Presence of cardiomyocytes exhibiting Purkinje-type morphology and prominent connexin45 immunoreactivity in the myocardial sleeves of cardiac veins

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BACKGROUND Pulmonary vein (PV) myocardium is a known source of atrial fibrillation. A debated question is whether myocardial extensions into caval veins and coronary sinus (CS) have similar properties. No studies have documented specific pacemaker and/or conducting properties of human extracardiac myocardium.

OBJECTIVE The purpose of this study was to characterize the histology and immunohistochemical features of myocardial sleeves in the wall of cardiac veins.

METHODS Sections of 32 human hearts were investigated. Specimens of PVs, superior caval vein (SVC), CS, sinoatrial and atrioventricular nodes, and left ventricle were stained with Best's Carmine for selective staining of intracellular glycogen. Anti-connexin45 (Cx45)– and Cx43–specific antibodies were used to determine the conduction properties of extracardiac myocardium.

RESULTS Myocardial sleeve was found in the wall of PVs of 15 of 16 hearts, 21 of 22 SVCs, and 8 of 8 CSs. Bundles of glycogen-positive cardiomyocytes exhibiting pale cytoplasm and peripheral myofibrils were observed in the venous sleeves. Strong Cx45 and weak Cx43 labeling was detected in the extracardiac myocardium. Similar staining pattern was observed for the pacemaker and conduction system, whereas ventricular myocardium exhibited prominent Cx43 and no Cx45 immunoreactivity.

CONCLUSION Myocardial fibers of PVs, SVC, and CS exhibit morphology similar to that of Purkinje fibers and are enriched in glycogen. We provide data for the first time on prominent positive staining for Cx45 in the extracardiac myocardium, indicating its potential pacemaker and/or conducting nature.

KEYWORDS Cardiac muscle sleeve; Caval vein; Connexin45; Coronary sinus; Glycogen; Immunohistochemistry; Pulmonary vein; Purkinje-type morphology

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Introduction

Myocardial sleeves of pulmonary veins (PV) play a critical role in the mechanism of atrial fibrillation (AF). Macroscopic features of these areas were described previously.[1–3] During the last decade, growing attention has prompted investigation into the microscopic properties of the extracardiac myocardial sleeves.[2,4–6] Perez-Lugones et al[7] documented the presence of cardiomyocytes exhibiting ultrastructural morphology of P-cells and Purkinje fibers (PFs) in the wall of human PVs. Although it is accepted that atrial tachyarrhythmias are frequently triggered from caval veins and coronary sinus (CS),[8–12] limited data have been published about the macroscopic[3] and microscopic morphology[13,14] of the myocardial sleeves of these regions. Moreover, there is a general lack of research on the immunohistochemical characterization of caval and CS myocardial sleeves.

Immunohistochemical markers to distinguish working myocardium and pacemaker or conducting cells have been established. In addition to several determinants of conduction in the heart, such as HCN4 and HNK-1 (CD57), connexin (Cx) isoforms, of which gap junction channels are composed, are also proteins characteristic of cardiac pacemaker tissues. Cx40, Cx43, and Cx45 are found differentially expressed in cardiomyocytes at various sites, which determine the characteristics of conduction velocity.[15] Cx43 is present throughout the working myocardium, whereas Cx40 is confined to the atrial myocardium and the ventricular conduction system. Cx45 is predominantly expressed in the impulse generating

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and conduction system but is present in substantially lower amounts in working myocardium.[16,17]

Methods

Human tissues

Thirty-two adult human hearts were removed from cadavers at 12–72 hours postmortem age that were maintained at 1°–5°C until fixation. The ages of deceased individuals ranged from 60 and 81 years. Their medical histories were unknown. Prior to death, donors had provided written consent for the use of their bodies for education and research (Willed Whole Body Program). The work was approved by the Regional and Institutional Committee of Science and Research Ethics, Semmelweis University (Research Ethics committee approval 122/2016).

Because of technical reasons, the heart could be excised together with PVs in only 16 of 33 subjects and with superior caval vein (SVC) in 22 of 33 subjects. The excision was extended into the lung hilum in the case of PVs, above the level of the azygos vein in the case of SVC, and as far as the orifice of great cardiac vein in the case of CS. The veins were then separated from the atria at the level of their ostia and cut transversely. CSs of 8 of 33 subjects were suitable for further tissue processing. Tissue samples were obtained from the sinoatrial and atrioventricular nodes, the atria, the anterior wall of the left ventricle, and the interventricular septum. Specimens were fixed in either 4% formaldehyde, or in 70% ethanol or methanol. After dehydration in graded concentrations of alcohol, tissue samples were embedded in paraffin, and 3- to 4-μm sections were made.

