

Morphological varieties of the Purkinje fiber network in mammalian hearts, as revealed by light and electron microscopy

Noriaki Ono¹, Takeshi Yamaguchi², Hajime Ishikawa³, Mitsue Arakawa⁴, Naohiko Takahashi¹, Tetsunori Saikawa¹, and Tatsuo Shimada⁵

¹Departments of Cardiovascular Science and ⁵Health Sciences, Faculty of Medicine, Oita University, Oita;

²Department of Anatomy, Faculty of Medicine, Kanazawa University, Kanazawa; ³Institute of Cetacean Research, Tokyo; and ⁴College of Nursing, Hyogo University, Akashi, Japan

Summary. Purkinje fibers in mammalian hearts are known to comprise the following three groups depending on their structure: group I found commonly in ungulates, group II in humans, monkeys and dogs, and group III in rodents. The aim of the present study was to document precisely the cytoarchitecture of a network of Purkinje fibers in different species by light and electron microscopy. Light microscopy of silver impregnated tissues revealed the reticular fibers ensheathing individual Purkinje strands consisting of 2-8 cells in both the ungulates (i.e., sheep and goats) and cetaceans (whales and dolphins) while they encircled each Purkinje cell in the primates (humans and monkeys), carnivores (dogs and seals), and rodents (rats). Scanning electron microscopy of NaOH digested tissues showed the ungulates (group I) to have a Purkinje fiber network composed of Purkinje strands; the cells in the strands were oval and made side-to-side and/or end-to-end connections. The Purkinje fiber network in the primates and carnivores (group II) was delicate and complicated; the Purkinje cells were usually cylindrical and connected end-to-end, the exception being their polygonal or stellate shapes at the bifurcations. Purkinje cells in the rodents

(group III) resembled ventricular cardiac myocytes in cytoarchitecture. Morphologically, whales and seals respectively belonged to Purkinje cells of group I and group II. These findings indicate that the structural variety of the Purkinje fiber network may reflect the conducting function and be related to the phylogeny of the mammalian species.

Introduction

In 1845, J. E. Purkinje first discovered a net of gray, flat, and gelatinous fibers in the subendocardium of ventricles of the sheep heart. He considered this to be something like the cartilage tissue, but finally concluded that it was a special motor apparatus consisting of muscular tissues. Among the various findings concerning the physiological significance of Purkinje fibers, Tawara (1906) demonstrated that Purkinje fibers ran within muscular trabeculae as the terminal ramifications of the cardiac conduction system and formed a large reticular network in the hearts of humans, dogs, cows and sheep.

The network of Purkinje fibers was also visualized by injection of India ink into the thick connective tissue sheath of cow Purkinje fibers, which enabled the macroscopic demonstration of the extensiveness of Purkinje networks (Cardwell and Abramson, 1931; Ansari *et al.*, 1999). Thereafter, delicate networks of Purkinje fibers was stereoscopically demonstrated in dog and sheep hearts by a modified periodic acid Schiff (PAS) method, since Purkinje cells have rich glycogen particles (Otsuka *et al.*, 1966; Shimada *et al.*, 1992). Furthermore,

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Address for correspondence: Prof. Tatsuo Shimada, MD, Department of Health Sciences, School of Nursing, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Yufu, Oita-ken, 879-5593, Japan
Tel & Fax: +81-97-586-5032
E-mail: tshimada@med.oita-u.ac.jp

scanning electron microscopy (SEM) using chemical digestion techniques made it possible to visualize the three-dimensional structure of the Purkinje fiber network in sheep, goats, and cows, and reveal that Purkinje strands consist of bundles of several oval cells and form a delicate network in these species (Canale *et al.*, 1986; Shimada *et al.*, 1983, 1984, 1986; Murakami *et al.*, 1986). SEM also showed that the three-dimensional architecture of the intercalated disks in Purkinje fibers differs completely between sheep and humans (Zhang *et al.*, 1976; Shimada *et al.*, 2004). In addition, Purkinje strands in the sheep heart are surrounded by the reticular fiber sheath, which probably functions as an insulator as well as a mechanical support (Morita *et al.*, 1991). Based on the mesh structure of Purkinje fibers in the sheep, three-dimensional virtual models of the Purkinje fibers have recently been produced in order to understand the electrophysiological properties of the heart (Iriji *et al.*, 2008) and employed to visualize the human Purkinje fiber network.

A previous review by Canale *et al.* (1986), on the other hand, indicated that the morphology of Purkinje cells in mammals show great variation between species, so that the Purkinje fiber network could be divided into three groups depending on the size and ultrastructure of Purkinje cells. Group I was found in ungulates such as sheep, goats, and cows, group II was in humans, monkeys, dogs and cats, and group III was in mice, rats and rabbits. Recently, studies using electrocardiograms have been done in hearts of multiple mammalian species, from the mouse to whale (Noujaim *et al.*, 2004). However, systematic studies clarifying the overall cytoarchitecture of the Purkinje fiber network in different species remain to be made. Moreover, only a little information is available concerning the morphology of Purkinje cells in aquatic mammals (Asami, 1958; James *et al.*, 1995).

Thus, the present study demonstrates the three-dimensional architecture of the Purkinje fiber network in various mammalian species including whales and seals by light and electron microscopy and discusses the structure and function from a viewpoint of comparative histology.

