Tissue structure and connexin expression of canine pulmonary veins

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Abstract

Objective: Rapid electrical activity in pulmonary veins (PVs) has been proposed as a mechanism for focal atrial fibrillation. The way in which the myocardial sleeve inside PVs can form a substrate for focal activity is not well understood. Therefore, we have studied tissue structure and connexin distribution at the veno-atrial transition in the dog. Methods: In adult mongrel dogs, the anatomy of the PV area was studied. Tissue structure in individual veins was assessed in formalin fixed sections using Masson’s Trichrome staining. Gap junction protein distribution was examined using antibodies against connexin40 (Cx40) and connexin43 (Cx43). The ultrastructure of myocytes in myocardial sleeves was studied using electron microscopy. Results: Individual PVs in the dog had a gross morphology similar to that observed in the human, with myocardial sleeves extending into the veins for 4–20 mm. In all veins examined, myocytes in myocardial sleeves had a normal atrial morphology and anti-desmin staining pattern. Cx43 was expressed throughout the sleeve at levels comparable to normal atrial myocardium. By contrast, Cx40 expression was lower in myocardial sleeves than in the rest of the left atrium. Myocytes in the sleeve, which were ultrastructurally similar to normal atrial myocytes, were predominantly organized in a circumferential pattern. Conclusions: PVs in the dog and various canine models of heart disease will be a suitable model for (patho)physiology of the veno-atrial transition. Myocytes in myocardial sleeves are similar to normal atrial myocytes. The circumferential orientation of these myocytes may provide a substrate for rapid circular reentry.

Keywords: Electron microscopy; Gap junctions; Histo(patho)logy; Supraventr. arrhythmia; Veins

This article is referred to in the Editorial by B. Kwak et al. (pages 703–705) in this issue.

1. Introduction

Atrial fibrillation (AF) is the most common of atrial arrhythmias, occurring in 0.4% of the general population. Many patients with AF are elderly [1] or have associated heart disease, with a 40% incidence in patients with congestive heart failure [2] and with a strong correlation to the extent of atrial dilatation [3,4]. However, 30% of patients presenting with AF have no underlying structural heart disease (so-called lone AF or idiopathic AF) [5].

Recent clinical studies have demonstrated that AF can be caused by a rapidly firing focus in the atrium, and it is thought that a large proportion of lone AF is due to focal AF [6]. In some cases, the arrhythmia originated from the crista terminalis or superior vena cava [7,8], but in the vast majority, the foci are located in one of the PVs [7,9]. A large portion of patients with PV foci do not have underlying structural heart disease [7–11]. Even patients without AF seem to have the potential substrate for arrhythmias originating from PVs [12]. The basis for the unique electrophysiologic properties (higher incidence of focal arrhythmias, presence of PV potentials, exit block) of this region is not known.

Various studies have demonstrated that atrial tachycardias originating in PVs can be treated with radiofrequency ablation [13–15]. However, this procedure has been shown...
to have a considerable recurrence rate of 15–45% [10,11,16] and may cause serious complications like pulmonary stenosis [17,18], indicating the need for more targeted treatment strategies.

Anatomical studies of the PVs have shown that myocardial tissue extends into the PVs [19–21]. The mechanism by which this myocardial sleeve can form a substrate for rapid ectopic activity is unclear. In theory, (micro)reentry [22] and/or spontaneous automaticity [23] may be involved.

Conduction around the veno-atrial transition will depend in part on gap junction channels, which are responsible for cell-to-cell coupling and consist of connexin proteins (reviewed in [24]). In general, sparse connexin expression is seen in structures with low conduction velocity exhibiting spontaneous automaticity like the sinoatrial [25,26] and atrioventricular [27] nodes. By contrast, high levels of expression are seen in rapidly conducting tissues like the ventricular conduction system and the working myocardium [24]. The gap junction proteins connexin40 (Cx40) and connexin43 (Cx43) are known to be abundantly expressed in the atrial myocardium [28,29] but connexin expression in the PV area has not been studied.

Several canine models of heart disease have been developed, including models of chronic atrial fibrillation [30], congestive heart failure [31] and chronic atrial dilatation [32]. Alterations in histology and electrophysiology of the atrial myocardium have been described in both rapid atrial pacing [30,33], and congestive heart failure models [31]. This makes the dog heart an ideal model to study PV histology and physiology and the impact of various cardiac diseased states on PVs. Here, we report on pinned to rubber pads, frozen rapidly in liquid nitrogen and study PV histology and physiology and the impact of For immunohistochemistry, PVs from 4 dogs were

2. Methods

2.1. Animals

Eight adult mongrel dogs weighing 25–30 kg were anesthetized by intravenous injection of 20 ml pentobarbital (50 mg/ml) together with 5 ml heparin (1000 IU/ml). Excised hearts, with the lungs attached, were retrogradely perfused through the aorta with cold cardioplegic solution for 5 min. The area between the left atrium and the lungs was dissected to expose the PVs. After removing epicardial fat and the pulmonary artery, the PVs were cut close to the lungs.

