Demonstration of Connective Tissue Sheaths Surrounding Working Myocardial Cells and Purkinje Cells of the Sheep Moderator Band

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Summary. Morphological studies were carried out to delineate the characteristics of connective tissue sheaths surrounding working myocardial cells and Purkinje cells in the moderator band of adult sheep hearts, using a series of techniques including silver staining, immunohistochemistry, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For SEM, tissue blocks were treated with 2N NaOH at room temperature to digest cellular elements.

Individual working myocardial cells were ensheathed by thin argyrophil fibers (reticular fibers), while fascicles of 4-8 Purkinje cells (Purkinje strands) were encircled by rather thick reticular fibers. Some collagen fibers were located between masses of myocardial cells as well as between the Purkinje strands. Immunohistochemical analyses indicated that reticular fibers directly surrounding both myocardial cells and Purkinje strands showed moderately positive reactions for anti-type I collagen and intensely positive reactions for anti-type III collagen.

Deserves particular note is that the three-dimensional architecture of the reticular sheaths varied widely at different places. The thin reticular sheaths surrounding each myocardial cell consisted of fibrils which were arranged in a coarse network and directed circularly along the long axis of cells. By contrast, the sheaths enclosing Purkinje strands were thicker, and their reticular fibrils were woven into a compact “felt-like” texture. The functional significance of these connective tissue sheaths is also discussed.

It is widely agreed that the contents of connective tissues in the mammalian heart vary with species, region, age and pathological state (Tawara, 1906; Holmgren, 1907; Borg and Caulfield, 1981; Pearlman et al., 1982; Robinson et al., 1983; Sato et al., 1983; Dolber and Spach, 1987; Smolich et al., 1990). By definition at light and electron microscopic levels, epimysium is the connective tissue sheath surrounding the entire muscle, endomysium surrounds individual myocardial cells, and perimysium connects the epimysium to the endomysium and surrounds groups of myocardial cells (Robinson et al., 1983). Previous authors unequivocally proposed that the epimysial collagen network prevented overstretching of the myocardial cells. Although the precise function of the endomysium remains to be clarified, its nutritional role (Holmgren, 1907) and viscous-elastic properties (Nagel, 1935; Borg and Caulfield, 1981) have been noted. In addition, collagenous septa between myocardial cells or masses of myocardial cells have been discussed in association with the transverse conduction velocity, and it has been suggested that thick septa (so-called perimysium) provide insulating boundaries for discontinuities of excitation (Sommer and Johnson, 1968; Spach and Dolber, 1985, 1986; Dolber and Spach, 1987).

It is well established that the heart conduction system and working myocardium show morphologically and physiologically contrasting features (Viragh and Challice, 1973; Canale et al., 1986). Purkinje cells thus were recently confirmed to differ markedly from myocardial cells in size, ultrastructure and arrangement (Viragh and Challice, 1973; Shimada et al., 1983, 1986). Although the conduction system has been characterized by its large contents of connective tissues (Tawara, 1906; Truex and Copenhaver, 1947), no systematic morphological studies of connective tissue sheaths surrounding Purkinje cells have been made. In this context, our attention in this study is focused on the morphological differences between the connective tissue sheaths of Purkinje cells and those of myocardial cells.

In the present study, the sheep moderator band bridging the space between the right side of the interventricular septum and the anterior papillary...
muscle was examined since it possesses both myocardial and Purkinje cells of typical structure. In order to facilitate the three-dimensional observation of the connective tissue sheaths, the cardiac tissues to be examined were treated with NaOH at room temperature. Such a treatment has been shown by OHTANI (1987) to effectively remove cellular elements while leaving connective tissue fibers intact. In addition, the connective tissue sheaths were also studied using silver-impregnation method, transmission electron microscopy (TEM) and immunohistochemical techniques.

MATERIALS AND METHODS

Twenty adult sheep of both sexes weighing 30-40 kg were used in the present study. The hearts were excised under anesthesia. The moderator band was removed from the right ventricle and subjected to the procedures indicated below.

Light microscopy

Specimens were cut into slices 3-5 mm thick with razor blades and fixed in 10% formalin containing 2% calcium acetate or a periodate-lysine-paraformaldehyde (PLP) fixative (McLEAN and NAKANE, 1974), dehydrated, cleared and embedded in paraffin. Thick sections (6 μm) were made and stained with a series of techniques: hematoxylin, organge G and phosphomolybdic acid-aniline blue staining (HOPA), and a silver impregnation procedure (Bielschowsky-Gomori method) for identification of reticular fibers (GOMORI, 1934; Montes et al., 1980).