Materials and Methods

All experimental procedures were done in accordance with the guidelines of the Physiological Society of Oita University, Japan, for the care and use of laboratory animals, which follow the guidelines of the National Institute of Heart (USA). Whale hearts were obtained from whales caught under a Special Permit in the Antarctic, through the Second Phase of the 2005/6 Japanese Whale Research Program.

Hearts of ungulates (5 sheep and 2 goats), primates (4 humans and 5 Japanese monkeys), carnivores (3 dogs and 2 seals), rodents (5 rats), and cetaceans (2 dolphins and 4 whales) were used as materials. The whales were *Balaenoptera bonaerensis* for investigation, of which two were fetal hearts. The endocardial regions containing Purkinje fibers were removed from the right and left ventricles and subjected to the procedures described below.

Stereo-microscopic examinations

Ventricular tissues in the hearts of sheep, humans, and whales were fixed in a mixture of 0.5% periodic acid and 10% formalin, immersed in a modified Schiff solution (Otsuka *et al.*, 1966), and photographed using a stereo-microscope (Leica, Wild M10, Germany).

Light microscopic examinations

Specimens were cut into 5–10 mm squares, fixed in 10% formalin or 4% paraformaldehyde in a phosphate buffer (pH 7.4), dehydrated, and embedded in paraffin. Some of the 7 μ m sections were stained with hematoxylin and eosin or Bielschowsky-Gomori silver staining for the identification of reticular fibers and observed under a light microscope (Nikon, Tokyo).

Scanning electron microscopic examination

For SEM, cardiac tissues were cut into approximately 5.0 \times 5.0 \times 0.5 cm blocks and fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde (Karnovsky's fixative) in a 0.1M cacodylate buffer (pH 7.4). Since Purkinje fibers are situated beneath the endocardium and concealed by the reticular fiber sheath, the endocardial endothelium was removed by mechanical scraping with the finger tip (for the ungulates, whales, humans, and seals), or by immersion in 6N NaOH for 10 min 60°C followed by ultra-sonication (38kHz) for an additional 1 min (for monkeys, dogs, and rats). Then both the subendocardial connective tissue and the reticular fiber sheath were further digested by immersion of the samples in 6N NaOH for 10 to 20 min at 60°C. After these NaOH-treated blocks were checked under a stereo-microscope, they were washed throughout in a saline solution, placed in 1% osmium tetroxide, 1% tannic acid, and 1% osmium tetroxide for 1 h each, dehydrated in an ascending series of ethanol, and then dried by the t-butanol freeze drying method. The dried specimens were coated with gold and examined at 15kv in a Hitachi S-800 scanning electron microscope (Hitachi, Tokyo).

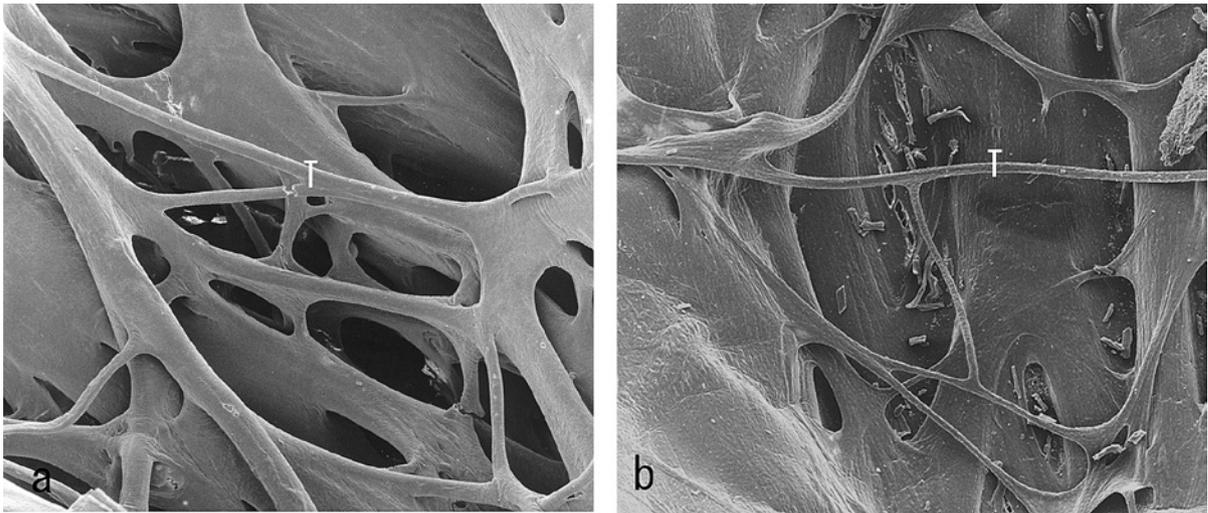


Fig. 1. SEM images showing the luminal surface of the left ventricle in human (a) and rat (b) hearts. Muscular trabeculae (T) of various sizes form a reticular network. a: $\times 20$, b: $\times 40$

Results

General survey of Purkinje fibers

From macroscopic observations, the luminal surface of the right ventricle appeared relatively smooth in all species examined, but the lower half of the left ventricle had a network of muscular trabeculae in humans, monkeys, and rats. The size of the mesh ranged from 1 mm to 10 mm. These networks were also observed three-dimensionally under low magnification by SEM (Fig. 1).

