

Ultrastructural and immunocytochemical study of the leptomeremes in the mouse cardiac muscle fibre

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Summary. The three-dimensional configuration and chemical composition of leptomeremes in the mouse ventricular cardiac muscle fibre were electron microscopically and immunocytochemically investigated using semithin sections and specific antibodies against actin, the intermediate filament proteins desmin and vimentin, and the actin-binding proteins α -actinin, filamin and vinculin. The leptomeremes appeared columnar in shape and periodically segmented by electron-dense disc-like septa. The electron-lucent areas between these septa were composed of fine interlinked filaments running obliquely to the major axis of the leptomere. Actin was localized in the electron-dense lines of the leptomeremes but not in the fine filaments. No reaction was, however, detected for desmin, vimentin, vinculin, filamin and α -actinin. The present results suggest, therefore, that the leptomeremes may not have a contractile function.

Key words: Leptomeremes, Mouse heart, Actin, Immunocytochemistry, Stereopairs

Introduction

Leptomeremes are unique intracellular structures first reported in the avian skeletal muscle by Ruska and Edwards in 1957. The name refers to its short, slender sarcomere-like appearance. Since their discovery, these structures have been observed in various types of muscle fibres, such as the human extraocular skeletal muscle (Mukono, 1966), intrafusal muscle fibres of frog (Katz, 1961) and rat (Ovalle, 1972), cardiac muscle fibres of hen, pigeon (Bogusch, 1973) and mouse (Thoenes and Ruska, 1960; Myklebust and Jensen, 1978; Ono et al., 1978), in cultured skeletal muscle cells (Miranda and Godman, 1973; Askanas

et al., 1978), and in myopathic skeletal (Lake and Wilson, 1975) and cardiac muscle fibres (Fujita et al., 1979). These structures are composed of periodically-arranged electron-dense narrow bands bridged by fine filaments, and are usually located among Z-lines and between the sarcolemma and the Z-line in the striated muscle fibres (Ruska and Edwards, 1957; Thoenes and Ruska, 1960; Katz, 1961; Miranda and Godman, 1973; Askanas et al., 1978; Myklebust and Jensen, 1978). Their physiological role and biochemical composition, however, are still unclear. In the present study, the three-dimensional configuration of the leptomeremes in the mouse cardiac muscles was examined using stereo pairs of semithin sections, and their biochemical characteristics were immunocytochemically determined.

Materials and methods

Procedure for Electron Microscopy

Mice of ddY strain, aged 0, 1, 2, and 7 days and 1, 6, and 12 months, were employed. The animals were perfused through the left ventricle, under sodium pentobarbital anaesthesia, with physiological saline followed by 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, for 10 min. The hearts were excised, cut into small blocks, and further fixed in the same fixative for 1h at 0-4 °C. The tissue blocks were washed in the above buffer for 20 min, and postfixed in 1% OsO₄ in 0.1M cacodylate buffer, pH 7.3, for 1h at room temperature. After washing in the buffer for 20 min, the specimens were dehydrated in a series of graded ethanols and propylene oxide, and embedded in Spurr's low viscosity epoxy resin (Spurr, 1969). Ultrathin and 1 μ m-thick semithin sections were cut in an Ultracut E ultramicrotome (Reichert AG, Vienna, Austria), and stained with uranyl acetate and lead citrate. Ultrathin sections were observed in a JEM-100S electron microscope (JEOL Co., Tokyo, Japan) operated at 80 kV, and semithin sections were examined in an H-700H

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electron microscope (Hitachi Co., Tokyo, Japan) accelerated at 200 kV.

Procedure for Immunocytochemistry

Preparation of Tissue

Mice of ddY strain aged 6 and 12 months were employed. The heart was perfused through the left ventricle with ice-cold physiological saline followed by ice-cold 2% paraformaldehyde in 0.1M PBS, pH 7.3. The heart was extirpated, cut into small blocks and fixed in the same fixative for 1h at 0-4 °C, followed by washing in 0.1M PBS for 30 min. In order to block the free aldehyde groups, the tissues were immersed in 50mM NH₄Cl in 0.1M PBS for 30 min, and washed in 0.1M PBS for 12h. The tissues were dehydrated in graded ethanols a progressively lower temperatures, down to -35 °C (Carlemalm et al., 1982), embedded in Lowicryl K4M and polymerized under UV irradiation in a low temperature polymerization apparatus (Dosaka EM, Co., Kyoto, Japan) for 24h at -35 °C, and for a further 2 days at room temperature. Ultrathin sections were collected on Parlodion-carbon-coated nickel grids.