Transmission electron microscopy

The tissue pieces containing both myocardium and Purkinje bundle were dissected out, placed in a Karnovsky's fixative at 4°C for 2 h, and postfixed in cacodylate buffered (pH 7.4) 2% osmium tetroxide-0.5% potassium ferrocyanide at 4°C for 2 h. They were dehydrated in a graded series of ethanol and embedded in epoxy resin. Thin sections were cut, stained with tannic acid and lead citrate, and viewed and photographed under a JEOL-100CX transmission electron microscope.

Immunohistochemistry

For light microscopic examinations, tissue blocks were fixed in the PLP fluid at 4°C for 2 h and were deparaffinized and subjected to the immunogold-silver method (TAKITA et al., 1990). Briefly, the sections were treated with rabbit anti-type I or anti-type III collagen antibodies diluted 1:2000 with PBS for 2 h at room temperature. They were then incubated with goat anti-mouse 5 nm gold conjugate (Jansen Life Sciences Products, Piscataway, NJ) diluted 1:40 in PBS for 1 h at room temperature. Finally, the sections were physically developed for 40 min at 20°C.

For electron microscopic examinations, tissues were fixed in the PLP fixative and embedded in Lowicryl K4M. Ultra-thin sections were incubated with rabbit anti-type I or anti-type III collagen antibodies for 1 h at room temperature, washed in PBS and immersed in immunogold (5 nm) solution for 1 h at room temperature. They were then rinsed in PBS and stained with aqueous uranyl acetate and examined by TEM. Control specimens were treated with goat-anti-mouse IgG gold-tagged antibodies.

Scanning electron microscopy (SEM)

The moderator band was fixed in Karnovsky's fixative for 3 h or longer at 4°C. After a short rinsing in 0.1 M cacodylate buffer (pH 7.4), the tissues were immersed in a 2N NaOH (OHTANI, 1987) or 2N KOH solution for 5-7 days at room temperature and were rinsed 4 times in physiological saline. They were then put in a 40% dimethylsulfoxide solution and freeze-cracked with a razor blade in liquid nitrogen (TOKUNAGA et al., 1974). Following a brief immersion in Karnovsky’s fixative, the tissue pieces were washed thoroughly in a physiological saline solution containing 5% Tween 20 for 2 h at 40°C, placed in a 1% aqueous solution of tannic acid for 2 h and postfixed with cacodylate buffered 2% osmium tetroxide for 2 h. They were dehydrated in graded concentrations of ethanol, and dried by the critical point drying method. The specimens were sputter-coated with gold, and viewed in a H-S800 scanning electron microscope. Some of the moderator bands were cut transversely along the long axis with a TC-2 tissue sectioner (Sorvall), and were then treated with NaOH. These specimens were mainly used for SEM observation at low power magnifications.

RESULTS

General structure of the moderator band

The right limb of the atrioventricular system is known to descend down the ventricular septum, and to enter the septomarginal trabecula (moderator
Fig. 1. Interior aspect of the right ventricle of the sheep heart. The moderator band (arrow) is seen to connect the right surface of the interventricular septum with the base of the anterior papillary muscle.

Fig. 2. Light micrograph of a transverse section of the moderator band stained with HOPA. The region of Purkinje cells (P) possesses more abundant collagen fibers (stained blue) than that of myocardial cells (M). ×230

Figs. 3 and 4. Sections of the moderator band impregnated with silver (Bielschowsky-Gomori method). The connective tissue sheaths surrounding both myocardial cells (M) and Purkinje strands (P) are stained a deep black color, while collagen fibers distributed between masses of myocardial cells (M) as well as between Purkinje strands (P) are stained a brownish color. Fig. 3: ×450, Fig. 4: ×230
Fig. 5. TEM images of myocardial cells (M) and Purkinje cells (P). A few reticular fibrils (arrow) with diameters of approximately 40 nm are distributed around each myocardial cell (a). Purkinje strands are surrounded by a thick layer of reticular fibrils (R) (approximately 40 nm in diameter) (b). Collagen fibrils (C) of a large diameter (60–80 nm) are found outside the thick sheaths (b). ×23,000

Fig. 6. Light micrographs of the moderator band stained with immuno-gold silver method for type I (a) and III (b) collagens. The connective tissue sheaths (arrows) surrounding Purkinje strands (P) and myocardial cells (M) show moderately positive reactions for type I collagen and intensely positive reactions for type III collagen. C collagen fibers. ×150
band) (TAWARA, 1906; TRUEX and COPENHAVER, 1947) (Fig. 1). The moderator band was observed here as it contains a region of Purkinje cells (Purkinje bundle) and that of working myocardial cells. The Purkinje bundle was usually encountered immediately beneath the endocardium, and less frequently within the myocardium. In cross sections of the moderator band, Purkinje and myocardial cells were identified with certainty under both the light and electron microscopes. Thus, Purkinje cells were characterized by their large size, marginal distribution of myofibrils and abundance in glycogen. In contrast, small size, even myofibrillar distribution and the paucity of glycogen were the predominating features of the myocardial cells. Purkinje cells were interconnected with junctional complexes such as desmosomes and gap junctions, always constituting conspicuous muscular fascicles (Purkinje strands).

