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ORIGINAL PAPER

Immuno-histochemistry and three-dimensional architecture of the intermediate filaments in Purkinje cells in mammalian hearts

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Abstract In mammalian hearts, Purkinje cells varied greatly in morphological appearance in different species, and were divided into three groups. Bovine Purkinje cells corresponding to group I were a large size, and had a few myofibrils and abundant intermediate filaments throughout the cytoplasm. The aim of the present study was to clarify the more detailed distribution and three-dimensional architecture of intermediate filaments in Purkinje cells. The hearts in various mammals including humans were investigated by both immuno-histochemistry and scanning electron microscopy (SEM).Immuno-histochemical studies demonstrated that sheep Purkinje cells in group I had a great number of intermediate filaments of 10 nm positive for desmin antibody. Purkinje cells in group II (humans, monkeys and dogs) and group III (mice) were somewhat larger or smaller in size than myocardial cells, but also showed a strong positive reaction for desmin antibody. The saponin or NaOH treatment of cardiac tissues in sheep and humans enabled us to view intermediate filaments by SEM three-dimensionally. Intermediate filaments in sheep Purkinje cells formed a considerably delicate network, and were distributed throughout the cytoplasm. In contrast, those in human Purkinje cells were lower in density, and were present around the nucleus and between myofibrils. It was concluded that a delicate network of intermediate filaments in Purkinje cells of mammalian hearts acted as the cytoskeleton to maintain intercellular stability.

Keywords Intermediate filaments · Desmin antibody · Purkinje cells · Mammalian heart · Imuno-histochemistry · Electron microscopy · Cytoskeleton · Desmin

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Introduction

The attention has been recently directed to the anatomy of cardiac conduction system and Purkinje fibers [1, 2]. Pak et al. [3] found experimentally that effects of catheter ablation were different between dogs and swine, and concluded that this was due to some factors including the distribution and structure of Purkinje fibers. It is now an accepted fact that the Purkinje fibers varied greatly in cell size and structure in different species [4–6]. Previously, we demonstrated that the cytoarchitecture of the Purkinje fiber network in humans resembled to that in dogs, but showed considerable differences from the sheep or mouse Purkinje network [7].

Based on transmission electron microscopic (TEM) findings, Purkinje cells in mammalian hearts were divided into three groups [6, 7]. First group Purkinje cells seen in ungulates (sheep, goats, bovines, cows and horses) were considerably larger in diameter than

working cardiac myocytes, and were easily identified by the presence of a few myofibrils and a great number of glycogen particle. Second group Purkinje cells seen in humans and dogs were somewhat larger, and third group Purkinje cells seen in rodents (rats and mice) were smaller. It was not always easy to identify Purkinje cells in both second and third groups, since they were also somewhat similar in appearance to the working myocardium. On the other hand, immuno-histochemical studies have demonstrated that Purkinje cells in the bovine heart possessed a great number of intermediate filaments throughout the cytoplasm [8, 9]. However, it remains to be unclear whether or not Purkinje cells in the second and third groups have abundant intermediate filaments. In general, intermediate filaments in Purkinje cells of the ungulates were not visible by means of TEM and SEM because of the presence of a great number of glycogen particles. Accordingly, there seem to be no publications describing the more detailed distribution and three-dimensional architecture of intermediate filaments in Purkinje cells. Recently, the cytoskeletal filaments of 10 nm in Bachmann bundle cells have been observed by utilizing the NaOH maceration method under SEM [10].

In this study, morphological properties of intermediate filaments of Purkinje cells in mammalian hearts are clarified by utilizing immuno-histochemical and SEM techniques, to identify definitely Purkinje cells and to provide fundamental morphological findings for studies of the cardiac rhythm.

Materials and methods

All experimental procedures were in accordance with the guidelines for Animal Experimentation of Oita University, Japan, for the care and use of laboratory animals. Human hearts were taken in part by autopsy from four adult subjects (aged from 32 to 55 years old) without any histories of heart disease. Hearts of group I (5 sheep), group II (4 humans, 3 monkeys, 3 dogs) and group III (3 mice) were used as materials. The endocardial regions containing Purkinje fibers were removed from the right and left ventricles, and subjected to the procedure indicated below.

