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A micro-anatomical model of the distribution of myocardial endomysial collagen

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Summary. Myocardial connective tissue probably provides passive support for regulating heart tensile strength and stiffness and ultimately for controlling heart mechanics through its endomysial part. However, endomysial collagen micro-arrangement is still a matter of debate. In order to define the fine distribution of left ventricle endomysial collagen, we applied the NaOH-scanning electron microscopy (SEM) maceration method (one of the techniques of choice for studying collagen micro-arrangement) to rabbit heart. Gomori-reticulum staining was used for correlated light microscopy (LM) observations. The SEM-NaOH method allowed isolation of collagen by removing other extracellular matrix components and cells and preserved collagen structure and position. Endomysial collagen appeared arranged in *laminae* that delimited the *lacunae* that were left empty by macerated myocytes and small vessels (mostly capillaries). These *laminae* were formed by reticular fibers, as confirmed by LM observations of Gomori-reticulum-stained samples, and were organized in irregularly meshed networks made of thin (single) and thick (composed) filaments. In longitudinal views, collagen *laminae* extended the entire length of *lacunae*. In transversal views, the cut surface of the *laminae* appeared to be made of collagen bundles. These observations provide an updated microanatomical view of endomysial collagen distribution, which integrates previous studies. This model is based on the evidence that collagen *laminae* enveloped the surface of small vessels and myocytes. Thus, a type of myocyte-myocyte or capillary-myocyte "laminar connection" anchored to the entire cell length here is emphasized, rather than a type of "strut connection" anchored to defined loci, as usually described. This structure explains better how endomysium may provide the necessary support for heart compliance and protection against overstretch.

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Introduction

Heart myocytes and capillaries are enmeshed in a net of connective tissue organized in different levels, respectively: epimysium, the layer of connective tissue surrounding myocardium; perimysium, associated with groups of myocytes; and endomysium that surrounds and connects each individual muscular cell. Thus, collagen is an essential component of myocardial connective stroma, largely composed of type I and III fibrillar collagen (Weber, 1989; Weber et al., 1994; Caulfield and Janicki, 1997; Burlew and Weber, 2000; Gazoti Debessa et al., 2001). Collagen arrangement probably has the significance of preserving heart micro-architecture and chamber geometry, maintaining the correct myocyte alignment and possibly contributing to the control of myocardial contraction. In fact, several reports have emphasized that the architecture of myocardial collagen fibers, in particular of the endomysium, may be involved in the regulation of the mechanical activity of the heart (Gay and Johnson, 1967; Robinson et al., 1983, 1988; Doering et al., 1988; Weber et al., 1988, 1994; Weber, 1989; MacKenna et al., 1994; Dai et al., 1996; Omens et al., 1997; Yang et al., 1997; Icardo and Colvee, 1998; Kunzelman et al., 1998; Rossi et al., 1998; Lutgens et al., 1999; Shirani et al., 2000; Zimmerman et al., 2000).

The endomysial collagen arrangement has been described as a weave network surrounding each individual myocyte and connecting adjacent myocytes and capillaries, through bundles of collagen called "struts" (Caulfield and Borg, 1979; Borg and Caulfield, 1981; Borg et al., 1982; Robinson et al., 1983; Abrahams et al., 1987; Caulfield et al., 1992; Caulfield and Janicki, 1997; Redington et al., 1998; Sanchez-Quintana et al, 1999). However, there is disagreement about the morphology or even the presence of myocyte-myocyte and myocyte-capillary struts (Ohtani et al.,

1988; Morita et al., 1991; Icardo and Colvee, 1998; Rossi et al., 1998). In addition, the visualization of heart collagen organization was also affected by the interference posed by numerous and different cellular elements of the cardiac tissue. For this purpose the use of scanning electron microscopy (SEM) after NaOH maceration (Ohtani, 1987) may represent a very effective method in demonstrating the real three-dimensional (3-D) organization of the collagen fiber network, as previously successfully revealed in numerous tissues, including pancreas (Ohtani, 1987), liver (Ohtani, 1988, 1992), umbilical cord (Vizza et al., 1996), and cardiac tissue (Ohtani et al., 1988; Morita et al., 1991; Icardo and Colvee, 1998; Rossi et al., 1998; Macchiarelli and Ohtani, 2001). The SEM-NaOH maceration method allows the isolation of collagen fibers, preserving their structure and position, by the elimination of extracellular matrix and all tissue cellular elements.