In the surface view of the sheep ventricles stained by the modified PAS method, a delicate network of subendocardial Purkinje fibers was clearly visualized under a stereoscopic microscope (Fig. 2a, b). However, the Purkinje fiber network in other animals—including humans—was not clearly observed with this method. When the thick subendocardial connective tissue of the whale ventricle was digested with the NaOH treatment, the Purkinje fiber network became visible (Fig. 2c).

In sections stained with hematoxylin and eosin, Purkinje cells were found to be distributed not only in the subendocardial layer but also within the muscular trabeculae. However, the morphological feature of the cells greatly varied depending on the animal species: Purkinje cells in both the ungulates (sheep and goats) and the cetaceans (whales and dolphins) were clearly identified by their light cell bodies with one or two nuclei and a few peripherally-placed myofibrils. They were

found not only in the subendocardium but also within the myocardium. In contrast, human Purkinje cells showed a light appearance and had relatively scarce myofibrils as compared with working cardiac myocytes. It was somewhat difficult to identify Purkinje cells in monkeys, dogs, seals and rats clearly.

In silver-impregnated sections, reticular fibers were stained deep black while collagen fibers were brownish. In all animals, each working cardiac myocyte was surrounded by a reticular fiber sheath (Fig. 3). In contrast, the reticular fiber sheaths in the Purkinje fibers varied in configuration by species. In sheep, goats, dolphins, and whales, the sheath surrounded Purkinje strands consisting of bundles of 2–8 oval cells (Fig. 3a, b) whereas the sheath surrounded individual Purkinje cells in other animals, including humans (Fig. 3c, d). In addition, connective tissue elements such as collagen fibers were present between Purkinje fibers and the working myocardium (Fig. 3a, b, c).

Purkinje cells in sheep and goats were considerably larger in size than ventricular myocytes (Fig. 3a). In addition, the histological appearance of Purkinje cells in whales and dolphins was almost identical to that in sheep (Fig. 3b). Human Purkinje cells were apparently larger in size than working cardiac myocytes (Fig. 3c) while monkey Purkinje cells were smaller in diameter than ventricular myocytes. Purkinje cells in dogs and seals were slightly larger in size as compared with ventricular myocytes. Those in rats were found in the muscular

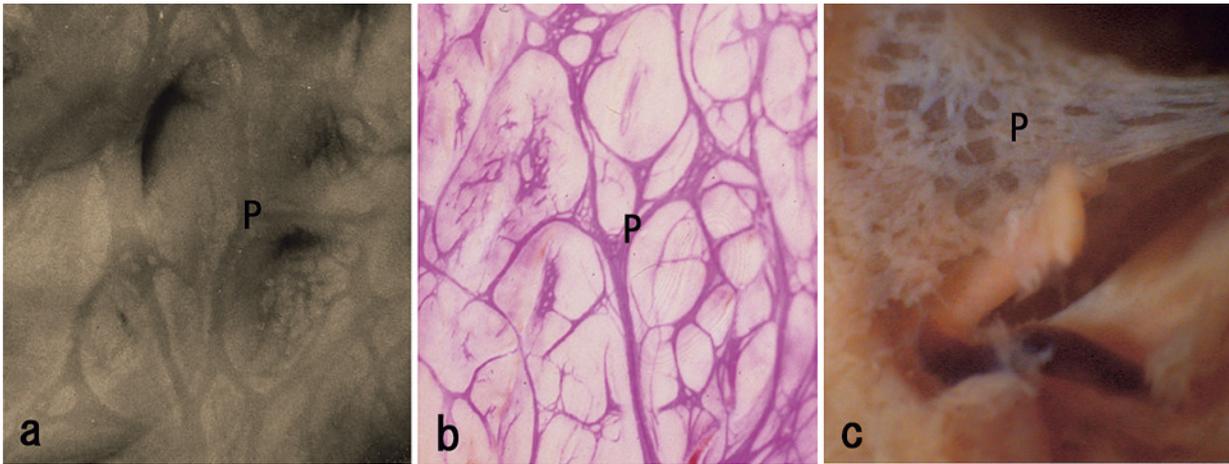


Fig. 2. Stereoscopic micrographs of the Purkinje network (P) of the left ventricle in a sheep (a, b) and fetal whale (c). A delicate network of Purkinje fibers can be seen. a: Non-staining, b: modified PAS reaction, c: NaOH-treatment. $\times 50$

