Laminin Isoforms in Human Extraocular Muscles

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Purpose. To determine the laminin isoform composition of the basement membranes (BMs) in the human extraocular muscles (EOMs) and relate it to the fact that EOMs are spared in laminin α2-chain–deficient congenital muscular dystrophy.

Methods. Samples from adult human EOMs and limb muscle were processed for immunocytochemistry, with monoclonal antibodies against laminin chains (Ln) α1 to -5, β1 and -2, and γ1. Neuromuscular junctions (NMJs) were identified with acetylcholinesterase reaction. The capillary density was measured in sections stained with anti-Lnα5.

Results. The extrasynaptic BM of the EOM muscle fibers contained Lnα2, -β1, -β2, and -γ1, and, in contrast to limb muscle, it also contained Lnα4 and -α5, to some extent. The distinct laminin composition of the EOMs was confirmed by the presence of Lutheran protein, an α5-chain–specific receptor not found in limb muscle. At the NMJs, there was increased expression of Lnα4 and expression of Lnα2, -α5, -β1, -β2, and -γ1 was also maintained. The capillary density was very high (1050 ± 190 capillaries/mm^2) in the EOMs and significantly ($P < 0.05$) higher in the orbital (1170 ± 180 capillaries/mm^2) than in the global (930 ± 110 capillaries/mm^2) layer.

Conclusions. The human EOMs showed important differences in laminin isoform composition and capillary density when compared with human limb muscle and muscles of other species. The presence of additional laminin isoforms other than laminin-2 in the BM of the extrasynaptic sarcolemma could partly explain the sparing of the EOMs in Lnα2-deficient congenital muscular dystrophy. (*Invest Ophthalmol Vis Sci.* 2004;45:4233–4239) DOI:10.1167/iovs.04-0456

The extraocular muscles (EOMs) are structurally and functionally highly specialized[1–9] and have therefore been considered a separate skeletal muscle class or allotype.[10] Microscopically, the EOMs are characterized by small muscle fibers of several types that are loosely arranged in a bed of abundant connective tissue with a rich supply of nerves and vessels. Another particular feature is the presence of multiple innervation in some fiber groups. The most striking feature of the EOMs, however, is their distinct behavior in disease. They are selectively spared in congenital myopathies such as Duchenne muscle dystrophy and dystrophinopathies and are selectively involved in other neuromuscular disorders, such as oculopharyngeal muscular dystrophy, Miller-Fischer syndrome, and Grave's ophthalmopathy.

The uniqueness of the EOM allotype has been elucidated recently at the whole-muscle RNA level in rodent[9,11–14] and monkey.[15] However, further characterization of the molecular basis of the EOM allotype at the cellular level is needed before we can fully understand the structural organization that makes these muscles so unique, in particular with respect to their selective sparing/involvement in neuromuscular diseases. Substantial data[16,17] suggest that the selective sparing of the EOMs in dystrophic *mdx* mice relies on constitutive properties (most likely involving the ECM and the cytoskeleton), rather than on molecular adaptations to the absence of dystrophin, and thereby emphasize the need of a thorough characterization of the EOM allotype at the structural level. The purpose of the present study was to characterize the composition of the basement membranes in the EOMs with respect to content of laminin chains, important components of the extracellular matrix (ECM) that play both structural and signaling roles.[18–21]

Skeletal muscle fibers are surrounded by a continuous basement membrane (BM) that includes the folds of the neuromuscular junction (NMJ) and the myotendinous junction (MTJ). The major noncollagenous components of the BM are the laminins.

Laminins are glycoprotein trimers composed of an α-, a β-, and a γ-chain. There are five different laminin (Ln)α-chains (α1-5), 3 Ln β-chains (β1-3) and 3 Ln γ-chains (γ1-3) known at present. Different combinations of the chains can form >14 laminin isoforms.[22] The different laminin chains have complex patterns of expression that in some cases are tissue specific and developmentally regulated. The laminins interact with the underlying cells via cell surface receptors, such as integrins and dystroglycan complex, and thereby influence cell fate and gene expression and participate in cell–ECM communication. An intact link between the ECM and the cytoskeleton is necessary for the structural integrity of muscle fibers. Defects in any of the elements of this link (e.g., collagen, laminin, sarcoglycans, integrin, dystrophin, desmin) are known to cause muscle dystrophy.[23–28]

The predominant laminin in the BM of muscle and peripheral nerve is Ln-2 (α2β1γ1).[29,30] Mutations in the Lnα2-chain in humans lead to congenital muscular dystrophy, characteristically affecting the limb and trunk muscles, but sparing the EOMs.[24,31]