Thus, in the present study, the 3-D distribution of the endomysial collagen in rabbit left ventricle was studied through the application of the SEM-NaOH maceration technique in order to provide an actual morphological basis for better understanding the role of this network in heart activity.

Materials and methods

The cardiac tissue of six adult rabbits -*Oryctolagus cuniculus*- was used (the investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health -NIH Publication nº 85-23, revised 1996-)

Anesthesia was induced by injection of pentobarbitone sodium (Nembutal®, Abbot) into the marginal ear vein of the animals, subjected to median sternotomy. Blood was washed out by perfusion through the aorta of 0.9% phosphate-buffered saline (PBS) at room temperature. Then, perfusion-fixation was performed via the same route with a solution of 2.5% glutaraldehyde in 0.1M PBS.

The left ventricle of each animal was isolated, and cut transversally (3 animals) or longitudinally (3 animals) at the middle part. The samples were kept in 2.5% glutaraldehyde in 0.1M PBS for 48 hours and then prepared according to the following protocol (Ohtani et al., 1988). 1. Washing in 0.1M PBS solution for 2 hours. 2. Maceration in 2N NaOH (9.5%-10% NaOH in distilled water) at room temperature (25-30 °C) for 3-7 days. 3. Digestion in distilled water at room temperature for 3-10 days or until the samples were pale and transparent. 4. Impregnation in 1% tannic acid (in 0.1M PBS) for 2 hours. 5. Washing in 0.1M PBS for 1 h. 6. Post-fixation 1% OsO_4 in distilled water for 1-3 hours. 7. Washing in 0.1M PBS for 1 hour. 8. Dehydration in an ascending series of graded alcohols. 9. Freeze cracking in liquid nitrogen with a razor blade followed by critical-point drying. 10. Coating with platinum. Observations were made with a Hitachi S4000 field emission scanning electron microscope operating at 4-10 kV.

Blood vessel and myocyte *lacunae* were classified taking into account the shape and size of the spaces left empty by maceration (Izumi et al., 1984; Kawai et al., 1984; Vahouny et al., 1985; Hossler et al., 1986; Rossi, 1998).

Light microscopy (LM)

Specimens of the left ventricle were cut into longitudinal or transversal slices, fixed in 10% buffered formaldehyde in 0.1M PBS, dehydrated through an ascending series of graded ethanol, cleared and embedded in paraffin. Sections of about 5 μm in thickness were performed and then stained with a silver impregnation technique (Gomori reticulum technique) to evidence reticular fibers. The sections were observed and photographed with a Zeiss III RS photomicroscope.

Results

As seen by SEM, the NaOH maceration method caused the selective removal of all cells and of extracellular matrix components except collagen. Collagen fibers appeared well preserved and regularly distributed in their general organization. Low power SEM views and LM sections showed a structure made up of collagen *laminae* arranged in a honeycomb-like fashion. Cracked samples allowed the ready visualization of flat *laminae* that delimited emptied spaces (*lacunae*) (Fig. 1). The *lacunae* corresponded to the areas occupied by small vessels (mostly capillaries) and myocytes before maceration. Therefore, when seen in transversal sections, and according to measurements and courses of *lacunae*, we could mainly identify: a) small *lacunae* of about 8-15 μm in diameter, corresponding to capillary spaces and b) large *lacunae*, of about 15-25 μm in diameter corresponding to myocyte *lacunae* (Fig. 2a). Furthermore, collagen *laminae* also delimited spaces wider than those above described and had varying size (diameter ranging between 40-200 μm). These latter spaces could be related to vessels (arterioles or venules) removed by maceration (Fig. 1). LM observations of Gomori reticulum-stained samples showed that collagen *laminae* were formed by reticular fibers (Fig. 2b). As seen by SEM, these fibers were arranged in a fine network that surrounded each *lacuna*, as was well shown in longitudinal sections (Fig. 3). At high magnification, each *lamina* appeared formed of thin and thick filaments. Thin filaments had a diameter of 40-70 nm; each of them could be identified as a single collagen fibril. Thick filaments presented a diameter of 200-500 nm and were composed of a conspicuous bundle of numerous collagen fibrils. The thin and thick filaments were arranged in an irregularly meshed network. The size of the meshes of this network was variable, ranging from 150 nm to 600 nm. Some meshes, probably due to artifacts, were of a size wider than that above described.