Light microscopic examinations

Specimens were cut into 5–10 mm square, fixed in 10 % formalin or 4 % paraformaldehyde in a phosphate buffer (pH 7.4), dehydrated, and embedded in paraffin. 7 μm sections were stained with routine hematoxylin and eosin. They were also immuno-stained with rabbit anti-desmin antibody (DAKO Corporation), immersed in rabbit IgG gold (5 nm) solution (Amersham Pharmacia Biotech, UK), physically developed [11], and observed under a light microscope (Nikon, Tokyo).

Electron microscopic examinations

For immuno-histochemistry, small tissue blocks including Purkinje cells in the sheep heart were fixed in periodate-lysine-paraformaldehyde (PLP) fixative, dehydrated and embedded in London resin (LR-white). Ultrathin sections were immuno-stained by the IgG gold-silver method using anti-desmin antibody, stained with only uranyl acetate and examined in a JEOL 100CX.

To visualize intermediate filaments by SEM, the sheep false tendon fixed in 1/4 Karnovsky's fixative was immersed in 40 %DMSO solution, freeze-cracked, and treated with 0.3 % saponin solution for 3 h at 37 °C [12]. On the other hand, remaining paraffin blocks of human hearts for light microscopy were also used for SEM. They were deparaffinized with xylene, hydrated, and fixed again in Karnovsky's fixative, and immersed in 2 N NaOH at 37 °C for 3 h to expose myofibrils and intermediate filaments [11]. All specimens were placed in 1 % osmium tetroxide, 1 % tannic acid and 1 % osmium tetroxide for 1 h each, dehydrated in ethanol of ascending concentrations and then dried by the t-butyl alcohol freeze drying method. The specimens were coated with gold, and examined at 25 or 15 kV in a HFS-II or Hitachi S-800 scanning electron microscope (Hitachi, Tokyo).

Results

General survey of Purkinje cells

In sections stained with hematoxylin and eosin, ventricular myocardial cells had abundant myofibrils with intense eosinophilia. Purkinje cells were usually found to be distributed in the subendocardium of ventricles. Purkinje cells in group I (Sheep) formed strands consisting of bundles of several oval cells, which were considerably larger in diameter (40–60 μm) than working cardiac myocytes and had a few peripherally-distributed myofibrils (Fig. 1a). Thus, sheep Purkinje cells were easily identified. However, it was not always easy to identify Purkinje cells in group I and II, because they were also similar in appearance. In this paper, Purkinje cells showing a clearly distinguishable profile were displayed. Purkinje cells in group II (humans) were somewhat larger in diameter (about 20 μm) than myocardial cells, and showed a light appearance due to the small number of myofibrils (Fig. 1b). Purkinje cells in group III (mice)

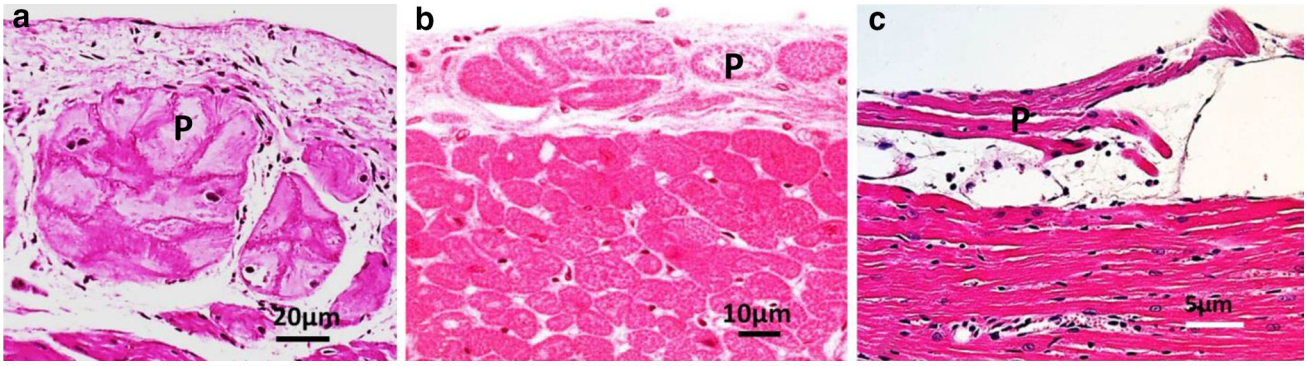

Fig. 1 Light micrographs of Purkinje cells (P) situated in the subendocardium in the sheep (**a**), human (**b**) and mouse (**c**) hearts. Sheep Purkinje cells are considerably large in size and have a few peripherally-distributed myofibrils. In contrast, human Purkinje cells are somewhat larger, showing a little light appearance as compared with ventricular myocardial cells. Mouse Purkinje cells with a small size run within muscular trabeculae

were smallest among three groups (about 12 μm), showing a somewhat reduced eosinophilia (Fig. 1c).