The α3-chain of laminin is characteristically present in the epithelial BMs, and in muscle it is found only in the blood vessels.[21,32] Lnα4 is present in capillaries, in muscle blood vessels and, during fetal development, it also surrounds myotubes.[33] In contrast, Lnβ1 and Lnγ1 are rather ubiquitous.[19]

Data on the composition of the BMs and on the distribution of laminin chains on the human EOMs are lacking, to the best of our knowledge. However, such data may be relevant to

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TABLE 1. Data on the Antibodies Used for Immunocytochemistry

Antibody	Specificity	Short Name	Reference
163DE4	Laminin α1-chain	Anti-Lnα1	37
5H2*	Laminin α2-chain	Anti-Lnα2	38
BM-2	Laminin α3-chain	Anti-Lnα3	39
FC10	Laminin α4 chain	Anti-Lnα4	33
4C7*	Laminin α5-chain	Anti-Lnα5	40
DG10	Laminin β1-chain	Anti-Lnβ1	41
C4	Laminin β2-chain	Anti-Lnβ2	42
113BC7	Laminin γ1 chain	Anti-Lnγ1	43
4A10	Tenascin-C	Anti-tenascin	44
MCA-1982†	Lutheran glycoprotein	Anti-Lu	45

* Purchased from Novocastra Laboratories, Newcastle Upon Tyne, UK.

† Purchased from Serotec Ltd. Oxford, UK.

elucidate further the selective sparing of the EOMs in muscular dystrophies involving elements of the ECM-dystroglycan complex[34,35] as well as to characterize some of the special features of the EOM allotype (e.g., multiple endplates on a single muscle fiber and rich vascularization).

MATERIALS AND METHODS

Sixteen EOM samples were obtained either at autopsy or after enucleation, from seven male donors (ages 17, 27, 34, 54, 82, 86, and 87 years) and one female donor (age 26 years) with no previously known neuromuscular disease. Six samples were taken from the rectus superior muscle, five from the rectus lateralis muscle, two from the rectus inferior, two from the rectus medialis, and one from the obliquus superior muscle. Samples from the biceps brachi and the first lumbrical and quadriceps muscles were also obtained at autopsy and used for comparison. All samples were obtained according to the ethical recommendations of the Swedish Transplantation Law, with the approval of the Medical Ethics Committee, Umeå University, and in compliance with the Declaration of Helsinki.

The samples were mounted on cardboard and rapidly frozen in propane chilled with liquid nitrogen and stored at −80°C until used. Series of 80 cross sections, 5 μm thick, were cut from each muscle sample on a cryostat (Reichert-Jung, Vienna, Austria).

Histochemistry

NMJs were detected histochemically using the acetylcholinesterase reaction.[36]

Immunocytochemistry

The sections were processed for immunocytochemistry with previously characterized monoclonal antibodies (mAbs), each recognizing a different laminin chain (Table 1). An mAb against tenascin was used to confirm the location of the MTJs.[44] The tissue sections were air dried for 15 to 30 minutes, rehydrated in PBS for 5 minutes, and incubated with 5% normal rabbit serum (DakoCytomation, Glostrup, Denmark) for 15 minutes, to inhibit unspecific staining. The sections were then