Primary antibodies

Anti-actin antibody: mouse anti-actin antibody IgM class clone JLA 20 (Oncogene Science Inc., Uniondale, NY, USA); anti-desmin antibody: mouse anti-desmin antibody IgG1 class clone DE-U-10 (Sigma Chemicals Co., St. Louis, MO, USA); anti-vimentin antibody: mouse anti-vimentin antibody IgG1 class Clone V-9 (Sigma Chemicals Co., St. Louis, MO, USA), or goat anti-vimentin polyclonal antibody (ICN Biomedicals, INC., Irvine, CA, USA); anti-vinculin antibody: mouse anti-vinculin antibody IgG1 class clone hVIN-1 (Sigma Chemicals Co., St. Louis, MO, USA); anti-filamin antibody: mouse anti-filamin antibody IgG1 class clone FIL-2 (Sigma Chemicals Co., St. Louis, MO, USA); anti- α -actinin: mouse anti- α -actinin antibody IgM isotype (Sigma Chemicals Co., St. Louis, MO, USA).

Secondary antibodies

Goat anti-mouse IgM antibody conjugated with 10 nm colloidal gold (Zymed Laboratories, Inc., San Francisco, CA, USA); goat anti-mouse IgG antibody conjugated with 5nm colloidal gold (Sigma Chemicals Co., St. Louis, MO, USA); rabbit anti-goat IgG antibody conjugated with 10 nm colloidal gold (Zymed Laboratories, Inc., San Francisco, CA, USA).

Immunostaining

Ultrathin Lowicryl sections were preincubated in 0.02M Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) at room temperature, and then washed in 0.02M TBS for 15 min. Incubation with the

first antibodies, diluted at adequate concentrations in 0.02M TBS containing 1% BSA, was performed for 60 min at room temperature in a moist chamber. After washing with 0.02M TBS for 15 min, the sections were reacted with the secondary antibodies, diluted in 0.02M TBS containing 1% BSA, for 60 min and then washed in 0.02M TBS for 15 min. This was followed by fixation in 2% glutaraldehyde in 0.02M TBS for 15 min, and washing in 0.02M TBS for 15 min. The sections were stained with uranyl acetate and lead citrate, and observed in a JEM-100S electron microscope (JEOL Co., Tokyo, Japan) operated at 80 kV.

Results

The leptomeres were composed of periodically-arranged electron-dense lines alternating with electron-lucent areas of 25-45 nm and 120-200 nm in width, respectively, together forming a striated pattern which extended for 0.5-2 μ m in length and 0.1-0.4 μ m in width (Figs. 1, 2).

Leptomeres were often observed in the cardiac muscle fibres of both ventricles, but they were never found in atrial cardiac muscle fibres. In longitudinal section, the leptomeres were usually located among the myofibrils, flanked by two mitochondria, at the level of the Z-line. Their axis was usually perpendicular, and occasionally parallel or oblique to the axis of the myofibrils, and they were always connected with the Z-line (Figs. 1, 2). Leptomeres were sometimes also found just beneath the sarcolemma, in which case, their axis was parallel to the surface of the myocardial cell, and connected the Z-line to the sarcolemma (Fig. 3). In cross section, the leptomeres were also situated among myofibrils and bound to Z-lines (Fig. 4). Occasionally, these structures were randomly distributed in the vicinity of the intercalated disc (Fig. 5). In such instances, they were always connected to the Z-band or to the mat of electron-dense material immediately subjacent to the sarcolemma at one end. Leptomeres were never observed independently in the cardiac muscle fibre.

Sometimes these structures were also seen in close apposition to T-tubules (Fig. 6), and to the terminal cisternae and the reticular portion of the sarcoplasmic reticulum (Figs. 1, 2).

The high electron-dense lines had a Z-line-like appearance and exhibited a fuzzy pattern which resembled that seen at the joint between actin filaments and the Z-line (Figs. 2, 3). Its electron density was, however, higher than that of the Z-line. The electron-lucent areas were made up of fine filaments of about 5 nm in diameter running obliquely to the major axis of the leptomeres.

Three-dimensional views of 1 μ m semithin sections revealed that the leptomeres were for the most part columnar or parallelepiped structures, divided into periodical segments by electron-dense, disc-like septa (Figs. 8, 8'). The fine filaments in the

Leptomeres in mouse cardiac muscle

electron-lucent areas ran obliquely to the major axis of the leptomere, in many directions, forming meshes (Figs. 9, 9'). Around the leptomere there were closely apposed mitochondria and T-tubules, but it could not be confirmed whether the leptomeres were connected to them.

Leptomeres were hardly ever found in young mice up to one month. However, in mice aged between 6 and 12 months, these structures were frequently observed, and

were clearly more abundant in 12 month-old than in 6-month-old mice. No morphological differences could be recognized between the leptomeres of young and adult mice (Fig. 7).

Immunocytochemistry

The results of the immunocytochemical study are summarized in the Table 1.

Table 1. Localization of actin, actin-binding proteins, and intermediate filaments in leptomeres in the mouse cardiac muscle fibre.