Immuno-histochemistry for anti-desmin antibody

In light microscopic observations examined by the immunogold-silver method, sheep Purkinje cells (group I) showed a stronger positive reaction for anti-desmin antibody as compared with the working myocardium (Fig. 2a). A stronger positive reaction in Purkinje cells appeared as a clump of precipitate, while a weak positive reaction in the working myocardium was found in close proximity of Z bands and intercalated disks (not demonstrated). As well as a large cell type of Purkinje cells in sheep hearts, Purkinje cells in group II such as human, monkey and dog hearts also exhibited a stronger positive reaction as compared with myocardial cells (Fig. 2b, c, d, e). A surprising observation was that a smaller cell type of mouse Purkinje cells (group III) also showed a strong positive reaction (Fig. 2f). In addition, Purkinje cells showing a strong positive reaction were found to run in the muscular trabeculae of the left ventricle in human and mouse hearts (Fig. 2c, f). Thus, all three groups of Purkinje cells examined by us showed a strong positive reaction for desmin antibody. The intermediate filaments positive for anti-desmin antibody appeared to be distributed throughout the cytoplasm in Purkinje cells.

Since PLP used in the present TEM study was a weak fixative, glycogen particles disappeared, and intermediate filaments with diameter of 10 nm in sheep Purkinje cells were clearly observable at the TEM level. Consequently, silver particles showing a positive reaction for desmin antibody were detected on intermediate filaments (Fig. 3a). The intermediate filaments were seen not only in areas rich in glycogen, but also near desmosomes of the intercellular junction (Fig. 3a, b). They appeared to form delicate networks with spaces of 100–220 nm.

Scanning electron microscopic findings

Scanning electron microscopic observations of the DMSO fractured surface of the sheep heart failed to view intermediate filaments in Purkinje cells (Fig. 4a). In this study, the treatment of freeze-fractured cardiac tissue with saponin resulted in removal of not only intracellular matrix, but also glycogen particles, and enabled us to view intermediate filaments in addition to myofibrils and mitochondria in sheep Purkinje cells three-dimensionally by SEM (Fig. 4b). Myofibrils were slender, ranged from 1 to 2 μm and mainly distributed peripherally (Fig. 5). Mitochondria, which were oval in shape and 1.0–1.5 μm in diameter, were scattered in the cytoplasm (Fig. 5). A considerably compact network of intermediate filaments with a very complicated pattern was distributed throughout the cytoplasm (Figs. 5, 6). They entirely encircled nucleus, myofibrils and mitochondria (Figs. 4, 5). The spaces of networks ranged in diameter from 0.1 to 0.5 μm.

On the other hand, SEM images of human ventricles treated with 2 N NaOH clearly illustrated myofibrils, mitochondria and intermediate filaments in Purkinje cells three-dimensionally (Figs. 6, 7).Myofibrils, which were slender and ranged from 0.5 to 1.5 μm, were quite richer than those of sheep Purkinje cells (Fig. 7). Especially, a network of intermediate filaments was abundantly distributed in the central region around the nucleus (Fig. 6). They lay in close contact with mitochondria and myofibrils, and were also present between myofibrils (Fig. 7). In addition, remaining glycogen particles in the central glycogen are attached to intermediate filaments (Fig. 6). The density of the intermediate filaments was relatively loose, and the spaces ranged in diameter from 0.3 to 1.0 μm. The present SEM study illustrated that the density of intermediate filaments in sheep Purkinje cells was greater than that of human Purkinje cells.