Tissue processing for histology

For general histology, paraffin sections were stained with hematoxylin and eosin or trichrome. Intracellular glycogen was demonstrated by Best's Carmine stain, which is a stain specific for glycogen content. Best's Carmine staining was performed as described.[18]

Immunohistochemistry

For Cx45 immunohistochemistry, specimens were fixed in ethanol/methanol and embedded in paraffin. After deparaffinization and rehydration through graded alcohols, the slides were washed 3 times in phosphate-buffered saline (PBS). Heat-induced antigen retrieval method was applied using Tris-based (Target Retrieval Solution pH-9; Dako, Santa Clara, California, US) or citrate-based (Sigma, Rocklin, California, US; H-3300) antigen unmasking solution, respectively. For Cx43 immunohistochemistry, frozen sections were prepared. Specimens were embedded in Cryomatrix (Shandon, Thermo Fisher Scientific, Waltham, Massachusetts, US), frozen in liquid nitrogen, and stored in a deep freezer (–80°C). Cryosections 10-μm thick were mounted on poly-L-lysine coated slides, fixed in cold (+4°C) acetone for 10 minutes, and air-dried. Before immunostaining, the slides were rehydrated in PBS and, permeabilization with 0.3% Triton X-100 was performed for 40 minutes.

Cx45 and Cx43 immunostaining were performed as follows. Protein blocking was carried out for 15 minutes with 1% bovine serum albumin in PBS, followed by overnight incubation at 4°C with primary antibodies. Cx45 was detected using a rabbit polyclonal antibody (sc-25716; dilution 1:100; Santa Cruz Biotechnology Inc), and Cx43 was detected using a goat polyclonal antibody (sc-6560; dilution 1:50; Santa Cruz Biotechnology Inc). Secondary antibodies, which included biotinylated goat anti-rabbit immunoglobulin G and biotinylated horse anti-goat immunoglobulin G (Vector Labs) were used, followed by endogenous peroxidase activity quenching step using 3% hydrogen peroxide (Sigma) in PBS. After formation of the avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit; Vector), the binding sites of the primary antibodies were visualized by 4-chloro-1-naphthol (Sigma).

The sections were covered by aqueous Poly/Mount (Polyscience Inc, Warrington, PA) and examined using a Zeiss Axiophot photomicroscope. An automated whole-slide imaging system (3D-Histech, Budapest, Hungary) was used to visualize the sections.

Results

Myocardial sleeve of the PVs

Extensions of left atrial myocardium could be observed in the PVs of 15 of 16 hearts (94%) and formed bundles displaying various courses (Figures 1A and 1B). Bundles of large cardiac cells (median diameter 18.1 μm; interquartile range [IQR] 16.5–19.7 μm) resembling PFs based on their lightly stained cytoplasm and peripheral myofibrils were detected in the PVs of 14 hearts. Among these cardiomyocytes, a dense network of fine collagen bundles was present (Figures 1C and 1D). Best's Carmine staining confirmed that PV myocardium was enriched in cardiomyocytes containing abundant glycogen (Figure 1E). Intense Cx45 labeling was observed in the myocardial sleeve of PVs. The Cxs were clustered in intercalated discs (Figure 1F).

Myocardial sleeve of the SVC

Myocardial sleeve composed of fibers displaying a mainly spiral course was found around 21 of 22 SVCs (95%) (Figures 2A and 2B). In 1 case, some groups of myocardial fibers were present at the root of the azygos vein, but no cardiac cells were found in the portion distal to this point. Bundles of Purkinje-like cardiomyocytes (median diameter 29.4 μm; IQR 27.9–32.5 μm) embedded in connective tissue were identified in 20 SVCs (Figure 2C). Similar to the PVs, abundant intracellular glycogen content was found in SVC myocardium (Figure 2D). Intense Cx45 positivity with a pattern similar to that shown in Figure 1F was observed (Figure 2E). No Cx43 staining in the sinoatrial node, sparse labeling in the vicinity of the sinoatrial node (mixed population of atrial and pacemaker cells), and marked positivity were detected in the atrial working myocardium (data not shown).