	LEPTOMERE		NORMAL SARCOMERE			OTHERS
	Periodic dense line	Filaments	A-band	I-band	Z-line	
anti-actin	+	-	+	+	+	ICD (+)
anti-desmin	-	-	-	-	-	around the T-tubule (+)
anti-vimentin	-	-	-	-	-	around the nucleus (+)
anti-vinculin	-	-	-	-	-	ICD (+); both ends of Z-line (+)
anti-filamin	-	-	-	-	-	ICD (+); both ends of Z-line (+)
anti- α -actinin	?	-	-	-	?	

ICD: intercalated disc; +: positive; -: negative; ?: unclear.



Fig. 1. Longitudinal section of a ventricular cardiac muscle fibre of a 12-month-old mouse. The leptomeres are situated among Z-lines and flanked by mitochondria. Their axis is usually perpendicular to the axis of the muscle fibre. Bar= 0.5 μ m. x 30.000



Fig. 2. Leptomeres consist of periodically-oriented Z-line-like electron-dense lines bridged by 5 nm-thick filaments. Bar= 0.1 μ m. x 92,000



Fig. 3. A leptomere situated between myofibrils and the sarcolemma, connected with the Z-line. The Z-line-like lines exhibit a fuzzy pattern. Bar= 0.1 μ m. x 71,000



Fig. 4. Cross section of a ventricular muscle fibre showing two leptomeres. Note that these structures display the same morphological characteristics and continuity with the Z-line as in longitudinal section. Bar= 0.5 μ m. x 44,000

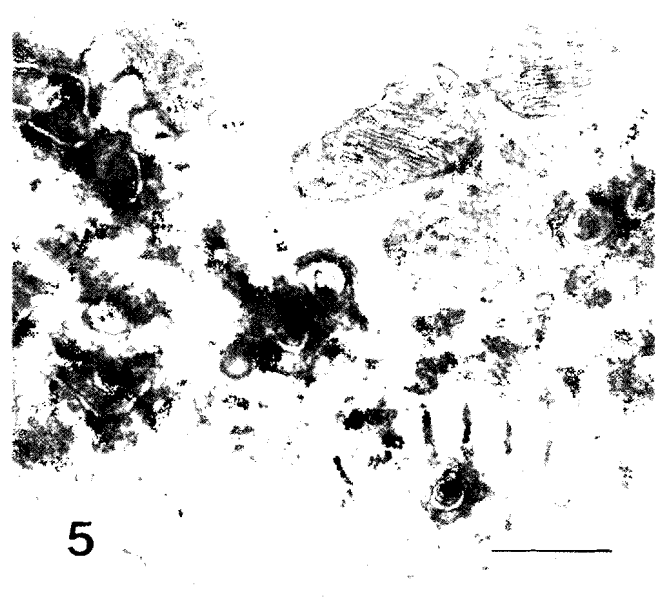


Fig. 5. Leptomeres are randomly distributed in the vicinity of the intercalated disc. Bar= 0.5 μ m. x 32,000



Fig. 6. A leptomere in close apposition with a triad. Electron-lucent areas (arrowhead) are seen in the fine filament areas among the electron-dense Z-line-like lines. Bar= 0.5 μ m. x 85,000

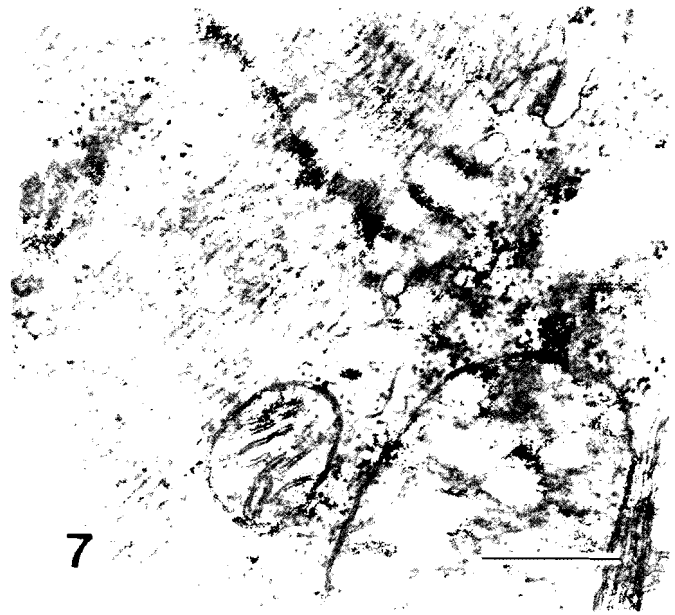
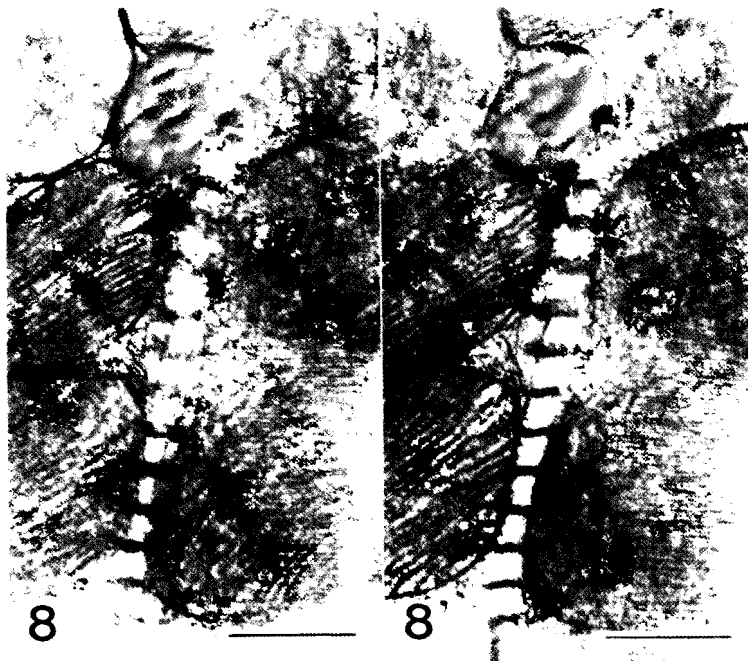