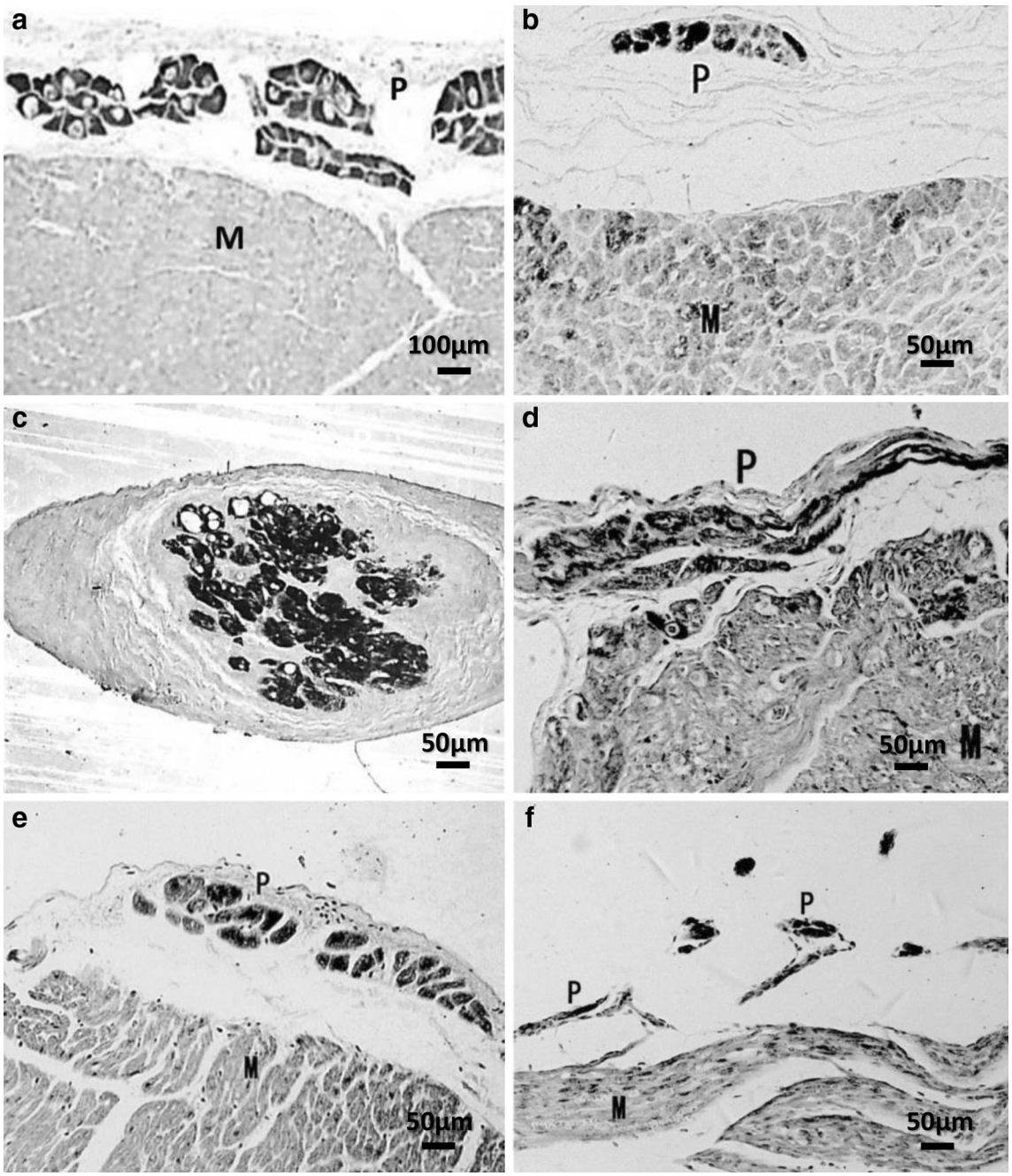

Fig. 2 Immuno-histochemical images of mammalian hearts by means of the immunogold-silver method. Sheep Purkinje cells (P) show a strongest positive reaction for anti-desmin antibody as compared with ventricular myocardial cells (M) (**a**). All Purkinje cells (P) seen in the human (**b**, **c**), monkey (**d**) and dog (**e**) mouse (**f**) hearts are also positive. Purkinje cells in humans (**c**) and mice (**f**) are also distributed in muscular trabeculae