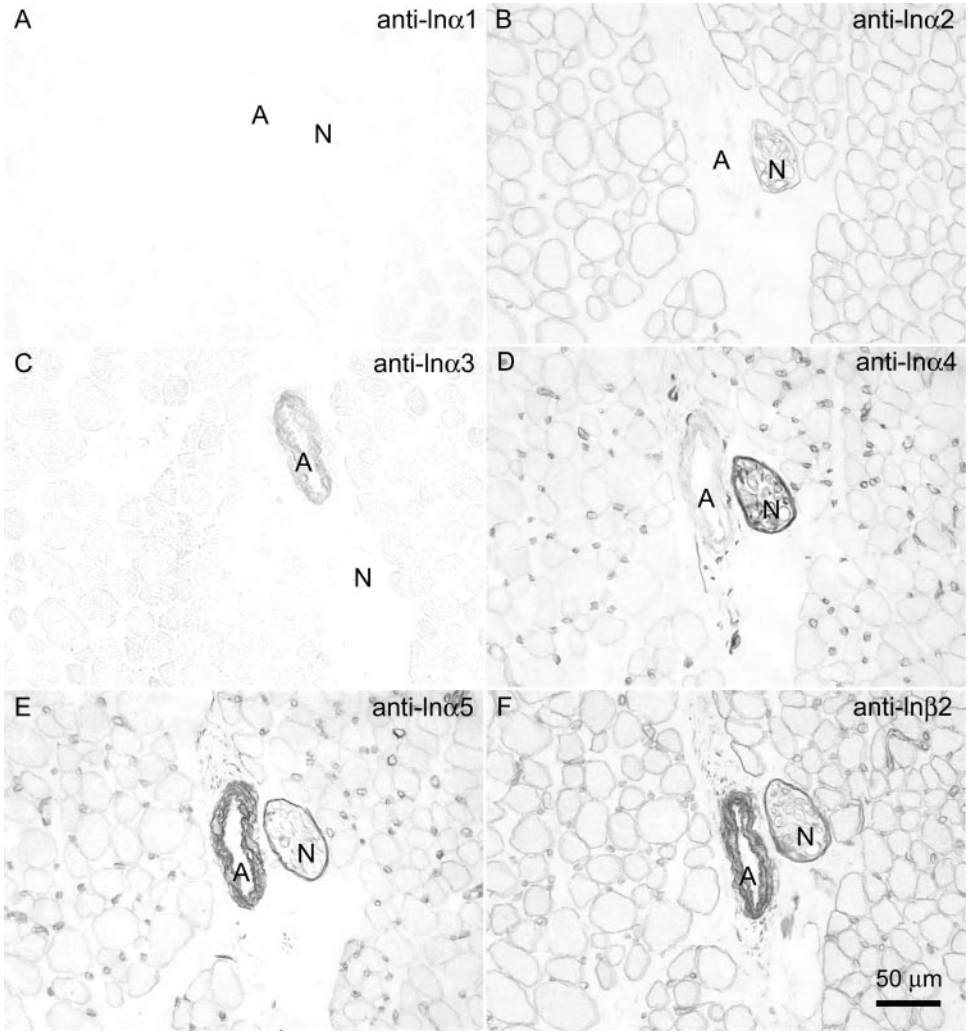

FIGURE 1. Photomicrographs of six cross sections from the global layer of a rectus superior muscle immunolabeled with (**A**) anti-Lnα1, (**B**) anti-Lnα2, (**C**) anti-Lnα3, (**D**) anti-Lnα4, (**E**) anti-Lnα5, and (**F**) anti-Lnβ2. A nerve (N) and an arteriole (A) are indicated. Anti-Lnα1 did not label any structure, anti-Lnα2 labeled fiber contours and nerves, and anti-Lnα3 labeled the arteriole only. Anti-Lnα4 labeled fiber contours weakly and capillaries and the nerve strongly. Anti-Lnα5 labeled fiber contours weakly and the capillaries, arterioles, and the perineurium more intensely, whereas anti-Lnβ2 labeled all the structures.