Fig. 7. A leptomere observed in a ventricular cardiac muscle fibre of a 2-day-old mouse. No fundamental morphological differences are found between the leptomeres observed in adult and in young mice. Bar= 0.5 μ m. x 36,000



Figs. 8 and 8'. Stereo pair of a leptomere in a 1 μ m-thick section. This columnar leptomere is segmented by a disc-like electron-dense septa. Bar= 0.5 μ m. x 35,000

Anti-desmin antibody

Desmin antibody reaction was positively found around T-tubules, but both the fine filaments and the Z-line-like electron-dense lines of the leptomeres were negative (Fig. 11).

Anti-vimentin antibody

Gold particles were distributed in the cytoplasm around mitochondria, nucleus, and on the myofibrils. They were also found in the nucleus, specially around the nucleolus (Fig. 13). No positive reaction was seen in the leptomeres (Fig. 12).

Anti-vinculin antibody

Positive labelling was observed at the intercalated disc, on the Z-line, and between the Z-line and the sarcolemma, which coincide with the position of costameres (Donowski et al., 1992). No reaction was, however, detected on the leptomeres (Figs. 14-16).

Anti-filamin antibody

Gold particle deposition was found at the periphery

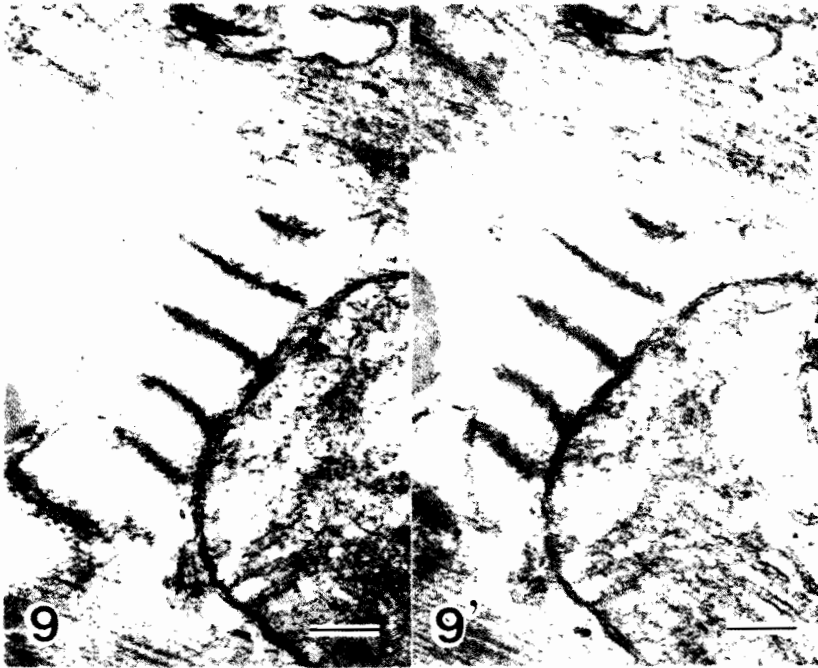
Anti-actin antibody

Positive labelling was detected on the actin filaments in the I-band and the A-band of the myofibrils, and at the intercalated disc. Gold particles were found on the Z-line-like electron-dense lines of the leptomeres, but the fine filaments of the leptomeres were negative (Fig. 10).

of Z-lines and on the electron-dense material of the intercalated disc. However, no positive labelling was observed in the leptomeres (Figs. 17, 18).