**Light microscopy of paraffin sections**

In cross sections of the moderator band stained with HOFA, structures were differentially stained: muscle cells, deep orange; elastic fibers, dark violet; collagen fibers, blue. Substantial amounts of collagen fibers were found in the subendocardial region, between the myocardium and Purkinje region and among the Purkinje strands (Fig. 2). The Purkinje strands were variable in size and consisted of 4-8 cells. In the myocardium, moderate amounts of collagen fibers were distributed between masses of myocardial cells. The connective tissue sheaths surrounding myocardial cells and Purkinje strands were not visible by this staining method. In the silver-impregnated sections, the connective tissue sheaths surrounding both myocardial cells and Purkinje strands were heavily stained a deep black color (Figs. 3, 4). This apparently indicates that these sheaths consist primarily of reticular fibers. In contrast, collagen fibers, which...
were less intensely silver stained to a brownish color, were found to be distributed between masses of myocardial cells as well as between Purkinje strands, appearing throughout the subendocardial region and in the adventitia of arteries (Figs. 3, 4). Whereas reticular fiber sheaths surrounded each myocardial cell, they were consistently present around Purkinje strands, but not between each Purkinje cell. In addition, these sheaths were more densely meshed and larger in thickness around Purkinje cells than around myocardial cells.

**TEM observations**

Connective tissue sheaths enclosing both types of cardiac muscle cells were examined by TEM in transverse thin sections of the moderator band. A few fibrils with diameters of approximately 40 nm were sporadically distributed around myocardial cells (Fig. 5a). In marked contrast, a large number of analogous fibrils (approximately 40 nm in diameter) were organized into a complex network around Purkinje strands, where they formed conspicuous connective tissue sheaths of a thickness as great as 1.5 μm (Fig. 5b). Blood capillaries and nerve fibers were located immediately outside these connective tissue sheaths. On the other hand, fibrils of larger diameter (60–80 nm) were found to be scattered between myocardial masses and between Purkinje strands (Fig. 5b).

**Immunohistochemistry**

Light microscopic paraffin sections from the moderator band were immunogold-silver stained for type I and type III collagens (Fig. 6). Intensely positive reactions for both collagen types were exhibited by a series of connective tissues located beneath the endocardial endothelium, between myocardial cell masses, between Purkinje cell strands and in the adventitia of...
arteries. The connective tissue sheaths surrounding Purkinje strands showed moderately positive reactions for type I collagen and intensely positive reactions for type III collagen. An appreciable intensity of reactions for type III collagen was also noted in association with the sheaths enclosing myocardial cells, but significant reactions for type I collagen were missing.

Further, electron microscopic Lowicryl K4M sections from the same tissue specimens were similarly immunostained for both collagen types, in order to supplement light microscopic histochemical data. Reticular fibrils ensheathing Purkinje strands and myocardial cells reacted to both gold labeled anti-type I and anti-type III collagen antibodies (Fig. 7). In addition, collagen fibrils present between Purkinje strands also reacted to both these antibodies. In all the control sections, the immunoreactions for these collagen types were virtually missing.

**SEM observations**

SEM examination of fractured undigested moderator bands enabled us to distinguish between larger Purkinje cells with fewer myofibrils and smaller myocardial cells with more numerous myofibrils (Fig. 8). Connective tissue sheaths surrounding Purkinje strands and myocardial cells were also observed by such examination. Detailed three-dimensional studies of these sheaths were, however, not feasible because of the coexistence of such cellular elements as muscle cells, elastic fibers and ground substances. Controlled digestion with NaOH of the fixed moderator band effectively removed all these coexisting elements, leaving microfibrillar components (collagen and reticular fibers) intact (Fig. 9). Using those SEM specimens which had undergone such a treatment, the three-dimensional architectures of the connective tissue sheaths were successfully demonstrated.