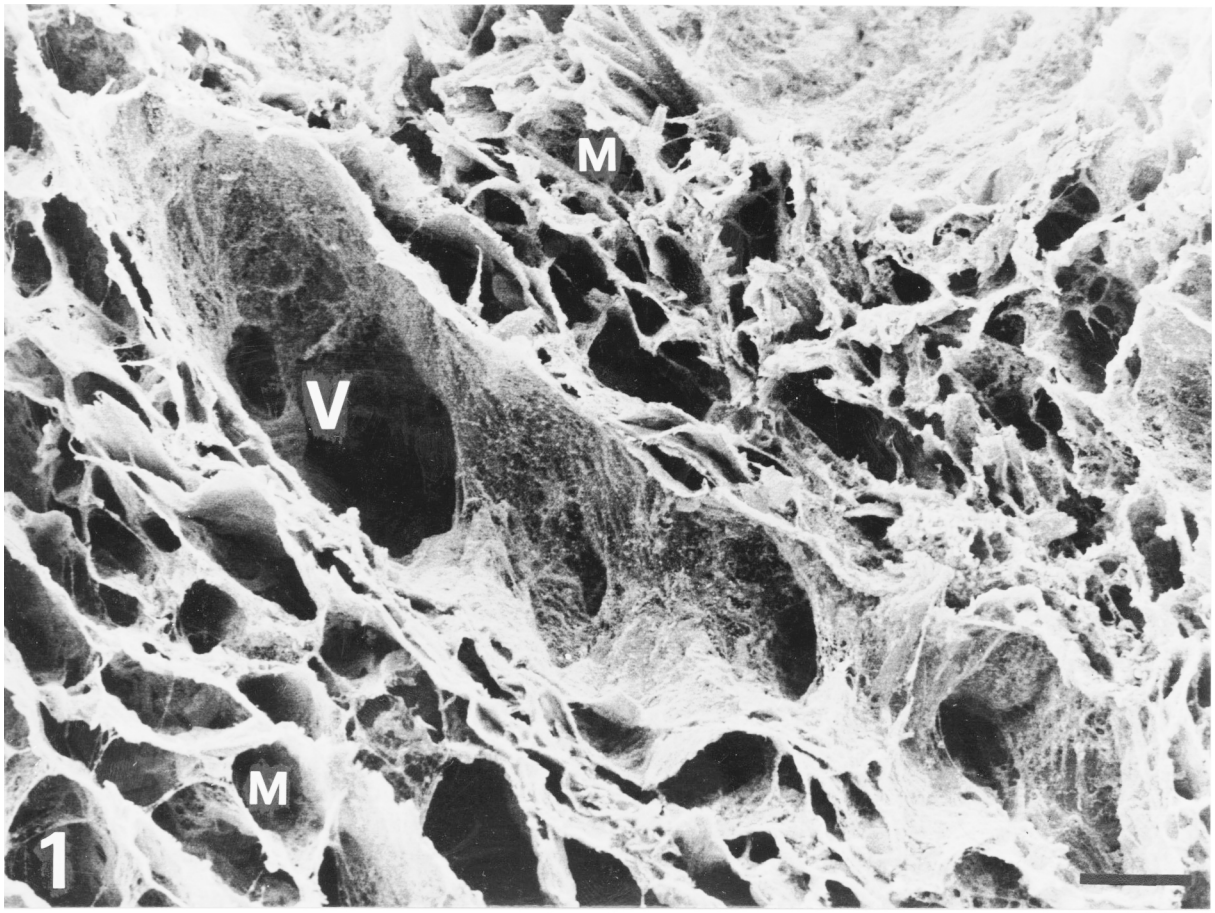

Fig. 1. Cracked section of rabbit left ventricle wall after NaOH maceration, illustrating the fine arrangement of the collagen. Note the numerous myocyte lacunae (M). Part of a large longitudinal section of a blood vessel lacuna is also shown (V). SEM. x 750. Bar: 21 μm.