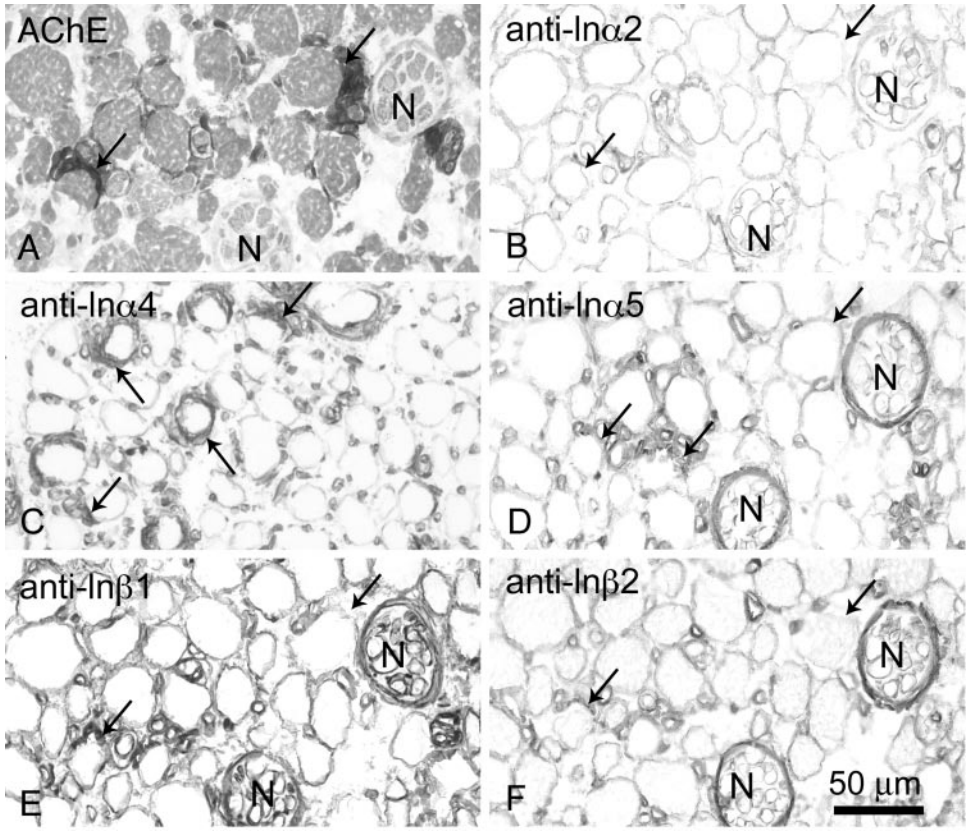

FIGURE 2. Photomicrographs of five cross sections (**A**, **B**, **D–F**) from a rectus superior and one from a rectus lateralis (**C**) muscle treated to show (**A**) acetylcholinesterase (AchE) activity or immunostained with (**B**) anti-Lnα2, (**C**) anti-Lnα4, (**D**) anti-Lnα5, (**E**) anti-Lnβ1, and (**F**) anti-Lnβ2. *Arrows*: NMJs detected by their AChE activity. N, nerves. The staining intensity was higher with anti-Lnα4 at the NMJs (*arrows*) than in the extrasynaptic portion of the fibers (**C**).

incubated overnight with the appropriate primary antibody at 4°C. The primary antibodies were diluted in PBS with 0.1% bovine serum albumin. Thereafter, the sections were washed in 0.01 M PBS and again incubated with normal rabbit serum for 15 minutes, followed by incubation with rabbit anti-mouse IgG (DakoCytomation) for 30 minutes at room temperature. After they were washed in PBS for 15 minutes, the sections were incubated with peroxidase mouse antiperoxidase complex (DakoCytomation) for 30 minutes and then washed in PBS for 15 minutes. Development of peroxidase was obtained by applying a solution containing 1 mg/mL of diaminobenzidine and H_2O_2 for 5 to 10 minutes, followed by rinsing in running water for 5 minutes. Finally, the sections were dehydrated in graded concentrations of ethanol and mounted (Pertex; Histolab Products AB, Gothenburg, Sweden). Control sections were processed as just described, except that the primary antibody was omitted. No staining was observed in the control sections. The sections were photographed under a microscope equipped with a charge-coupled device (CCD) camera (Nikon, Tokyo, Japan).

Capillaries

The number of capillaries was determined in representative areas of the orbital and global layer in sections from 5 EOM samples, stained with the antibody recognizing Lnα5-chain. The size of the areas chosen was 500 × 500 μm, except where muscle fascicles were too thin, and 200 × 200-μm areas were chosen instead. Altogether, 11 areas from the global layer and 14 areas from the orbital layer were chosen. All vessels with an outer diameter <15 μm were assumed to be capillaries according to the definition put forward by Jerusalem.[46] The mean capillary density was calculated for both the global and the orbital layer in all five muscles. The significance of the mean difference between the two layers was then analyzed, using a two-sample *t*-test (two-tailed). A total of 4861 capillaries (1977 in the global layer and 2884 in the orbital layer) were counted.

RESULTS

Extraocular Muscles

The staining patterns observed were identical in the orbital and global layers. No evidence of variation in the staining patterns was noted between the middle and the distal portions of the EOMs.

Anti-Lnα1 did not show immunoreactivity in any tissue structure in the sampled sections (Fig. 1A).

Anti-Lnα2 immunoreactivity was present in the fiber contours in the extrasynaptic and synaptic regions (Figs. 1B, 2B), and the MTJs (Fig. 3B). Anti-Lnα2 also stained the perineurium and endoneurium, but not the blood vessels (Figs. 1B, 2B). Anti-Lnα3 immunostained the blood vessels but not the capillaries, the muscle fibers, or the nerves in the EOMs (Fig. 1C). Anti-Lnα4 labeled the muscle fiber contours weakly extrasynaptically in most samples (Figs. 1D, 2C, 4), moderately at the NMJs (Fig. 2C) and MTJs. This mAb also stained the capillaries strongly (Figs. 1D, 2C, 4). Both the perineurium and the endoneurium were strongly labeled by anti-Lnα4 (Figs. 1D, 2C, 4).