Anti- α -actinin antibody

Positive staining for α -actinin was neither observed in normomeric myofibrils nor in leptomeres.



Figs. 9 and 9'. Stereo pair of a leptomere in a 0.25 μ m-thick section. The fine filaments are not arranged parallel to the major axis of the leptomere, but form meshes. Bar= 0.1 μ m. x 89,000

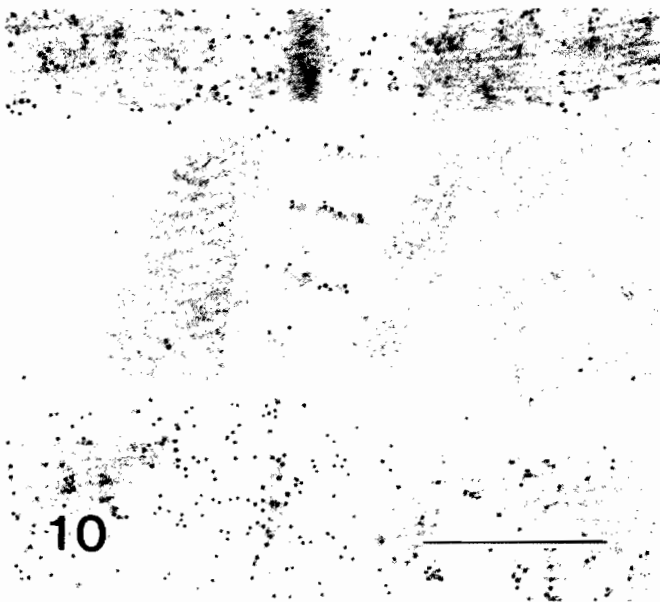


Fig. 10. Gold particle deposition indicating the presence of actin is detected on the Z-line-like dense lines of the leptomere and at the Z-band, A-band and I-band in the myofibrils. Bar= 0.5 μ m. x 48,000

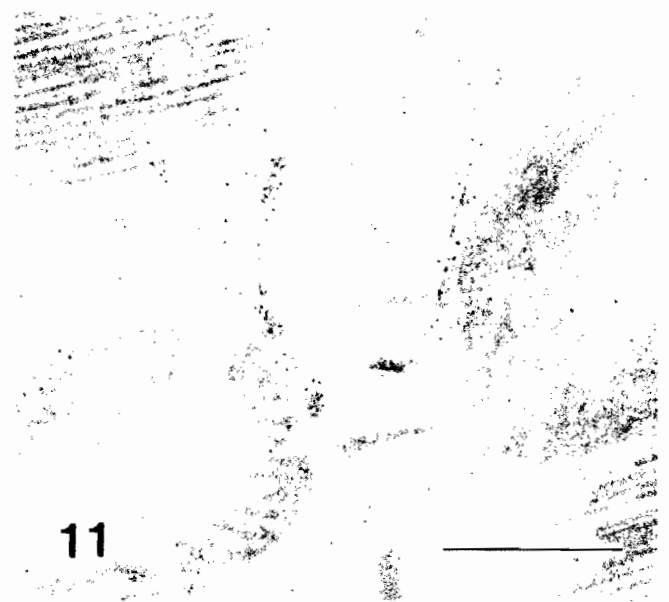


Fig. 11. Section treated with anti-desmin antibody and colloidal gold-conjugated secondary antibody. Gold particles are only observed along the T-tubules. Bar= 0.5 μ m. x 49,000

Discussion

The structures observed in the mouse cardiac muscle in the present study correspond to the «leptomeric fibrils» of Ruska and Edwards (1957), the «micro-ladders» of Katz (1961), the «leptomeric organelles» of Karlsson and Andersson-Cedergren (1968), Ovalle (1972, 1987) and Tidball and Andolina (1992), the «leptomeric bodies» of Miranda and Godman (1973), and the «zebra bodies» reported by Lake and Wilson (1975). Karlsson and Andersson-Cedergren (1968) described two types of leptomeres in the frog intrafusal muscle fibres: type I, which consisted of longitudinally-arranged fine filaments interrupted by dark disks with a periodicity of 187 nm and were connected with the Z-line of the normal sarcomeres, and type II, which was less frequent and consisted of granulated disks separated by shorter spacings of 30 nm (Karlsson and Andersson-Cedergren, 1968). Judging from their size and constituents, the leptomeres here described are thought to belong to type I; in our material, type II leptomeres were never observed. Previously-reported aggregates of misaligned leptomeres, referred to as «laminated structures» and

Leptomeres in mouse cardiac muscle

«leptomeric complex», in the human extraocular skeletal muscle (Mukono, 1966) and in hen cardiac Purkinje cells (Bogusch, 1975), respectively, were not encountered.