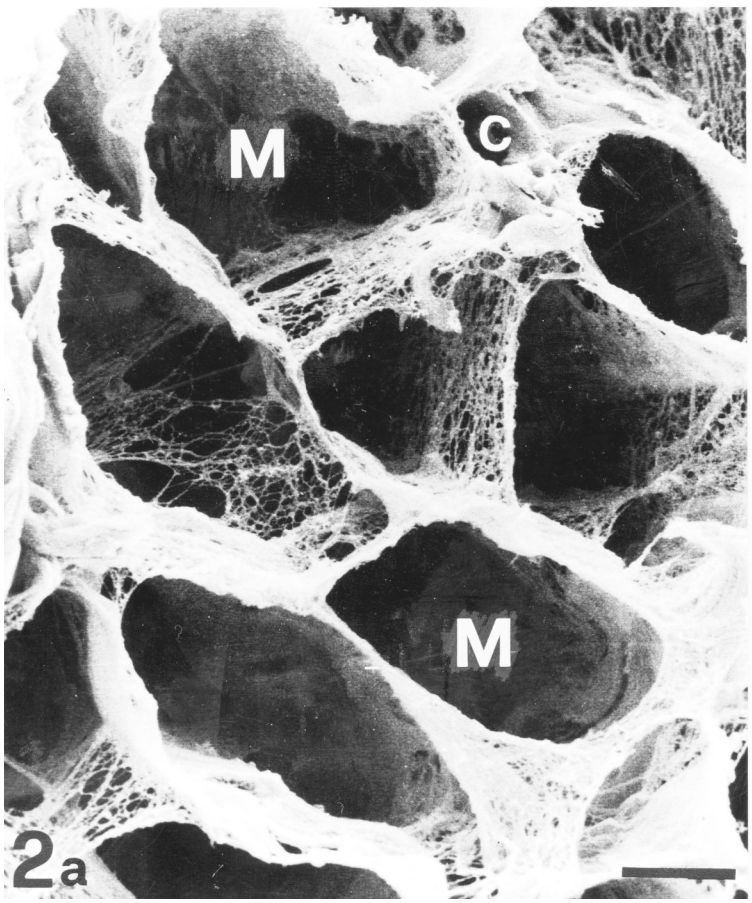

Fig. 2. a. Numerous myocyte lacunae (M) in cross-section are invested by a continuous layer of endomysial collagen. A capillary lacuna (C) can be also seen. SEM. x 2300. Bar: 6.5 μm. **b.** Section of rabbit left ventricular wall impregnated with silver, evidencing reticular fibers. These probably correspond to the fine net of collagen fibers detected in Figs. 1, 2a around myocyte lacunae and vessel lacunae. LM. Gomori reticulum staining. x 250. Bar: 50 μm.