Anti-Lnα5 immunoreactivity was moderate in the extrasynaptic fiber contours (Fig. 1E) and was slightly increased at the MTJs (Fig. 3C) but not at the NMJs (Fig. 2D). The capillaries and other blood vessels were strongly labeled with anti-Lnα5 (Figs. 1E, 2D, 5). The perineurium was clearly more strongly stained than the endoneurium (Figs. 1E, 2D).

Anti-Lnβ1 (Figs. 2E, 3D), anti-Lnβ2 (Figs. 1F, 2F, 3E, 5B) and anti-Lnγ1 (Fig. 3F) immunostained muscle fiber contours, capillaries, other blood vessels, perineurium, and endoneurium strongly. Anti-Lnβ2 labeled all fiber contours even at a very low concentration (mAb diluted 1:40,000) in the EOMs and in the limb muscle samples (Figs. 5A, 5B). The staining intensity appeared higher at the MTJs with anti-Lnβ2 (Fig. 3E) and anti-Lnγ1 (Fig. 3F) and, to a lesser degree, with anti-Lnβ1 (Fig.

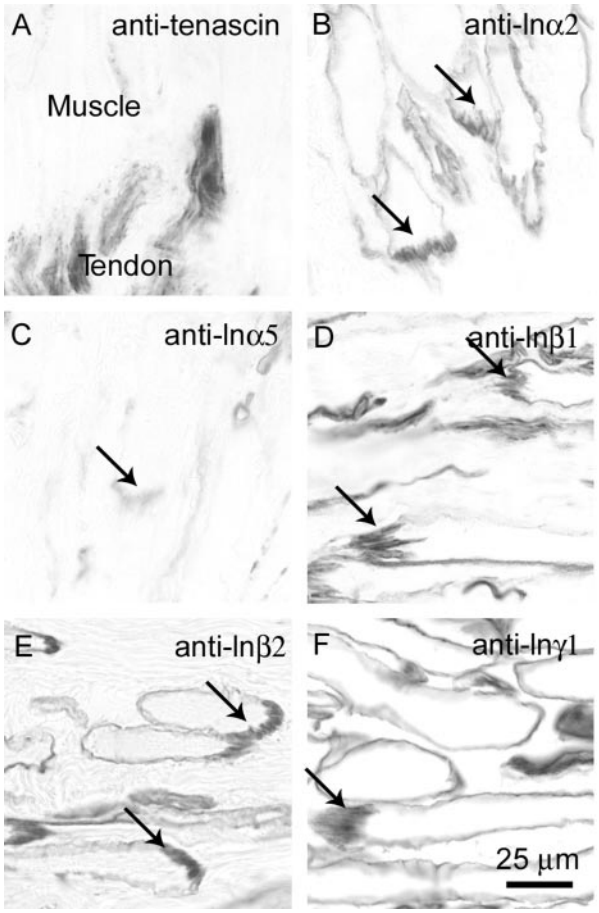

FIGURE 3. Transverse sections from the MTJ area of a rectus superior (**A–D**, **F**) and a rectus lateralis (**E**) muscle immunostained with (**A**) anti-tenascin, (**B**) anti-Lnα2, (**C**) anti-Lnα5, (**D**) anti-Lnβ1, (**E**) anti-Lnβ2, and (**F**) anti-Lnγ1. *Arrows*: some of the MTJs.

3D). The BM was as strongly labeled at the NMJs as it was extrasynaptically with anti-Lnβ1, anti-Lnβ2, and anti-Lnγ1 (Fig. 2).

Limb Muscle

The BM of the limb muscle fibers was either unlabeled (Fig. 5C) or weakly labeled with anti-Lnα5. We have observed variation in the amount of staining seen around muscle fibers of different skeletal muscles (Thornell L-E, unpublished observation, 1999) indicating intermuscle and interindividual variation in the amounts of the Lnα5 present. We tested the hypothesis that the EOMs differ from limb muscles in α5 chain composition by using an antibody against Lutheran protein. Lutheran blood group glycoprotein is a transmembrane receptor for the α5-chain,[47,48] present on the surface of cells and epithelia in various tissues that also contain α5-chain.