Early studies by Ruska and Edwards (1957) documented the presence of leptomeres in adolescent myotendon junction of the developing skeletal muscle of the thrush. Caesar et al. (1958) also reported similar patterns in the impulse conducting system of the sheep heart. These authors assumed the structures to be a prodromal pattern from which new normomeric myofibrils would arise (Ruska and Edwards, 1957; Caesar et al., 1958). Challice and Virágh (1973), on the



Fig. 12. No reaction for vimentin is detected in the leptomeres. Bar= 0.5 μ m. x 66,000



Fig. 13. Weak anti-vimentin positive reaction is observed around the nucleus. Bar= 0.5 μ m. x 40,000

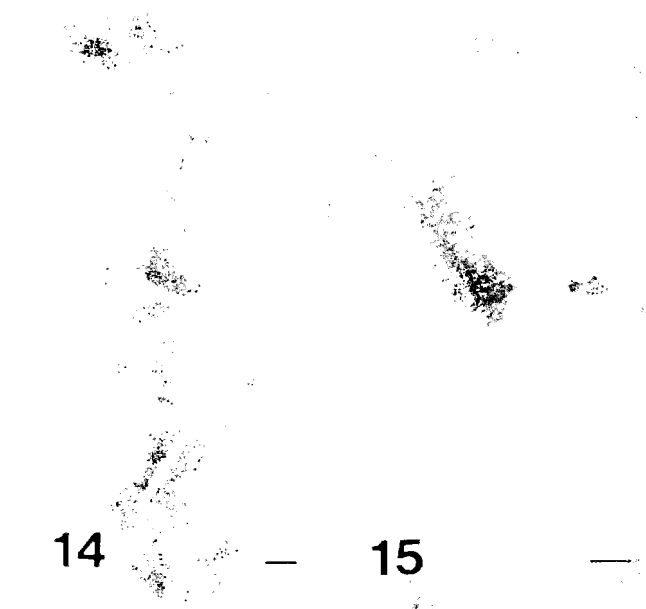


Fig. 14. Anti-vinculin antibody reaction can be seen on the amorphous electron-dense substance just beneath the sarcolemma of the intercalated disc. No reaction is noted in the desmosome. Bar= 0.1 μ m. x 41,000

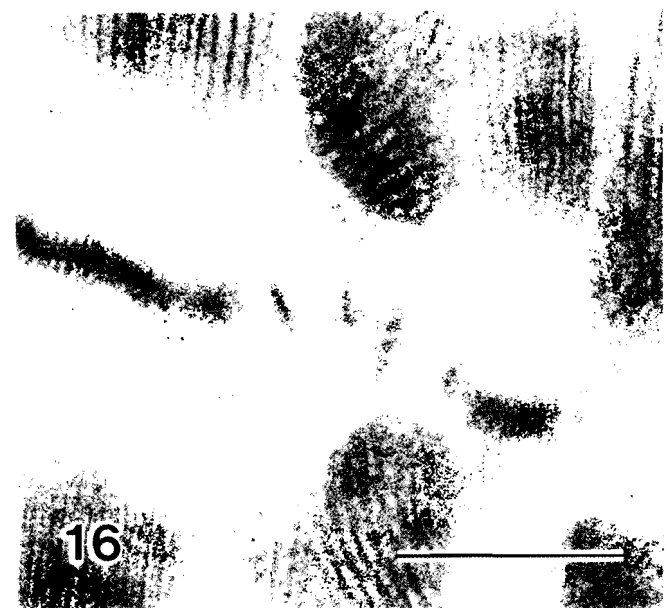


Fig. 16. This leptomere shows no deposition of the anti-vinculin antibody reaction. Bar= 0.5 μ m. x 61,000

Fig. 15. Gold particles showing the anti-vinculin antibody reaction are observed at both ends of the Z-band. Bar= 0.1 μ m. x 51,000

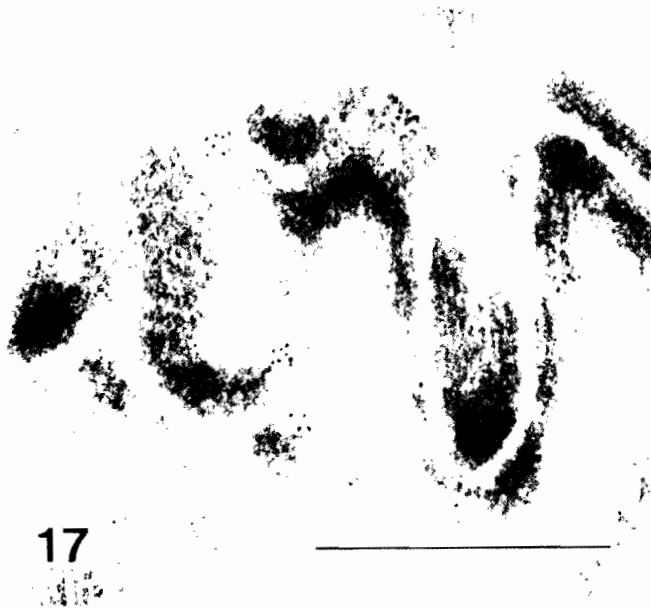
Leptomeres in mouse cardiac muscle

Fig. 17. Positive staining for filamin is detected on the amorphous electron-dense material beneath the sarcolemma of the intercalated disc. Bar= 0.5 μm . x 79,000

