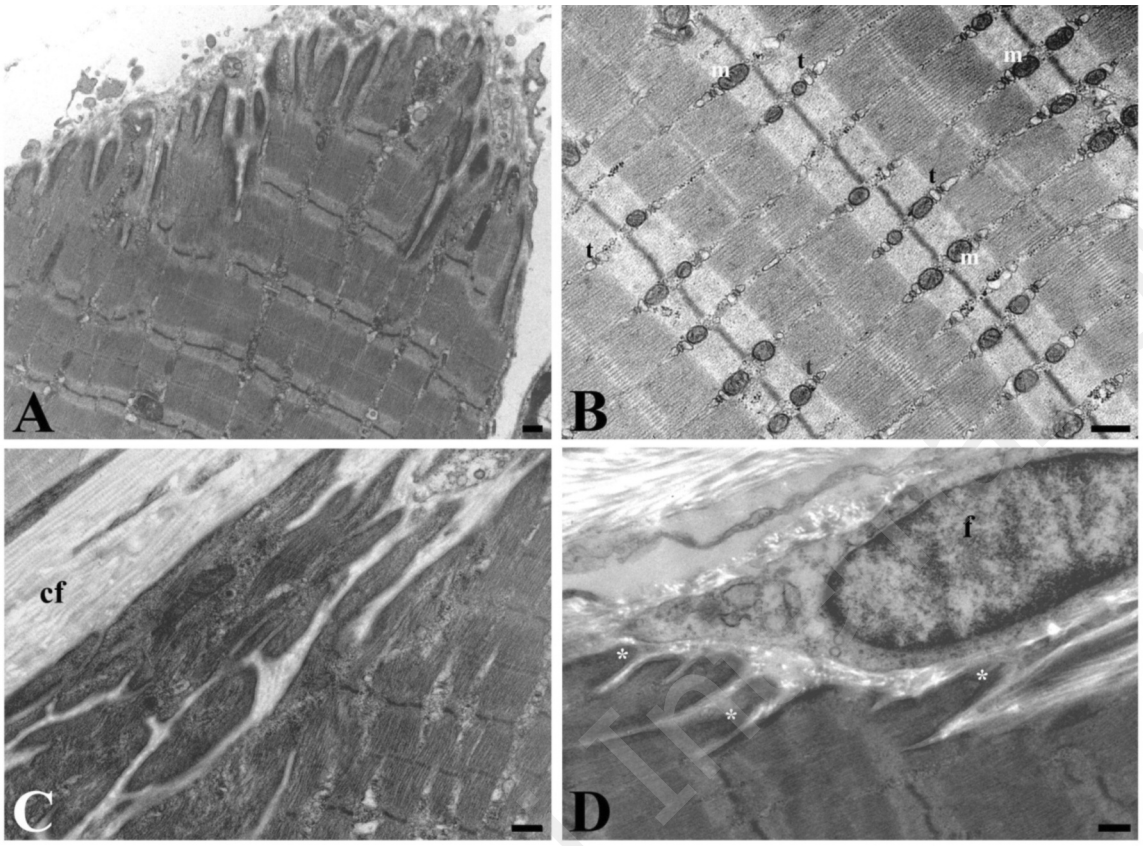

Figure 3. TEM observations of P30 rats. The muscle-tendon interface appears very folded with many long interdigitations (A). The muscle ultrastructure reveals a regular disposition of sarcomeres with aligned myofilaments and Z lines, both near the MTJ and far from it (A,B). The sarcoplasmic reticulum appears well organized and a lot of triads (t) are observable (B). In the tendon tissue, the collagen fibers appear parallel to the sarcomere direction, therefore they are distributed along the entire length of tendon interdigitations where they end at the muscle basal lamina level (C). Numerous electron-dense plaques (*) appear in the muscle tissue, between the terminal myofilaments and the sarcolemma (D).
Bars A, B, C, D: 0.5 μm.

fications of other structures like muscle basal lamina, tendon fibroblasts and myofilaments-ECM attachment. As we described, these adaptations appear to be strictly related with the development of muscle-tendon interface.

Although the MTJ has a key role in the contractile force transmission, it has been only occasionally studied in pathology. The muscle-tendon interface has been investigated in some dystrophies, for evaluating the effects correlated with the absence of dystrophinor others proteins[23,24].

The present work is a preliminary study on the MTJ morphogenesis, which amplifies the scientific knowledge about its role and modifications in specific physiological conditions. It should be viewed as the starting point for further studies, which are in progress in our laboratory. In particular, we just demonstrated how the MTJ can be modified during aerobic exercise, atrophy generated by disuse and specific protocols for atrophy prevention[1,19]. We plan to further characterize MTJ biochemical and structural changes, highlighting their correlation with different levels of force, generated by different exercise protocols.

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