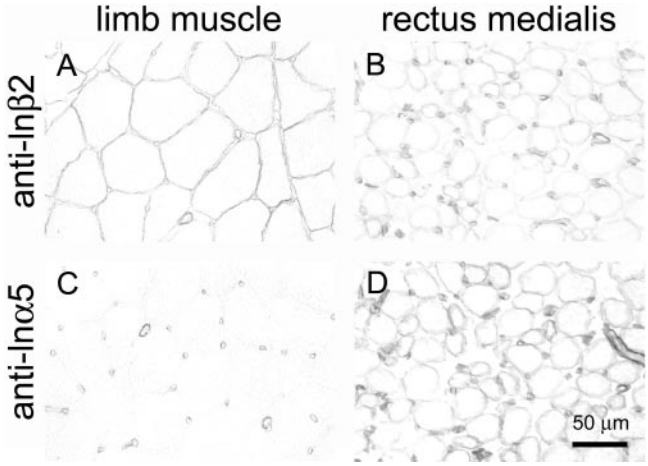

FIGURE 5. Photomicrographs of sections from a biceps brachi muscle (**A**, **C**) and a rectus medialis muscle (**B**, **D**) immunostained with anti-Lnβ2 (**A**, **B**) or anti-Lnα5 (**C**, **D**). All the fiber contours in both muscles were immunostained with anti-Lnβ2, whereas this isoform is absent from extrasynaptic BM of rodent skeletal muscle.[20] Only the BM of the fibers in the rectus medialis muscle was labeled with anti-Lnα5. A much higher capillary density in the rectus medialis was demonstrated by staining with anti-Lnα5.

Anti-Lutheran immunostained the contours of the fibers in the EOMs only, whereas it labeled capillaries, blood vessels and perineurium in both the EOMs and limb muscle (Fig. 6).

The laminin chain composition of human MTJs[37] and NMJs[30] have been determined previously. In addition, we observed staining of the NMJs with anti-LNα4 in adult limb muscle (Fig. 7).

Capillary Density

The capillary density in the EOMs, determined in sections processed with anti-Lnα5, was 1050 $\pm$ 190 capillaries/mm^2. The capillary density was significantly ($P < 0.05$) higher in the orbital layer (1170 $\pm$ 180 capillaries/mm^2) than in the global layer (930 $\pm$ 110 capillaries/mm^2).

DISCUSSION

The present study showed important differences in BM laminin isoform composition and capillary density between the human EOMs, human limb muscle, and muscles of other species. The presence of additional laminin isoforms other than laminin-2 in

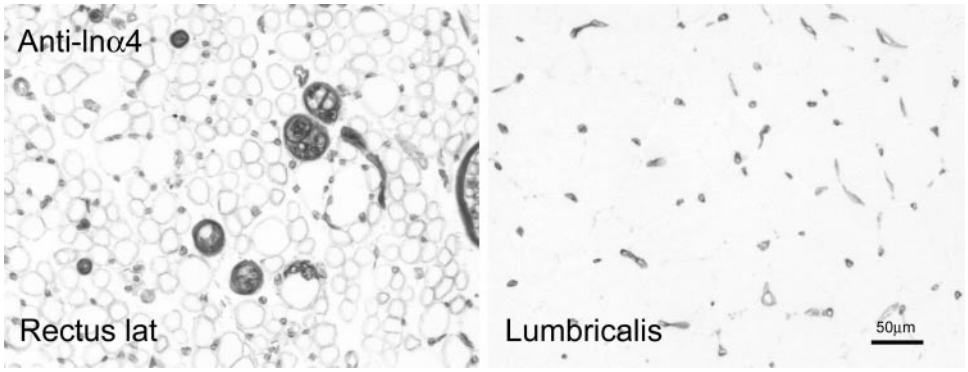

FIGURE 4. Photomicrographs of a rectus lateralis muscle and a lumbricalis muscle from the same subject mounted and sectioned together and immunostained with anti-Lnα4. The muscle fiber contours were labeled in the EOM, but not in the lumbrical muscle.

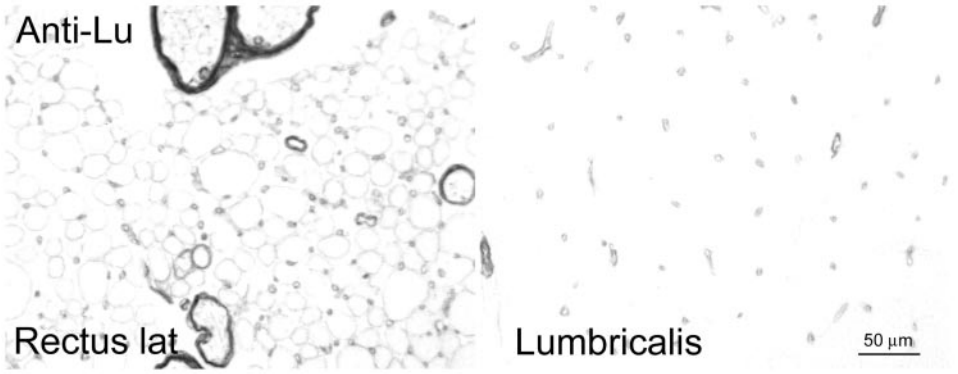

FIGURE 6. Photomicrographs of a rectus lateralis muscle and a lumbricalis muscle from the same subject mounted and sectioned together and immunostained with anti-Lu. The mAb reacted with the blood vessels of both muscles but only with the BM of the muscle fibers in the EOM.