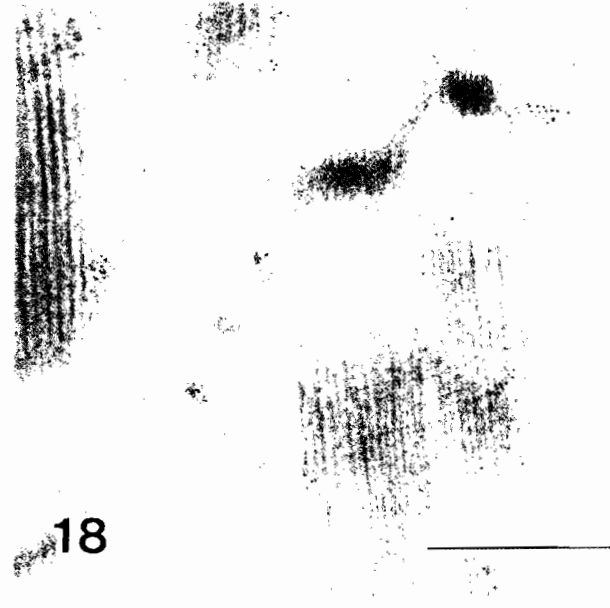


Fig. 18. Anti-filamin antibody reaction deposits can be seen at both ends of the Z-band, but not on the leptomere. Bar= 0.5 μm . x 51,000

other hand, noted the absence of leptomeres in embryonic or juvenile mammalian cardiac muscles, and later studies indicated that the occurrence of leptomeres seems to be a casual finding in the developing skeletal muscle (Bogusch, 1975). Leptomeres have been reported to occur mainly in ventricular cardiac muscles, including the specialized conducting system (Purkinje fibres and His' bundle), and especially in adult animals (Thoenes and Ruska, 1960; Virágh and Porte, 1973; Bogusch, 1975; Ono et al., 1978; Tidball and Andolina, 1992). In our materials these structures were hardly ever found in the ventricular cardiac muscle fibres of up to one-month-old mice, but they were much more frequent in cardiac muscles of 12-month-old than in 6-month-old mice. The appearance of these structures seems, therefore, to be related to aging.

In cultured skeletal muscle fibres, treatment with cytochalasin D, an inhibitor of actin polymerization (Miranda and Godman, 1973), or with 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation (Askanas et al., 1978), induced the formation of leptomeres. Except for the complicated laminated structures showing similar characteristics with leptomeres reported in the human extraocular skeletal muscle by Mukono (1966), leptomeres have never been described in human normal ordinary skeletal muscles. However, the appearance of leptomeres has been documented in various human pathological materials, including non-muscular tissues (Lake and Wilson, 1975; Fenoglio et al., 1976; Askanas et al., 1978; Silverman et al., 1978; Fujita et al., 1979; Carstens and Martin, 1986; Mirejovsky, 1990). In the experimental mouse heart treated with adriamycin and/or carnitine, increased myofibrillar disruption was

associated with an increase in leptomeres (Payne, 1982). These findings suggested that leptomeres may be pathological cytoplasmic inclusion bodies.

In the present three-dimensional observations of 1 μm sections, the leptomeres were columnar or parallelepiped in shape. However, in the literature, they have been mostly described as spindle-shaped structures, regardless of the plane in which the ordinary cardiac myofibrils were sectioned, i.e., longitudinally, transversely or obliquely (Thoenes and Ruska, 1960; Karlsson and Andersson-Cedergren, 1968; Myklebust and Jensen, 1978). Their spindle-shaped appearance may be due, as already pointed out by Thoenes and Ruska (1960), to either a tangential cutting of the curved tube or to an oblique cut of the straight tube in the sections. The leptomeres were divided into several compartments by Z-line-like electron-dense septa, with fine filaments running parallel to each other, not perpendicularly as described in most reports (Bogusch, 1975; Askanas et al., 1978), but diagonally to the Z-line-like septa as if twisted around the main axis of the leptomere. In ultrathin sections, therefore, a continuous network of fine filaments is to be observed if the cut is made through the central part of the leptomeres. However, if a tangential cut is made, an electron-lucent zone would be seen in the electron-lucent areas. This could explain the presence of a bright streak, about 15 nm wide, in the electron-lucent area between two adjacent dense septa (Gossrau, 1968; James and Meek, 1973; Bogusch, 1975).