Studies were performed according to the regulations for humane care and treatment of animals established by the National Institute of Health and locally monitored by the Animal Studies Subcommittee at Indiana University School of Medicine, Indianapolis, IN, USA.

2.2. Anatomy

The gross anatomy of the PV area was examined in all dogs. After cardioplegia, the epicardial fat pad was carefully removed and the PV area was spread out to expose the anatomy on the epicardial side. The preparations were subsequently injected with red and green dye through the right coronary and circumflex arteries, respectively, to define arterial supply of the veins. In some other preparations, the ventricles and the right atrial appendage and free wall were removed. The remaining left atrium was turned inside-out and filled with gauze. The tip of the left atrial appendage and the PVs were tied with suture and spread out to delineate the anatomy of the PV area from the endocardial side.

2.3. Histology

For histology, individual veins from 5 dogs were stored in 10% neutral buffered formalin solution. The tissue was embedded in paraffin and sectioned on a microtome in 4–5 μm thick longitudinal or transverse sections. Serial sections were stained with either hematoxylin–eosin stain or with Masson’s trichrome stain.

2.4. Immunohistochemistry

For immunohistochemistry, PVs from 4 dogs were pinned to rubber pads, frozen rapidly in liquid nitrogen and stored at −80 °C. After removal of the rubber pad, 10 μm thick longitudinal cryosections were cut and fixed to coverslips coated with 50 mg/1 poly-L-lysine. Rabbit polyclonal anti-Cx40 (Alpha Diagnostics Int., San Antonio, TX, USA) and anti-Cx43 (Chemicon Int., Temecula, CA, USA) were used to detect connexins. At high concentrations, the anti-Cx40 antibody cross-reacted with Cx43, as was evident from immunostaining of gap junctions between ventricular myocytes. At the dilution used in this study (1:4000), anti-Cx40 showed clear immunostaining of gap junctions between endothelial cells in the ventricle, but not between ventricular myocytes. For this reason, ventricular tissue was included in each experiment as a control.

In most experiments, connexin antibodies were used in combination with mouse monoclonal anti-desmin antibody (DAKO A/S, Denmark) to characterize myocyte orientation. To compare the distributions of Cx40 and Cx43, some sections were labeled with a combination of rabbit polyclonal anti-Cx40 antibody and mouse monoclonal anti-Cx43 (BD Transduction Laboratories).

Sections were permeabilized with 0.2% Triton-X100 in phosphate buffered saline (PBS) (1 h), incubated with 2% bovine serum albumin (BSA) in PBS for 30 min, and with
Fig. 1. Gross anatomy of the canine PV area. (A) Preparation in which the ventricles were removed below the AV ring and PVs were tied with suture and spread out. The epicardial fat pad overlying the PV area was removed. (B) Same preparation after injection of red and green food color dye into the right coronary and circumflex arteries, respectively. (C) Atrial preparation in which the left atrium was turned inside out and filled with gauze. (D) Computer generated outline of the same preparation, with the various groups of veins indicated: LI: left inferior; LS: left superior; RS: right superior; RI: right inferior; LAA: left atrial appendage. Within a group of veins, the largest veins with a separate myocardial sleeve was given the index one, the second largest the index 2 and the third largest the index 3. Thick blue lines indicate the end of the myocardial sleeve, determined by back-illumination of the preparation. (E) Average dimensions ($n=8$) for the veins indicated in D.
the primary antibodies overnight. Subsequently, sections were incubated with 2% BSA for 30 min, and for 2 h with a combination of the secondary antibodies goat-anti-rabbit FITC and donkey-anti-mouse Texas Red (Jackson Immunoresearch, West Grove, PA, USA). In negative controls, the first antibodies were omitted.

Tissue sections were viewed using a Leitz Laborlux S microscope equipped for fluorescence microscopy. Images were digitized using a Spot camera (Diagnostics Instruments).

2.5. Electron microscopy

Small tissue samples from the tip of myocardial sleeves of two dogs were fixed in 3% glutaraldehyde and postfixed with Osmium tetroxide (2%). One hundred-nm (100-nm) sections were cut on an ultramicrotome and stained with Uranyl acetate and Lead citrate. Specimen locations were confirmed by Toluidine blue staining of serial sections of the same specimens, viewed with conventional light microscopy. For comparison, samples from the myocardium of the left atrial appendage were studied. Myocardial ultra-thickness of myocardial sleeves gradually decreased (Fig. 2A, green line, shown at higher magnification for a