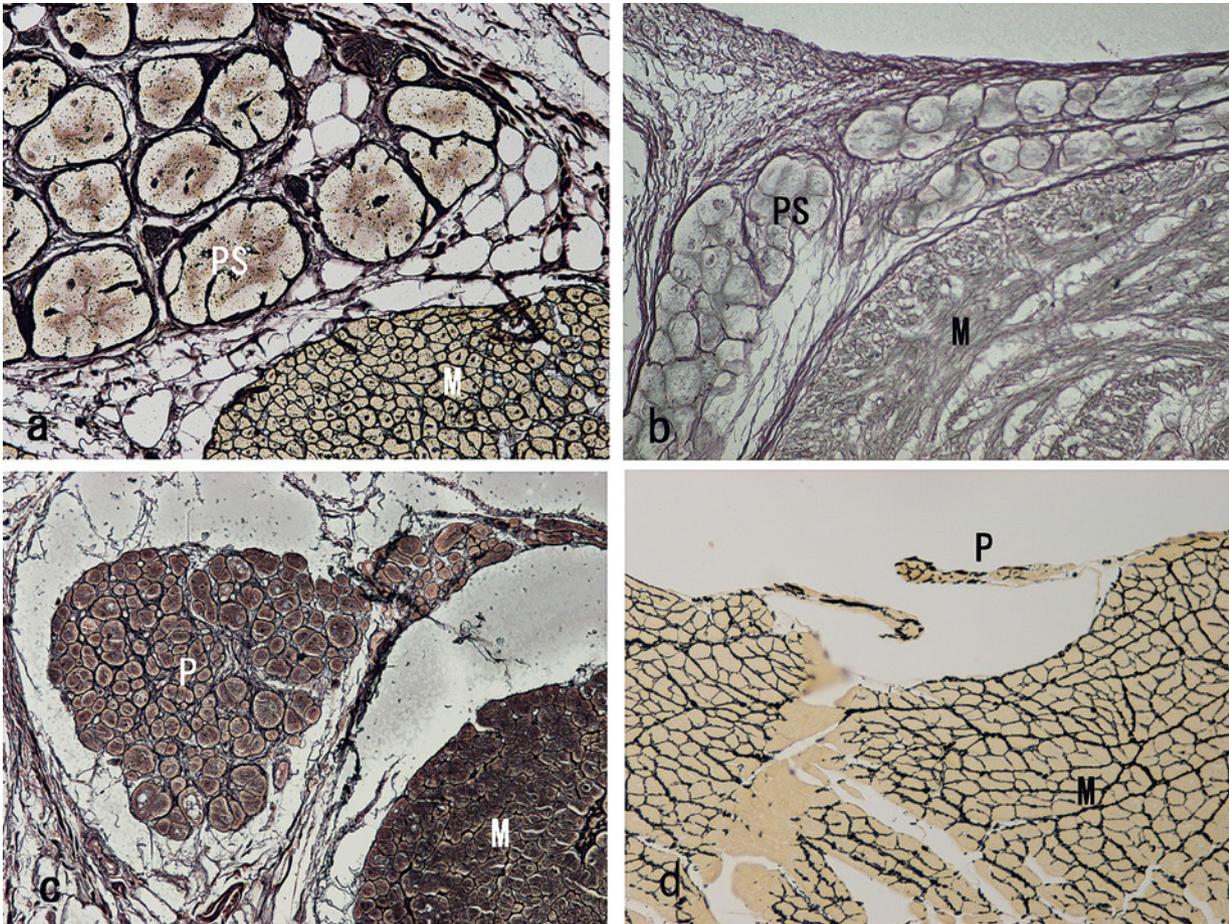


Fig. 3. Light micrographs of the ventricular endocardial region impregnated with silver. In the sheep (a) and whale (b) heart, Purkinje strands (PS) consisting of 2–8 cells are surrounded by the reticular fiber sheath whereas each Purkinje cell (P) in the human (c) and rat (d) is surrounded by the sheath. Individual ventricular myocytes (M) in all mammals are surrounded by the sheath. $\times 130$