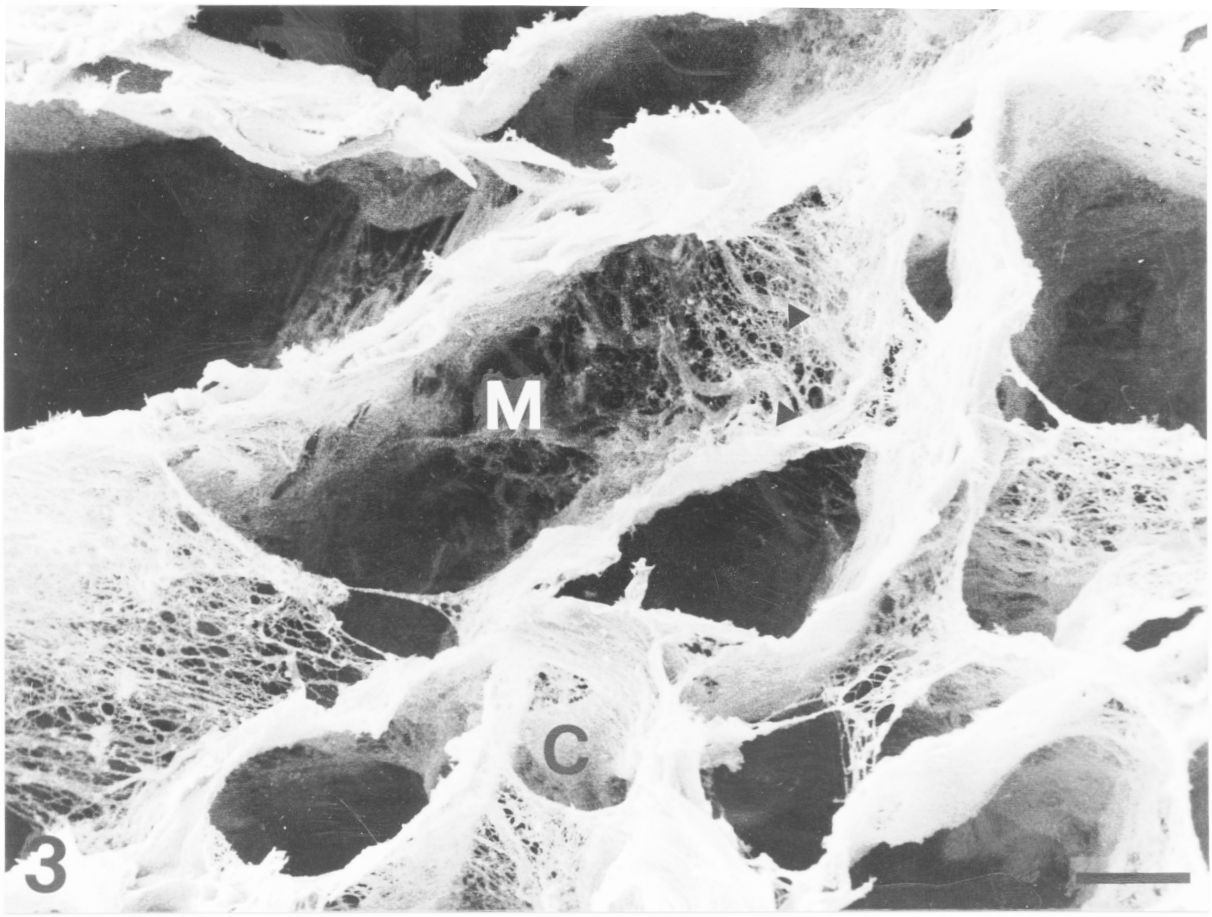

Fig. 3. Longitudinal cut of a myocyte lacuna (M) delimited by the endomysial collagen layer. Thick collagen filaments (arrowheads) are visible over this layer. A small capillary lacuna (C) surrounded by the endomysial collagen sheath is also seen. SEM. x 2,300. Bar: 6.5 μm.