the BM of the extrasynaptic sarcolemma could partly explain the sparing of the EOMs in Lnα2-deficient congenital muscular dystrophy.

Extrasynaptic BM

The present immunohistochemical data indicate that the extrasynaptic BM of adult human EOM muscle fibers contained Lnα2, -β1, -β2, and -γ1, and, to some extent, Lnα4 and -α5. Lnβ2 has traditionally been regarded as being absent from the extrasynaptic BM.[20] In the present study, Lnβ2 was detected with mAb C4, an mAb that has been suggested to cross-react with Lnβ1 at high concentrations.[49] The mAb C4 was diluted up to 1:40,000 in the present work, and the staining delineating EOM and limb muscle fibers remained remarkably stable, refuting a possible cross-reaction. Furthermore, Lnβ2 was also detected in the extrasynaptic BM of human limb muscle fibers, in a previous study.[50] Therefore, there is an important interspecies difference between human and rodent muscle with respect to the presence of Lnβ2 in the extrasynaptic BM.

In adult human skeletal and cardiac muscle the main isoform of the extrasynaptic BM is laminin-2 (α2β1γ1) formerly known as merosin.[19,20,29,33,37] Lnα1[40] and -α3 chains[41] have not been detected in the BM of human muscle fibers. Lnα4 chain is found in adult human smooth and cardiac muscle, but not in mature skeletal muscle fibers.[33] Lnα5 chain is present in epithelial BM and in endothelial tissues including capillaries but only at the NMJs of muscle fibers. Thus, the Lnα2 chain is the only α-chain normally found extrasynaptically in human limb muscle. Lnα2 is a chain of laminin-2 (α2β1γ1), -4 (α2β2γ1), and -12 (α2β1γ3). However, Lnγ3 is only present in epithelia and peripheral nerves,[52] which implies that laminin-2 and -4 are the only laminin isoforms of extrajunctional BM in skeletal muscle. The expression of Lnα4 and -α5 in the extrasynaptic BM of human EOM fibers described herein suggests the presence of additional laminin isoforms in the BM of these fibers. Lnα4 and -α5 are chains of laminin-8 (α4β1γ1), -9 (α4β2γ1), -10 (α5β1γ1), and -11 (α5β2γ1), and since LNβ1, -β2 and -γ1 were detected, theoretically all these laminins could be present in the BM of EOM fibers. The presence of Lnα5 chain receptor Lutheran on the surface of the muscle fibers of the human EOMs and its absence in limb muscle strongly confirms the distinct structural composition of the BMs of the human eye muscles.

This complex laminin isoform composition is a possible explanation for the sparing of EOMs in merosin-deficient congenital muscular dystrophy (CMD). CMDs are characterized by postnatal hypotonia, contractures, muscle weakness, and brain involvement.[52] Approximately 50% of the CMD cases are caused by mutations in LAMA2, the gene encoding the Lnα2 chain, resulting in complete or partial Lnα2 (merosin) deficiency.[24,31] Loss of BM stability and degradation of the extracellular framework has been demonstrated in Lnα2-deficient mice.[53] The EOMs are spared in this disease, but the exact mechanism for this is not completely known.[16] The presence of additional laminin isoforms, such as laminin-8, -9, -10, and -11 in the BM of the EOMs may be crucial for the maintenance of muscle fiber integrity in the absence of Lnα2 chain.

The presence of Lnα4, an isoform typical of developing myotubes,[33] in adult EOMs adds to the list of developmental protein isoforms (e.g., embryonic and fetal myosin heavy chains)[7,54] that these muscles retain and that are likely to be of major importance for their unique properties.