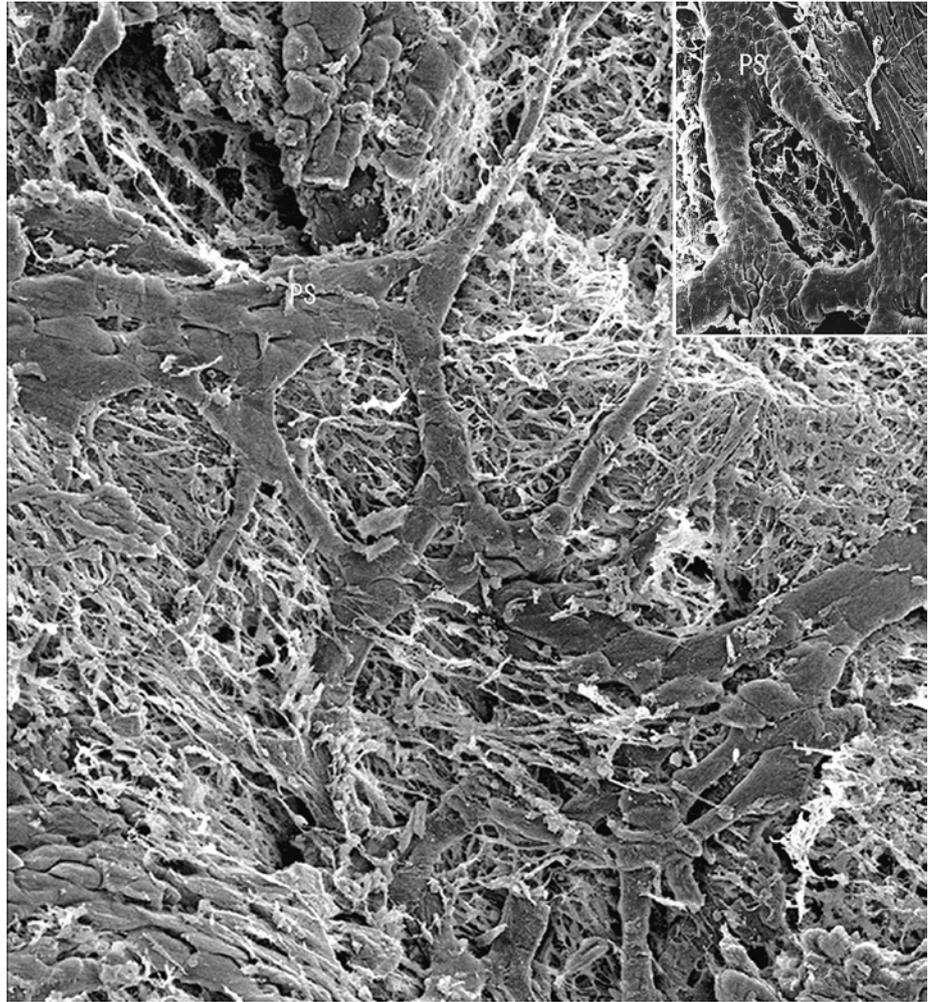


Fig. 4. SEM image showing the luminal surface of the left ventricle of the whale heart. The NaOH treatment enabled us to view the Purkinje network. $\times 260$. **Inset** shows a Purkinje network in the sheep heart. The Purkinje strands (PS) consisting of some oval cells form the network. $\times 110$

trabeculae and were apparently smaller in size than ventricular myocytes (Fig. 3d).

Three-dimensional architecture of the Purkinje network

It is difficult to observe Purkinje fibers by conventional SEM because the fibers are distributed in the subendocardial region and enveloped by the connective tissue sheath. Thus, in this study, the endocardial endothelium was removed by scraping it mechanically with the finger tip or by treating it chemically with NaOH followed by ultrasonication. The subendocardial connective tissue and the reticular fiber sheath were also digested by the NaOH treatment. The immersion of cardiac blocks in 6N NaOH solution for 10–15 min

at 60°C was effective for the removal of the connective tissue elements, thus enabling the three-dimensional visualization of the Purkinje fiber network by SEM (Fig. 4–6). However, the myocardium was not clearly observed since the connective tissue between Purkinje fibers and the myocardium was not digested. An additional immersion in 6N NaOH solution was sometimes necessary to expose both Purkinje fibers and ventricular myocytes by SEM (Fig. 7, 8).

The configuration of the Purkinje network slightly varied depending on the animal species. In goats and sheep, Purkinje strands consisting of 2–8 oval cells formed a reticular network (see also papers by Shimada *et al.*, 1983, 1986). The mesh of Purkinje fiber networks was relatively large in size, ranging from 200 μm to 1500 μm .

Whales also had Purkinje strands consisting of 2–8



Fig. 5. Purkinje network (P) and muscular trabeculae (T) in the human heart. Purkinje cells running in parallel within the trabeculae are continuous with a delicate network of polygonal or stellate cells. $\times 120$

oval cells forming a delicate network (Fig. 4), as also shown in a stereoscopic micrograph (Fig. 2c). The size of the mesh ranged between 20 and 80 μm . The Purkinje fiber network in the dolphin was almost the same as that of the whales when viewed by SEM. Thus, the Purkinje fiber network in the cetaceans was ultimately similar in configuration to that in the ungulates.

Low-power SEM images showed a network of muscular trabeculae in the human left ventricle (Fig. 1a). In NaOH treated specimens, the muscular trabeculae consisted of bundles of 4–8 cylindrical cells, which ran parallel to each other (Fig. 5). These trabeculae were continuous with a fine, complicated network of Purkinje cells (Fig. 5). The mesh size of the Purkinje network varied considerably, ranging from 20 to 300 μm .

In dogs, muscular strands consisting of 4–8 cylindrical

cells formed a rough network, whose meshes ranged in size from 200 to 500 μm (Fig. 6). These strands were continuous with a fine, complicated network of Purkinje cells (Fig. 6) similar to the human Purkinje network (Fig. 5).

Cytoarchitecture of Purkinje networks

In the present study, the treatment of the cardiac tissue with 6N NaOH for the 20 min was used for the direct visualization of both Purkinje cells and working cardiac myocytes by SEM because connective tissue elements such as collagen and reticular fibers could almost be digested with this treatment. In these specimens, there was no essential difference in the cytoarchitecture of the ventricular myocardium between species: the ventricular