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**Fig. 10.** SEM image of the fractured surface of the Purkinje region after NaOH-treatment. Purkinje strands (P) are encircled by thick sheaths (arrows) and abundant collagen fibrils (C) are distributed between Purkinje strands. ×350
Purkinje strands yielded much larger cavities than ordinary myocardial cells (Fig. 10). The cavities of the Purkinje strands, of varying size and often bifurcated, assumed a honeycomb-like appearance. The sheaths surrounding Purkinje strands were formed by densely meshed reticular fibrils running along the plane parallel to the surface of the strands (Figs. 11, 13a). In contrast, the sheaths of myocardial cells consisted of a looser network of corresponding fibrils as compared with their counterpart in Purkinje strands (Figs. 12, 13b). Most of the fibrils were arranged circularly along the long axis of myocardial cells. In addition, the collagen fibrils, which were distributed beneath the endocardial endothelium, between the myocardial masses and between the Purkinje strands, formed fibrillar bundles of sizes varying from 1-5 μm (Figs. 11, 12).

**DISCUSSION**

Purkinje cells are characterized by assembling into a fascicle consisting of 4-8 cells, called the Purkinje strand. It has been reported that each Purkinje strand is encircled by a connective tissue sheath, and that no connective tissue elements can be detected between individual Purkinje cells (JOHNSON and SOMMER, 1967). On the other hand, working myocardial cells have been shown to be surrounded individually by a comparable fibrous sheath (MORITA and SHIMADA, 1990).

Using picrosirius red staining, DOLBER and SPACH (1987) have recognized two types of collagenous septa in the cardiac papillary muscles of dogs and rabbits: thin collagenous septa which ensheathe myocardial...
cells for short distances, and thick collagenous septa which ensheathe groups of myocardial cells for long distances. By definition, it appears that these thin and thick collagen septa correspond respectively to the endomysium and perimysium. In the present study, the moderator band of sheep, which contains both Purkinje and working myocardial cells, were morphologically examined. The light microscopic and TEM results obtained here demonstrate that reticular fibrils directly surround Purkinje strands and myocardial cells while collagenous fibrils are dispersed between these strands and in the perimysium. In this context, it is interesting to note that as early as 1906, Tawara noticed the abundance of connective tissue elements in the heart conduction system, a feature he regarded as the most characteristic of this system.

It has been formerly held that collagen and reticular fibrils respectively contain type I and type III collagens exclusively (Fleischmajer et al., 1978; Montes and Junqueira, 1988; Junqueira et al., 1978). By the use of a recently updated immunogold technique, type III collagen has been shown to coexist with type I collagen on banded collagen fibrils in the skin, amnion, aorta and tendon after removal of the amino and carboxyl propeptides (Keene et al., 1987). In the present study on sheep moderator bands, analogous colocalization of these two collagen types was confirmed not only in association with collagenous fibrils but also with reticular fibrils.

SEM observations of the sheep moderator band subjected to 2N NaOH treatment revealed that the fibrous sheaths of myocardial cells and those of Purkinje strands markedly differ from each other in their thickness and density and course of the constituting reticular fibrils. The question now raised concerns what roles the connective tissues associated with myocardial cells and Purkinje strands might play, and why a large amount of connective tissues is present in

Fig. 12. SEM image of connective tissue sheaths surrounding myocardial cells. The sheaths are thin and consist of loose networks of reticular fibrils. C collagen fibrils between myocardial masses. ×3,600
the Purkinje region. SPACH and collaborators have suggested that thick and thin collagenous septa in the myocardium interrupt or modify lateral impulse propagation (SPACH and DOLBER, 1985, 1986; DOLBER and SPACH, 1987). The connective tissues of the Purkinje region have been speculated to function as an insulator (LATHROP and BAILY, 1977) which inhibits the escape of impulses out of the Purkinje system. This, along with the large diameter of Purkinje cells (SHIMADA et al., 1986) and the presence of prominent nexuses between the cells (SUGI and HIRAKOW, 1986), may be the morphological basis for effective conduction of the impulses. Throughout the mammalian species so far examined, the Purkinje system is one which is located subendocardially, and is necessarily susceptible to compression during heart beats. In these terms it can be alternatively assumed that the thick fibrous sheath of the Purkinje strand provides mechanical support which protects the Purkinje cells from external forces. Intermediate filaments and myofibrils retained by the cell may also serve this purpose (THORNELL and ERIKSSON, 1980; CANALE et al., 1983).

In conclusion, the connective tissue sheaths surrounding Purkinje strands were found to be thick and densely meshed by reticular fibrils. Substantial amounts of collagen fibers were observed between Purkinje strands. In contrast, individual myocardial cells were surrounded by a thin sheath consisting of looser networks of reticular fibrils. Moderate amounts of collagen fibers were distributed between masses of myocardial cells. It is reasonable to assume that characteristic architectures of connective tissue elements in the myocardium and Purkinje region reflect the functional aspects of the heart.

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REFERENCES


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