Discussion

Purkinje cells in mammalian hearts have been ultrastructurally classified into three groups as proposed by Canale et al. [6]. First group Purkinje cells seen in the ungulates such as sheep, goats and horses were oval in shape and are much larger in diameter than working cardiac myocytes. They were easily identified by the presence of a few peripherally-placed myofibrils, large central areas of glycogen particles and one or two nuclei. Second group Purkinje cells seen in humans, monkeys and dogs were less well different from working myocardial cells. They were fundamentally cylindrical in shape, somewhat larger in diameter and had somewhat less myofibrils and more

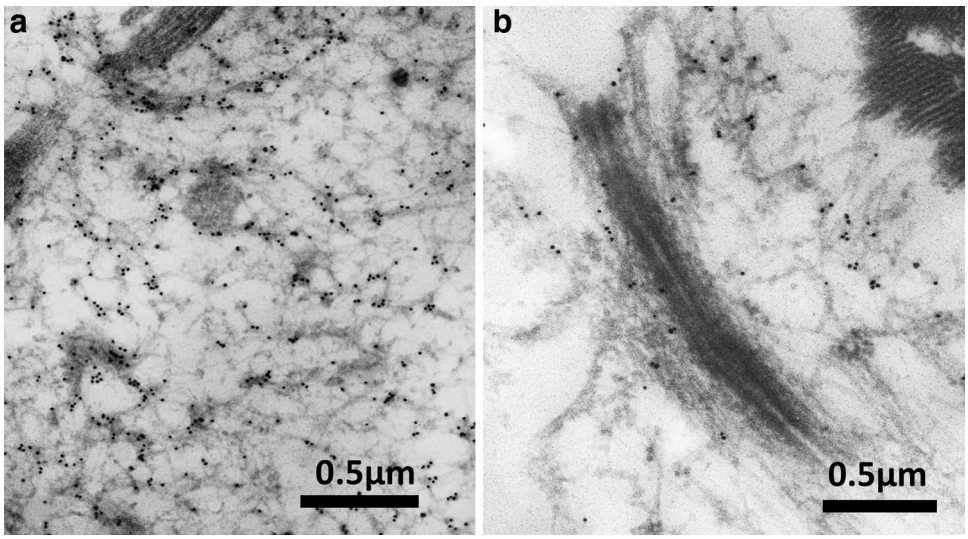

Fig. 3 TEM images of sheep Purkinje cells immuno-stained with desmin antibody. Silver particles showing a positive reaction are associated with intermediate filaments (IF) (**a**). The intermediate filaments are also in close contact with desmosomes (D) (**b**)

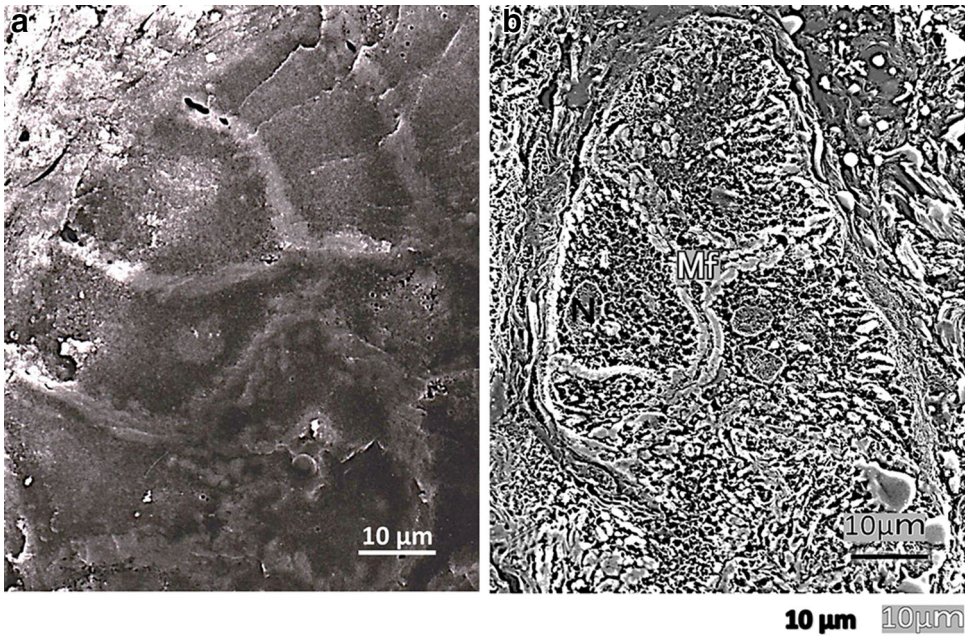

Fig. 4 SEM image of the fractured surface of sheep Purkinje cells. In untreated tissues (**a**), only myofibrils are identified in the periphery of the cytoplasm. In saponin-treated tissues (**b**), Purkinje cells can be clearly observed, because intracellular ground substance and glycogen particles have been digested. The intermediate filaments (IF) are distributed throughout the cytoplasm. *Mf* myofibril, *N* nucleus