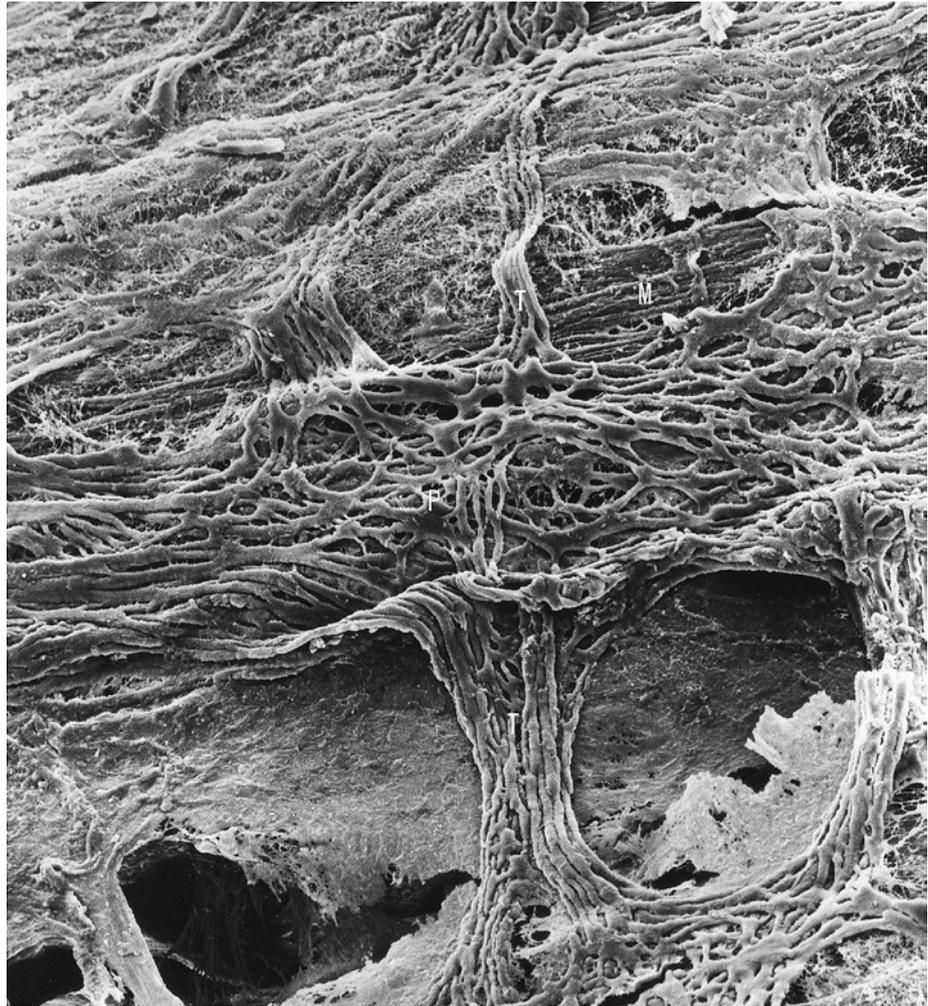


Fig. 6. Purkinje network (P) and muscular trabeculae (T) in the dog heart. The Purkinje network shows a considerably complicated cytoarchitecture while Purkinje cells in trabeculae run in a straight line. $\times 120$

myocytes were generally cylindrical in form and arranged in parallel, but often bifurcated and connected with adjacent cells to make a complex architecture (Fig. 7a, c, e, f, 8b). The myocytes were typically connected with one another in end-to-end fashion at the intercalated disks although they also occasionally made side-to-side connections along the lateral surface in rats (Fig. 8b).

In contrast, the Purkinje fiber network considerably varied in cytoarchitecture depending on the animal species. Purkinje cells in the whales and dolphins were fundamentally similar in cytoarchitecture to those in sheep and goats. These cells were oval or spherical and connected with adjacent cells with both end-to-end and side-to-side contacts (Fig. 4, 7a, b), thus forming Purkinje strands composed of 2–8 cells. The strands had large diameters ($30\ \mu\text{m}$ or more) and anastomosed in the

Purkinje fiber network.

Purkinje cells running within muscular trabeculae in humans and monkeys were generally cylindrical in form and arranged in parallel by making end-to-end connections (Fig. 5, 7d). However, some Purkinje cells showed a polygonal or stellate form in the Purkinje fiber network (Fig. 5) and at the Purkinje cell-ventricular myocyte junctional region (P-M junction) (Fig. 7c). The human Purkinje cells were larger in size (about $20\text{--}40\ \mu\text{m}$) than ventricular myocytes (about $10\ \mu\text{m}$).

In dogs, the cells in the Purkinje fiber network were usually cylindrical in form, but assumed a polygonal or stellate form at the bifurcations (Fig. 6, 7e). The cytoarchitecture of the network in dogs was fundamentally similar to that in humans. In seals, Purkinje cells were also larger in size than ventricular myocytes