The immunohistochemical method is a very useful tool to identify the chemical composition of the cytoskeleton (Stromer, 1992). Muscle tissue, on the

Leptomeres in mouse cardiac muscle

other hand, offers the possibility of using muscle cells themselves as a control to examine the affinity between the contractile proteins and their antibodies.

The fine filaments, measuring about 5 nm in diameter encountered in various cell types, including muscle cells, are generally considered to be actin filaments. In leptomeres, however, actin was localized in the Z-line-like electron-dense lines, but not in the fine filaments. These findings suggest that the 5 nm filaments in leptomeres may be a filamentous protein different from actin, and that the Z-line-like septa may be made up of a felt-like meshwork of either filamentous actin or actin monomers.

α -actinin is a constituent of Z-lines in the striated muscles and of dense bodies in the smooth muscles (Pollard and Cooper, 1986; Gerisch et al., 1991; Stromer, 1992). However, in spite of their similarity, this protein was not found in the Z-line-like dense lines in leptomeres. In the present study, positive labelling for α -actinin was never found in the Z-lines of I-bands nor in the electron-dense subsarcolemmal substance of the intercalated discs regardless of the concentration of antibodies, the fixation conditions and the pre- or post-embedding method employed. The fact that none of the four kinds of monoclonal anti- α -actinin antibodies employed (obtained from rabbit, bovine, porcine and chicken sources) yielded positive staining may be due to their species specificity. Tidball (1987) noted the molecular heterogeneity between the Z-lines at the ends of muscle cells and non-terminal Z-lines, namely, that the terminal Z-lines of myofibrils contain very few or no α -actinin compared with non-terminal Z-lines (Tidball, 1987). It is thus possible that the Z-line-like, electron-dense lines may also contain no α -actinin.

Intermediate filaments play a crucial role in the maintenance of the structural organization of the cardiac muscle fibre (Lazarides, 1980; Tokuyasu et al., 1983; Thornell et al., 1984). Desmin is a major component of the intermediate filaments in striated muscle, and extends transversely and longitudinally as a three-dimensional lattice encircling and interlinking the peripheral regions of Z-lines of individual myofibrils, and connecting the Z-lines with sarcolemma, sarcoplasmic reticulum and T-tubule (Lazarides, 1980; Tokuyasu, 1983; Thornell and Price, 1991; Stromer, 1992). Vimentin, an insoluble structural protein, has been reported to be localized in the intermyofibrillar and perinuclear sarcoplasm and to co-localize with desmin (Lazarides, 1980; Stromer, 1992). The localization of these two components described in the present study coincided with previous reports. However, no gold particles indicating the existence of desmin and vimentin were observed in leptomeres.

Filamin is a high molecular weight actin-binding protein which induces F-actin gelation and stimulates actin polymerization (Gomer and Lazarides, 1983; Kotliansky et al., 1986). The localization of this protein in mouse cardiac muscles coincided with that of previous reports (Kotliansky et al., 1981, 1986).

However, in leptomeres, filamin was not found. Vinculin, a 130 KD actin-binding protein, has been reported to localize at costameres, which are electron-dense plaques between the Z-line and the sarcolemma and in the intercalated disc of cardiac muscle fibres (Pardo et al., 1983; Shear and Bloch, 1985; Donowski et al., 1992). The present results regarding vinculin are in agreement with previous reports. In leptomeres, however, no positive staining was detected. The immunocytochemical results suggested that no contractile proteins as examined here are present in the leptomeres.

In the intrafusal muscle fibre, a close relationship has been observed between leptomeres and nerve terminals (Katz, 1961; Rumpelt and Schmalbruch, 1969). These authors suggested that leptomeres mechanically influence the sensory nerve. Apposition of leptomeres and nerve terminals has also been reported in cardiac muscle (Bogusch, 1975). However, in our materials, such a close relationship of these structures was not observed. From the fact that leptomeres locate in interfibrillar position connecting adjacent Z-lines or Z-line and sarcolemma, many authors assumed that these structures were involved in mechanical functions (Thoenes and Ruska, 1960; Katz, 1961; Karlsson and Andersson-Cedergren, 1968; Virágh and Challice, 1969; Rumpelt and Schmalbruch, 1969) such as stabilizing the cellular configuration (Bogusch, 1975) or increasing the rigidity of the cell (Virágh and Challice, 1969). The results of the present study lend support to the mechanical function. Moreover, they seem to indicate their relationship to aging and their pathological etiology, and suggest that they are not involved in the contractile activity of the cardiac muscle fibre.

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