abundant glycogen particles. Third group Purkinje cells in rats and mice were smaller in diameter than working cardiac myocytes, and were identified by the presence of somewhat less myofibrils and abundant glycogen particles. It may be suggested that morphological differences of Purkinje cells were closely related with species as well as animal sizes.

Physiologically, the conduction velocity of Purkinje cells was 1.7–1.8 m/s [13] compared to 0.4–0.9 m/s in working myocardium. In general, the conduction velocity was proportional to cell diameter as well as size and number of gap junctions [14, 15]. In addition, goat Purkinje fibers had large gap junctions with a considerably large size [16]. It is likely that first group Purkinje cells with a large size (ungulates) are fastest in conduction velocity among three groups.

In the working myocardium, it has been established that intermediate filaments, 10 nm in diameter, function as the intracellular cytoskeleton [8, 9]. TEM observations have illustrated that intermediate filaments are closely associated with Z bands, desmosomes and sarcolemma [6]. On the other hand, immuno-histochemical and TEM studies in the bovine heart have demonstrated that Purkinje cells show a stronger positive reaction for desmin antibody as compared with the working myocardium and that they are distributed throughout the cytoplasm [8, 9]. The present immuno-histochemical studies illustrated that sheep Purkinje cells also possessed a great number of intermediate filaments

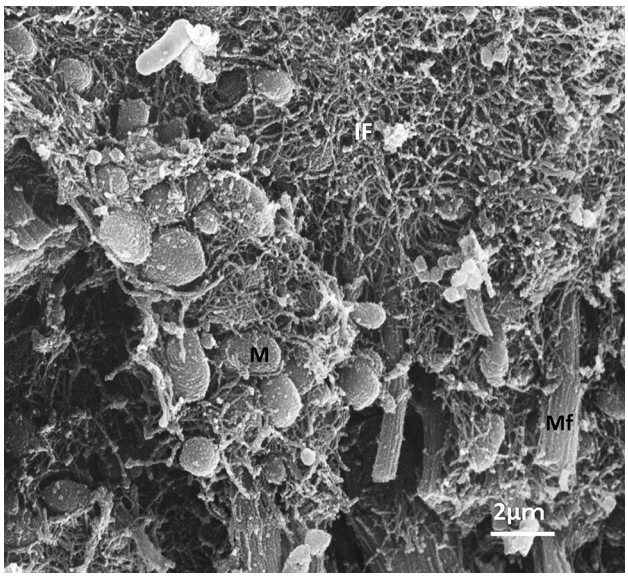

Fig. 5 Intermediate filaments (IF) seen in the large central area of sheep Purkinje cells. They form a greatly-delicate network, and also enmesh myofibrils (Mf) and mitochondria (M)

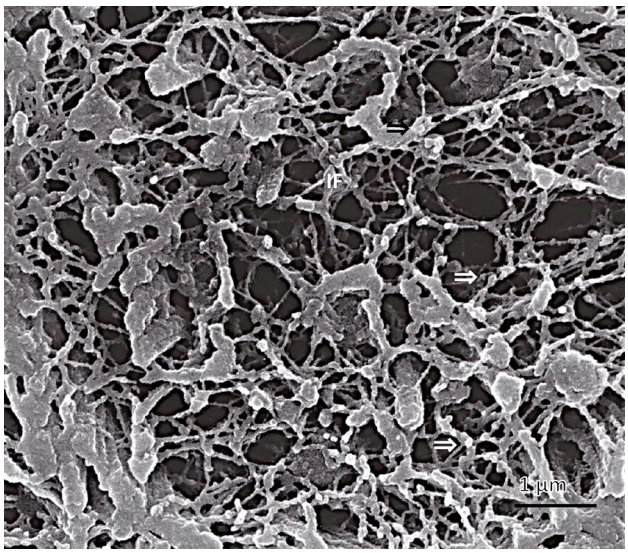

Fig. 6 SEM image of human Purkinje cells treated with NaOH. There are abundant intermediate filaments (IF) with a delicate network in the central region around the nucleus. The remaining glycogen particles (*arrows*) are attached to the filaments