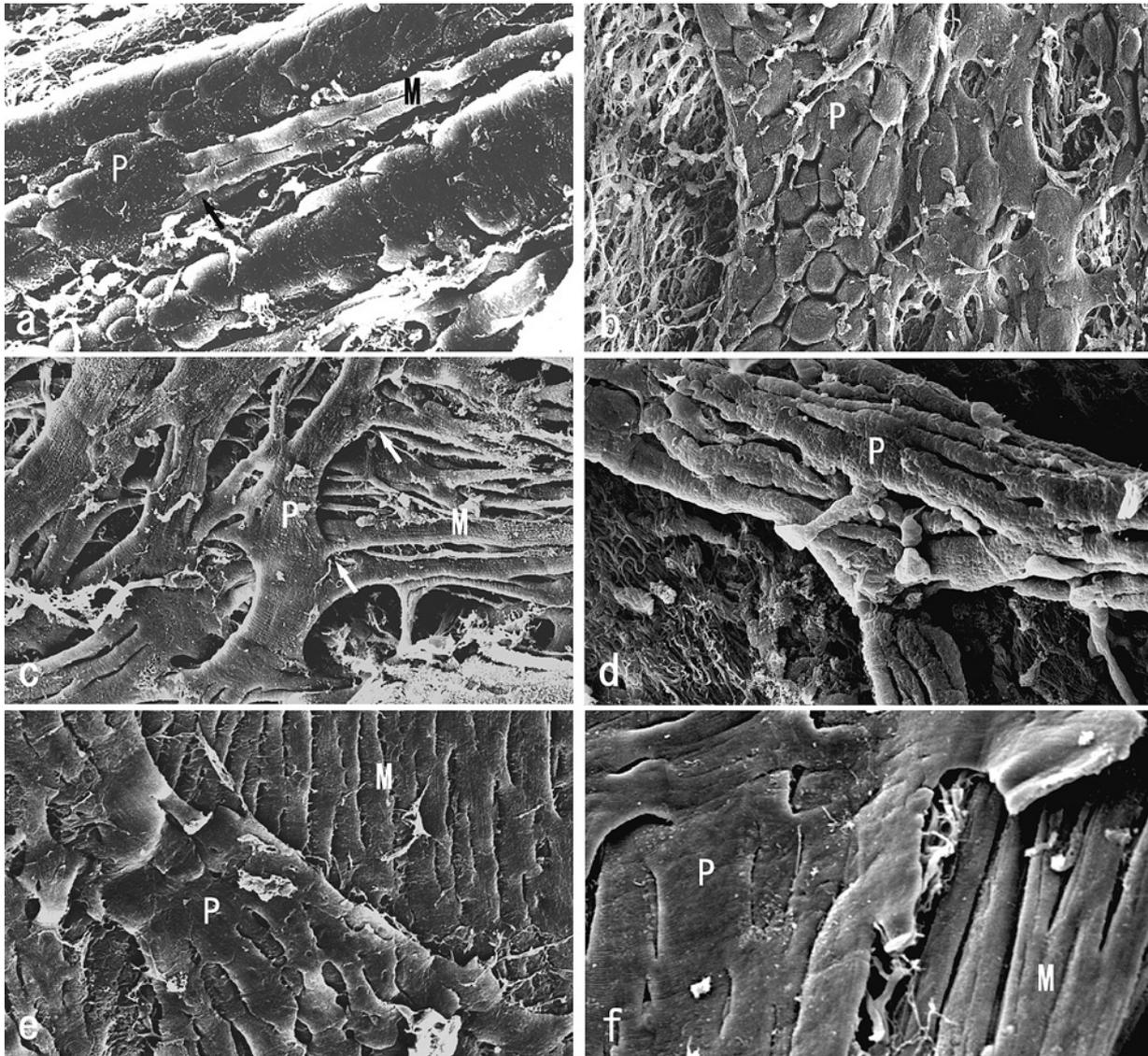


Fig. 7. Surface views of Purkinje cells (P) and ventricular myocytes (M). Purkinje cells in the sheep (a) and whale (b) are oval and considerably larger in diameter than ventricular myocytes. Purkinje cells in the human (c), monkey (d), dog (e) and seal (f) are usually cylindrical and are connected end-to-end with each other. At the human P-M junctional region (arrows), Purkinje cells are polygonal (c) and become larger. a: $\times 380$, b: $\times 300$, c: $\times 300$, d: $\times 900$, e: $\times 300$, f: $\times 380$

(Fig. 7f) and were usually cylindrical in form.

In rat ventricles, Purkinje cells had a cylindrical form, ran in parallel within muscular trabeculae (Fig. 8a), and were connected with ventricular myocytes in the endocardium (Fig. 8b). The rat Purkinje cells were

considerably smaller in diameter (about 8–10 μm) than ventricular myocytes (about 18–20 μm).