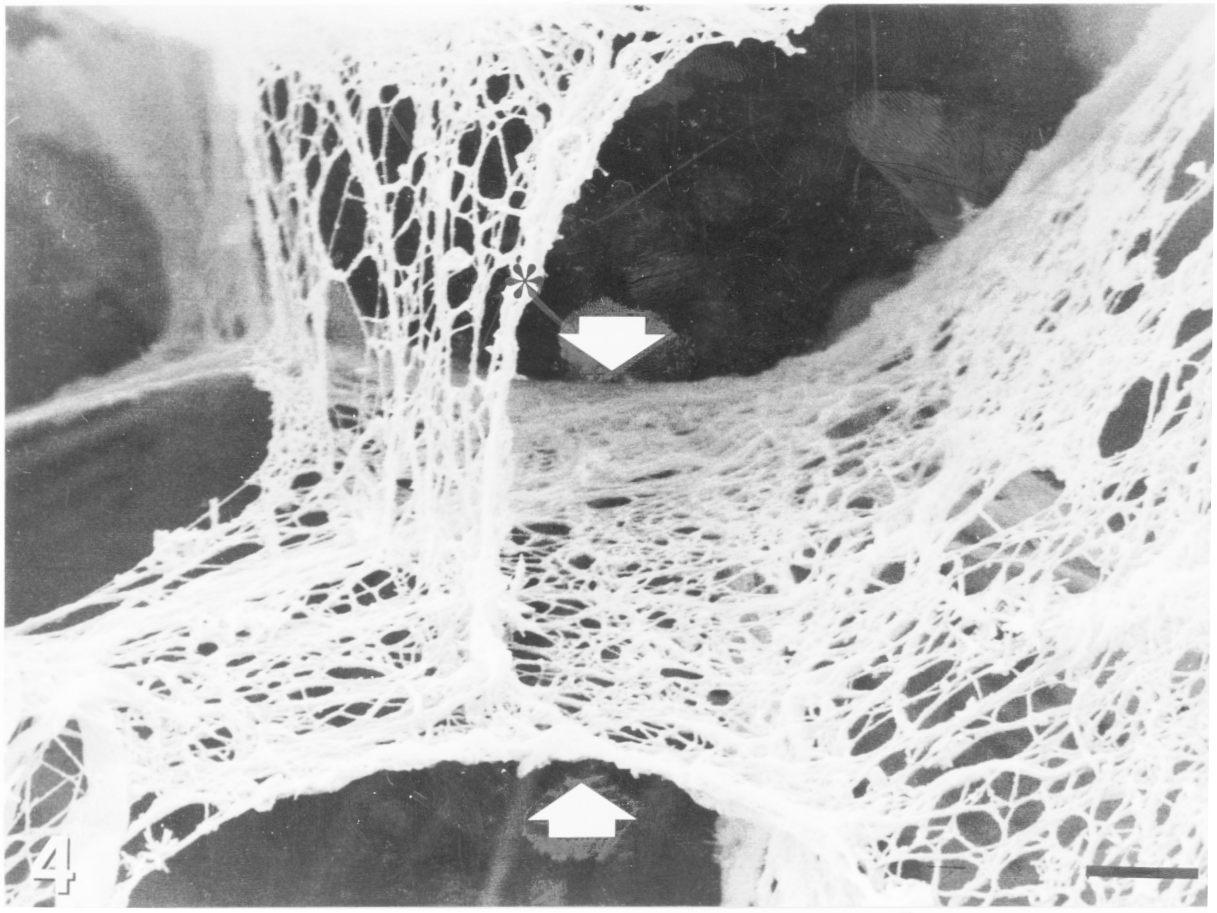

Fig. 4. A high-powered electron micrograph displaying the meshed arrangement of the collagen network of rabbit left ventricular endomysium. These fine fibers are seen to embrace a myocyte lacuna (arrows) and to be organized in irregular bundles (asterisk) at the cut edges of the laminae. Note the collagen network formed by thick (composed) and thin (single) filaments. SEM. x 8,400. Bar: 1.7 μm.

Frontal views of transversal sections showed that the cut edges of the *laminae* were constituted by irregular bundles of collagen (Fig. 4). The *laminae* were always monostratified, and delimited contiguous *lacunae*. Therefore, the endomysial *laminae* formed a continuous sheath that clearly covered at the same time the vessel and capillary *lacunae* and the myocyte *lacunae* (Figs. 2a, 4).

Discussion

The SEM-NaOH maceration method allows an optimal 3-D overview of the endomysial collagen organization in rabbit myocardium, overcoming the visual interference posed by extracellular matrix, myocytes and eventually by other cells or cellular fragments present in these areas (Ohtani et al., 1988; Morita et al., 1991; Icardo and Colvee, 1998; Rossi et al., 1998; Macchiarelli and Ohtani, 2001). Thus, we could acknowledge that this technique not only can clearly demonstrate the extensive collagen framework in the cardiac tissue (Ohtani et al., 1988; Morita et al., 1991; Icardo and Colvee, 1998; Rossi et al., 1998), but also may contribute to clarify the role of this extracellular matrix component in heart activity.

The collagenous connections between neighboring myocytes or between myocytes and vessels including capillaries have been previously termed struts. This term was suggested by Caulfield and Borg (1979) in order to describe isolated bundles of twisted collagen fibers interconnecting myocytes among them, or myocytes with capillaries. Indeed, the struts were described in light and electron microscopic studies (Borg and Caulfield, 1979, 1981; Caulfield and Borg, 1979; Borg et al., 1982; Perlman et al., 1982; Robinson et al., 1983, 1988; Icardo and Colvee, 1998; Rossi et al., 1998). Furthermore, a weave of collagen pericellular fibers or collagen fibril micro-threads, partially enveloping myocytes and capillaries, were also described as endomysial components (Robinson et al., 1983, 1988). The identification of struts may be related to the technique and point of observation employed, as was suggested in a SEM study on human papillary muscle (Icardo and Colvee, 1998) in which the struts were only recognized in macerated samples but not visualized with NaOH maceration. The struts were identified in human ventricular myocardium by a modified NaOH-maceration technique (Rossi et al., 1998).

According to recent observations (Macchiarelli and Ohtani, 2001), also in the present work, we observed that in rabbit left ventricle the endomysial collagen formed a wide layer that entirely wrapped each cardiac muscular fiber, like a lamina, and extended to neighboring myocytes, capillaries and larger blood vessels. These collagen *laminae* showed a finely meshed fibrillar micro-architecture. A pattern of laminar distribution of collagen similar to rabbit left ventricle was also described in human left ventricle myocardium (Ohtani et al., 1988), in sheep myocardial and Purkinje cells (Morita et al., 1991) and in human mitral papillary muscle (Icardo and Colvee, 1998) using maceration methods. Our results are also in agreement with LM studies in dog and rabbit myocardium (Dolber and Spach, 1987), in which thin collagenous septa uninterruptedly sheathing several myocytes on their entire length were described.