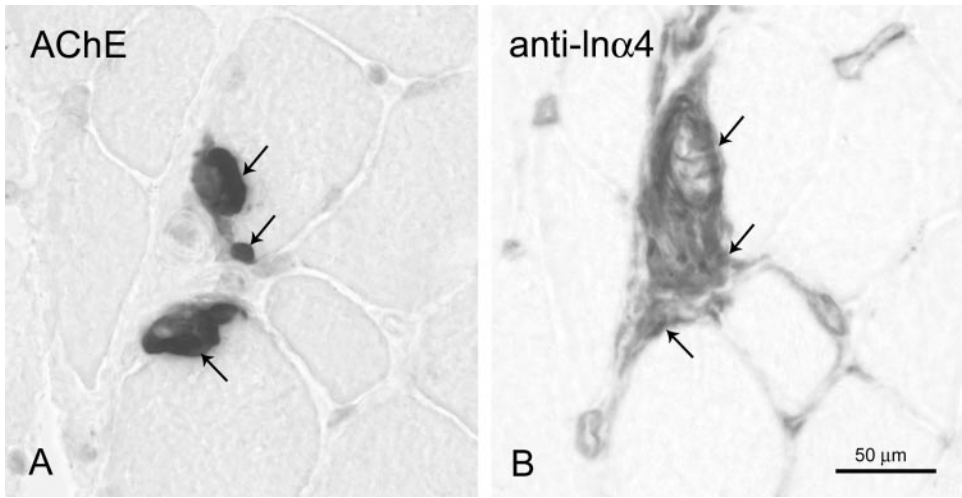

FIGURE 7. Photomicrographs of NMJs (*arrows*) in limb muscle identified by the localized acetylcholinesterase activity (**A**) and immunostained with anti-Lnα4 (**B**).

Neuromuscular Junction

At the NMJs we found increased expression of Lnα4 and also that expression of Lnα2, -α5, -β1, -β2, and -γ1 was maintained. In mice, laminin-4 ($\alpha2\beta2\gamma1$), -9 ($\alpha4\beta2\gamma1$), and -11 ($\alpha5\beta2\gamma1$) are present in the synaptic BM, and laminin-2 ($\alpha2\beta1\gamma1$) and -8 ($\alpha4\beta1\gamma1$) are found in the BM of the adjoining Schwann cells.[20] Lnα4 has not been detected in adult human limb muscle BM, although it is present during development,[33] but Lnα5 is expressed at the NMJs. Herein, we report the novel presence of Lnα4, even in the NMJ of human limb muscle. Lnα4 is crucial for proper synaptic localization.[55]

Myotendinous Junctions

The MTJs contained Lnα2, -α5, -β1, -β2, and -γ1 as described for skeletal muscle.[37,50,56] Lnα1 is found in developing MTJs but not for sure in adult human MTJs, which makes Lnα1 also a developmental isoform.[37] We could not detect Lnα1 in MTJs of the adult human EOMs. Thus, the EOMs show an independent regulation of the developmental laminin chains α1 and α4, given that Lnα1 was absent in the adult EOM, but Lnα4 was found in the extrasynaptic BM, as in developing muscle.[33]

Nerves and Blood Vessels

The perineurium was labeled by mAb against Lnα2, -α4, -α5, -β1, -β2, and -γ1. The endoneurium stained strongly with all these antibodies except anti-Lnα5, which only stained the endoneurium moderately. In mice, the endoneurium contains laminin-2 ($\alpha2\beta1\gamma1$) and the perineurium laminin-9 ($\alpha4\beta2\gamma1$) and -10 ($\alpha5\beta1\gamma1$).[20] In a large human peripheral nerve it has recently been demonstrated that the endoneurium contains Lnα2, -α4, -β1, and -γ1, whereas the perineurium displays Lnα3, -α4, -α5, -β1, -β2, and -γ1.[32] Our findings differ from those of Wallquist et al.[32] in that we found Lnβ2 in the endoneurium and no trace of Lnα3 in the perineurium in the EOMs. Therefore, there seems to be a difference between the laminin composition of large peripheral nerves (in this case the sural nerve) and small nerves close to their endpoints in the EOMs.

The capillaries were stained by mAbs against Lnα4, -α5, -β1, and -β2. Larger blood vessels were in addition also stained with the mAb against Lnα3, in accordance with previous results.[32]

The capillary were was higher in the EOMs than in any other previously reported human muscle—for example biceps brachi (440 ± 118 capillaries/μm^2) and first dorsal interosseus (375 ± 86 capillaries/μm^2), including the richly vascularized jaw muscles—for example, the masseter (813 ± 81 capillaries/μm^2).[57] The significantly higher capillary density of the orbital layer is in line with the higher oxidative enzyme activity[58] and higher overall vascular density of the orbital layer.[59]

Acknowledgments

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