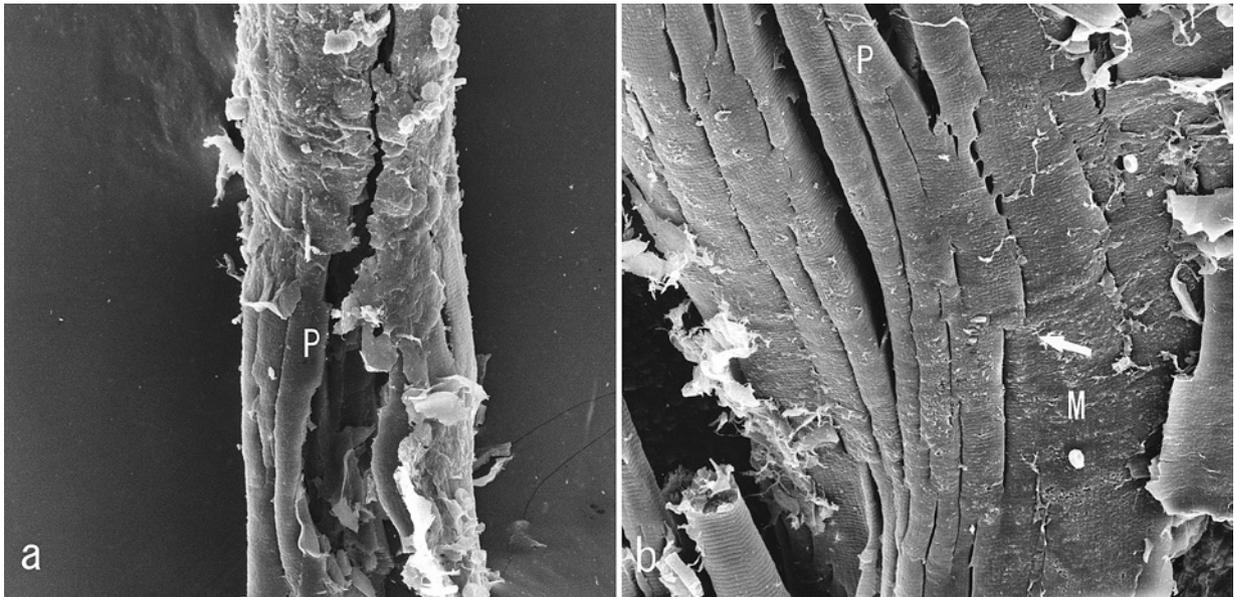


Fig. 8. Purkinje cells (P) and ventricular myocytes (M) of the rat heart. Purkinje cells are smaller in size than ventricular myocytes. **a:** Muscular trabeculae. **b:** Endocardial region. $\times 500$

Discussion

The Purkinje network, distributed in the subendocardial region in ventricles, conducts excitation from the right and left bundle branches to the ventricular myocardium. The present study revealed that the Purkinje fiber network in all the animal species examined had certain common features regarding the configuration and cytoarchitecture. On the other hand, we also showed that the Purkinje fiber network does vary in cytoarchitecture between species and can be divided into three groups, as introduced by Canale *et al.* (1986).

The representative of group I is the Purkinje fiber network in the ungulates. In the present study, it was only in ungulates (sheep and goats) that strands of 2–8 oval cells formed the Purkinje fiber network with relatively large meshes like a fish-net, as also shown in previous papers (Shimada *et al.*, 1983, 1986; Canale *et al.*, 1986). The Purkinje cells were much larger in size than ventricular myocytes, oval and connected with each other with end-to-end or side-to-side contacts. Electrophysiologically, it has been established that the conduction velocity is proportional to the size of cardiac muscle cells and their gap junctions. Because the Purkinje fibers in the sheep and bovine heart possess desmosomes and large gap junctions (Canale *et al.*, 1986;

Shimada *et al.*, 1986; Sugi and Hirakow, 1986), the Purkinje fiber network in ungulates is morphologically considered to have fast conduction properties. In addition, Purkinje strands consisting of some oval cells were encircled by a reticular fiber sheath which was thicker and denser than that surrounding each ventricular myocyte (Morita *et al.*, 1991). Thus, the cytoarchitecture of the Purkinje strands in ungulates may play an important role in the maintenance of the rapid and stable propagation of impulses, in companion association with the thick reticular fiber sheath.

For the Purkinje fiber network in group II, this is the first report that visualized a three-dimensional network of Purkinje cells in humans and dogs by SEM. Unlike sheep Purkinje cells, the cells in humans and dogs were usually cylindrical or fusiform in shape, ran in parallel, and made end-to-end contacts at the intercalated disks. These findings imply that the longitudinal propagation is the main route in humans and dogs. The human Purkinje cells were larger in size than ventricular myocytes and belong to fast (2–4 m/sec) cardiac fibers (Cranefield *et al.*, 1972). We also showed in humans and dogs that Purkinje cells with polygonal and stellate shapes formed a delicate and complicated network at the bifurcations, suggesting that the propagation of impulses appears to be successfully controlled at these sites of the Purkinje network.

In group III comprising rats and mice, Purkinje cells were very similar in appearance to ventricular myocytes (Canale *et al.*, 1986). Purkinje cells in the present study were considerably smaller in size than ventricular myocytes. We also showed the presence of large and small networks of muscular trabeculae in rat ventricles; Purkinje cells running within these trabeculae were cylindrical in shape, arranged in parallel, and were continuous with ventricular myocytes in the endocardium. These findings are in accord with the fact that the heart rate in rats and mice is very rapid, being above 250 and 700 beats per minute, respectively (Sawazaki, 1985; Noujaim *et al.*, 2004).

The present study also demonstrated that the Purkinje network in whales is fundamentally similar to that in the sheep, indicating that the cetaceans belong to group I. On the other hand, the network in seals resembled that in dogs or group II. Phylogenetically, cetaceans are closely related to ungulates, while pinnipeds such as seals are closely related to carnivores (Gibson *et al.*, 2005).

In conclusion, the present study precisely revealed the cytoarchitecture of the Purkinje fiber network in the heart of different animal species, especially by SEM. We demonstrated that the cytoarchitecture of the Purkinje fiber network can be divided into three groups—as introduced previously—and also showed that the morphological variations in each group may reflect the conducting function of the network as well as be concerned with phylogeny. On the other hand, the variations may also be related to the size of the heart. In the largest mammalian hearts in cetaceans and ungulates (group I), not only the subendocardial but also the intramural Purkinje networks participate in efficient expansion of the excitation throughout the ventricular wall (Ryu *et al.*, 2009). Accordingly, it is reasonable to consider that the subendocardial Purkinje fiber networks have relatively larger meshes in these animals. The relatively larger hearts in humans and dogs (group II) had abundant muscular trabeculae, including Purkinje cells. In addition, a fine and delicated Purkinje network may contribute to the rapid conduction of excitation to the subendocardial myocardium.

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