Our results seem to indicate that the endomysial collagen is distributed as a sort of lamina around myocyte and capillary surface. Likely, the common concept that endomysium is a continuos sheath, may be reinforced by these observations. In the present study, cell body and cell membrane specializations (such as functional complexes forming intercalated disks) were removed by maceration. Thus, we could not evaluate the relationships among collagen *laminae* and the intercalated disks. Indeed, it is reasonable to hypothesize that the endomysium may stop to the intercalated disks, creating discontinuity of the endomysium at this level as previously reported (Ohtani et al., 1988; Icardo and Colvee, 1998).

As revealed by LM in Gomori reticulum-stained samples, the *laminae* of endomysial collagen appeared constituted mainly by reticular fibers. A little amount of reticular fibers is also present in the outer layer of basement membrane, mainly constituted by type IV of collagen in the more conspicuous inner part (Timpl and Brown, 1996). Thus, on the basis of the above observations and taking into account the relative thickness of the endomysial *laminae* hereby showed in LM transversal sections, we could assume that these *laminae* likely include also the reticular fibers belonging to myocyte or endothelial cell basement membrane.

The structure hereby reported in rabbit left ventricle, provides an updated model of the 3-D endomysial collagen distribution that integrates previous observations. This model is based on the evidence that in the myocardial endomysium the collagen is arranged in a finely-meshed fibrillar texture to form wide *laminae* that cover myocyte and capillary surface. This distribution emphasizes a type of myocyte-myocyte or capillary-myocyte "laminar connection", probably anchored to the entire cell length, rather than a "strut connection" anchored to defined loci, as usually described (Fig. 4, Diagram 1).

The endomysium is the fine connective tissue intimately related to myocytes and neighboring capillaries. It probably plays a crucial role in the maintenance of myocardial integrity and performance (Borg and Caulfield, 1979, 1981; Robinson et al., 1983, 1988; Ohtani et al., 1988; Icardo and Colvee, 1998). We suggest that the pattern of laminar arrangement of the collagen endomysium here described in rabbit heart, and previously observed in other mammals (Ohtani et al., 1988; Morita et al., 1991; Icardo and Colvee, 1998), is probably representative of one of the factors that may contribute to provide a mechanical resistance to stretch (Ohtani et al., 1988), and at the same time to guarantee myocyte compliance. In addition, the laminar

arrangement of collagen fibers could avoid the slippage between myocytes, lateral cell deformation and even reduce blood vessel collapse during systole (Caulfield and Borg, 1979; Robinson et al., 1983; Factor and Robinson, 1988; Ohtani et al., 1988; Weber et al., 1988; Caulfield and Janicki, 1997). In fact, the existence of continuos connections between myocardial fibers and blood vessels may ensure a relative patency of capillaries during the systolic phase, allowing a continuos flow.

Today, in modern specialist text-books of cardiology (Hurst, 1994) the anatomical site and arrangement of the components of the model of cardiac muscle contraction as originally proposed by Hill (1938) are still considered uncertain. As it is well known, in this model the muscle contraction is caused by the action of three components: 1) a contractile element responsible for developing force and of shortening; 2) an *in series* elastic component which is passively stretched during contraction of the contractile element; and 3) an *in parallel* elastic component which provides tension during resting, but does not offer significant resistance during contraction (Diagram 2). On the basis of these considerations, it is possible that the model of laminar distribution of collagen that we suggest may provide a structural basis of the third element of Hill's model, e.g. the *in parallel* component.

The central role played by myocardial extracellular matrix (Gay and Johnson, 1967; Caulfield and Borg, 1979; Robinson, 1980; Borg and Caulfield, 1981; Borg et al., 1982; Abrahams et al., 1987; Dolber and Spach, 1987; Factor and Robinson, 1988; Caulfield and Janicki, 1997; Yang et al., 1997; Bishop and Lindahl, 1998; Icardo and Colvee, 1998), provides further evidence for the concept that a collagen network alteration may result in an impairment of myocardial functions, as observed in various cardiac disorders (Spach and Dolber, 1986; Doering et al., 1988; Weber et al., 1994; Le Grice et al., 1995; Caulfield and Janicki, 1997; Omens et al., 1997; Hanley et al., 1999). Indeed, left ventricular systolic dysfunction and pathological hypertrophy are associated with thickening of the collagen sheath and to a decreasing stress/strain relationship and a subsequent lowering stroke of volume (Weber et al., 1988; Shirani et al., 2000). Ultimately, activation of collagenolytic enzymes and disruption of collagen architecture were observed in association with ischemic heart disease and left ventricle failure (Spinale et al., 2000).