showing a strong positive reaction for desmin antibody. In general, these filaments were especially abundant in Purkinje cells of birds and larger mammals [6]. In the present SEM study, Purkinje cells in the first group such as sheep had a considerably compact network of intermediate filaments throughout the cytoplasm. This suggests that the intermediate filaments functioning as a cytoskeleton are indispensable elements to maintain the structural integrity

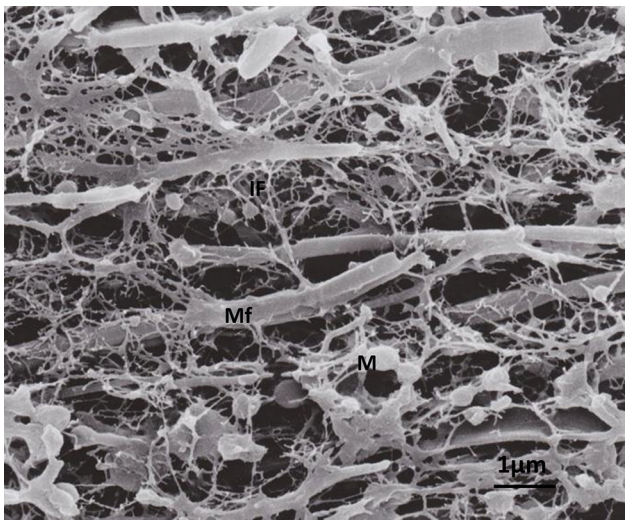

Fig. 7 The intermediate filaments (IF) in human Purkinje cells. A delicate network of the intermediate filaments is also distributed between myofibrils (Mf) as well as around myofibrils. *M* mitochondria

of Purkinje cells, because the cells were considerably large in size, and had peripherally-distributed myofibrils. What appearance does intermediate filaments in Purkinje cells of the second and third groups show? Purkinje cells were somewhat larger or smaller in size than myocardial cells. In this study, an immuno-histochemical reaction of Purkinje cells for desmin antibody was clearly stronger as compared with the working myocardium as demonstrated in Fig. 2. Furthermore, the present SEM observation illustrated that a relatively dense network of intermediate filaments in human Purkinje cells was distributed in a large central region around the nucleus, around myofibrils and between myofibrils. These morphological findings may suggest that Purkinje cells of all mammals including human being are tightly supported by the intracellular cytoskeleton such as intermediate filaments.

Although the intermediate filaments are one of the cytoskeleton modulating morphology and function of cells, cytoskeletal linkage to the organelles has not been unequivocally demonstrated. The present SEM study is the first to demonstrate three-dimensional characteristics of intermediate filaments in the Purkinje system. They lay enmeshed around nucleus, mitochondria and myofibrils with a dense and complicated network. According to recent studies, the cytoskeleton could modulate gene phenotype concerning the nucleus and regulate mitochondrial function such as mitochondrial shape, stretching, contraction and permeability of the mitochondrial outer membrane to ADP in myocytes [17]. The present data may also indicate that the intermediate filaments in Purkinje cells are in close relation with functions of the nucleus, mitochondria and myofibrils. Furthermore, glycogen particles in Purkinje

cells appeared to be closely clasped by a network of intermediate filaments in the present SEM images. In the past, there was an interesting report. The glycogen particles in Purkinje cells were less soluble in water than that of the working myocardium [18]. This may be due the fact that glycogen particles are closely associated with a dense network of intermediate filaments.

Conclusion

Purkinje fibers, which were the terminal ramification of the cardiac conduction system, were different in cell sizes, shapes and ultrastructures according to animal species. The present morphological study demonstrated that all three groups of Purkinje cells in mammalian hearts including humans had a great amount of intermediate filaments positive for anti-desmin antibody. It is concluded that the intermediate filaments in Purkinje cells act as the cytoskeleton to maintain the intracellular stability. We believe that the present immuno-histochemical technique using desmin antibody is very useful to identify Purkinje cells in the human, dog and rodent heart.

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