Since the physical arrangement of the collagen network in the heart, particularly its 3-D architecture

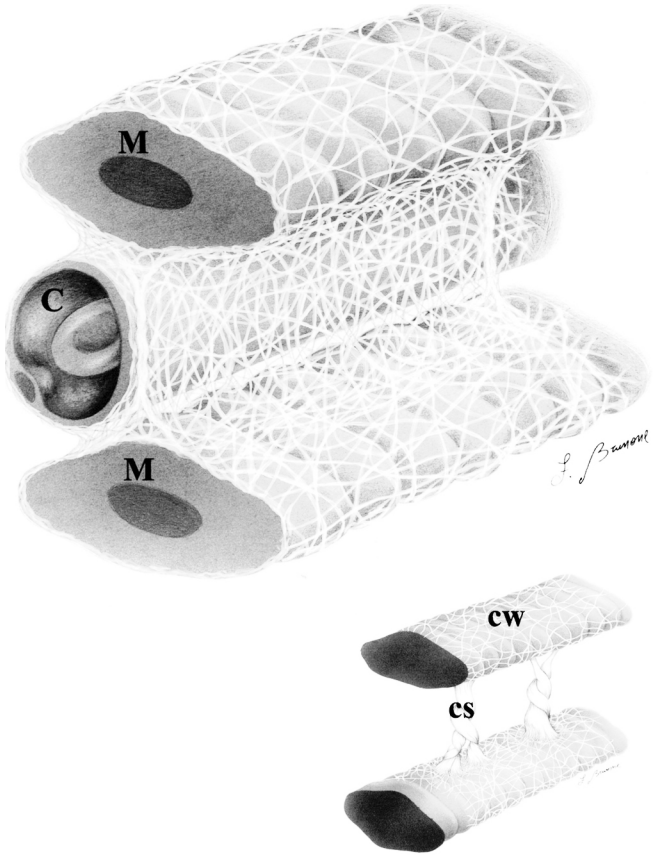

Diagram 1. The drawing proposes a revisited model of distribution of endomysial collagen in rabbit heart. The endomysial collagen fibers are organized in a layer enveloping myocytes and capillaries. The endomysial sheath spreads from one myocyte (M) to a neighboring one, like a lamina and extends along the full myocyte length. This lamina also completely wraps neighboring blood vessels (mostly capillaries) (C). **Inset.** Drawing modified from the diagrammatic representation of endomysial collagen architecture in mammals proposed by Robinson et al. (1983), indicating the collagen weave (cw) enwrapping the myocytes and the struts (cs) connecting one myocyte to another one by anchoring to defined loci.

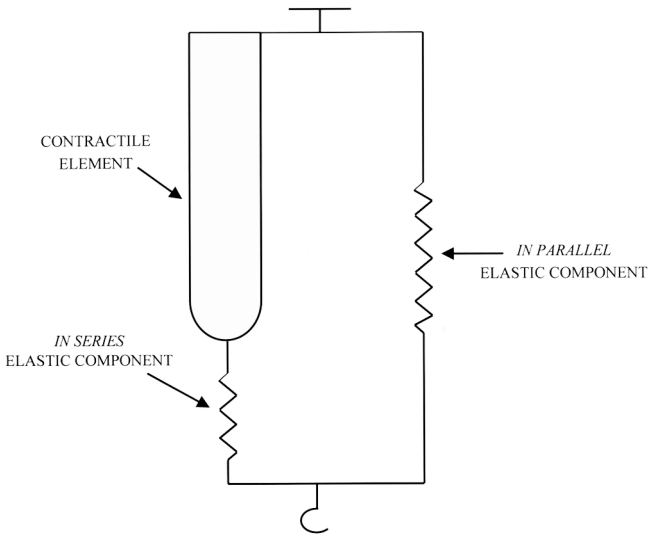

Diagram 2. This is a modified representation of the drawing proposed by Sonnenblick (1996), indicating Hill's model for muscle contraction. The three components that cause muscle contraction, the contractile element, the in series elastic component and the in parallel elastic component, are schematically illustrated.

(Omens et al., 1997; Yang et al., 1997), is relevant in understanding the basic structure of myocardial mechanics, further detailed studies could certainly provide an excellent guide for improving our knowledge on myocardium during normal and pathological conditions.

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