3. Results

3.1. Anatomy of the PV area

The gross anatomy of the PV area, after removal of the overlying epicardial fat pad, is depicted in Fig. 1A. The PV area in this preparation was perfused exclusively by the circumflex artery (green color in Fig. 1B). Fig. 1C shows a preparation from another dog, in which the left atrium and PVs were turned inside out to reveal the numerous PV ostia more clearly. On the endocardial side, some muscle bundles in the PV area were observed to have circumferential pattern around the four groups of PVs, visible in Fig. 1C around the left and right superior vein groups. The number of individual left atrial ostia, with separate myocardial sleeves, varied from 8 to 11. The left superior PV region had two separate ostia, LS, the vein closest to the left atrial appendage, and LS, both branching further than 20 mm from the left atrium. The other groups of veins branched closer to their left atrial ostia. The large left inferior PV LI branched close to the tip of the myocardial sleeve. In the right inferior region, a small vein with a separate myocardial sleeve (RII) was commonly present to

the left of a much larger PV (RI), the vein closest to the inferior vena cava. Veins in the right superior area were most tightly integrated into the lungs. This area consisted of three (groups of) veins, which all branched close to the end of the myocardial sleeve, with a more variable branching pattern than the rest of the PVs. In between the larger RS, and RS, a smaller vein RS, with variable branching pattern was normally present.

Fig. 1D shows the outline of the preparation in Fig. 1C. The ends of the myocardial sleeves, revealed by back illumination of the preparation, are indicated by solid blue lines. Typically, myocardial sleeves measured 4–20 mm from the ostium to the tip of the sleeve when the veins were stretched slightly. The longest sleeves were seen in left inferior PVs. Fig. 1E summarizes the dimensions of the major veins in the explanted heart, as viewed from the epicardial side.

3.2. Histological structure of the myocardial sleeve

From the left atrial myocardium towards the vein, the thickness of myocardial sleeves gradually decreased (Fig. 2A), ending in the rounded tip surrounded by fibrous tissue (Fig. 2A, green line, shown at higher magnification for a number of veins in Fig. 2B–G). Particularly at the tip of the myocardial sleeve, bundles of myocardial fibers were often observed to be separated by fibrous tissue (Fig. 2D). There was considerable variability in the shape of the tip, varying from a compact (Fig. 2E) to a loosely packed fiber arrangement (Fig. 2G). In rare instances, a series of several separate bundles of myocardial fibers was observed at the venous end of the myocardial extension (Fig. 2I).

The exact shape of sleeves varied between veins in individual dogs, and between the veins at comparable positions among dogs. No systematic differences between the veins at different positions were noted.

Within myocardial sleeves, sharp transitions in fiber orientation were sometimes observed (Fig. 2H, and 2A, epicardial side of the sleeve), with the pattern on the endocardial side extending to the tip of the sleeve. Particularly at high magnification (Fig. 2D), it was apparent that myocytes on the endocardial side of the myocardial sleeve were cut transversely in longitudinal sections. The observation that the length axis of individual myocytes was perpendicular to the length axis of the vein suggested that myocytes on the endocardial side are organized in a circumferential or spiralling pattern around the lumen of the veins.

The circumferential fiber arrangement was confirmed in
transverse sections of PVs (Fig. 3). Fig. 3A and B illustrate
a vein sectioned transversely, close to the tip of the sleeve.
At one end of the section, strands of myocytes are oriented
circumferentially around the lumen of the vein (Fig. 3A).

At the opposite side of the same section, sparse myocytes
were present with length axes perpendicular to the axis of
the vein (Fig. 3B, at higher magnification in 3C), probably
reflecting myocytes at the extreme tip of the sleeve, since

Fig. 3. Histology of the myocardial sleeve, transverse sections. (A, B and C) Section through a RI, vein, near the tip of the myocardial sleeve. A and B
were taken at opposite sides of the same section. C is a magnification of B. (D and E) RI, PV, transverse section through the middle of the sleeve,
illustrating circumferential fiber orientation on the endocardial side of the vessel wall. (F and G) Transverse section near the ostium of a LS$_2$ PV.
Fig. 4. Connexin expression in myocardial sleeves. (A, D and G): anti-Cx40 (green) and anti-desmin (red) immunofluorescence. (B, E and H): anti-Cx43 (green) and anti-desmin immunofluorescence (red). (C, F and I): anti-Cx40 (green) and anti-Cx43 (red) immunofluorescence. (A, B and C): tip of myocardial sleeves (RS₁, R₁, R₁ respectively), corresponding to the region indicated by the green line in Fig. 2A. (D, E and F): middle of myocardial sleeves (RS₁, R₁, R₁ respectively), corresponding to the region indicated by the red line in Fig. 2A. (G, H and I): left atrial free wall. Green strands at the bottom of panels A, B and C and at the middle of panel E were caused by elastin fiber autofluorescence and were also present in negative controls (first antibody omitted). For all panels, original magnification 400X.
completely disconnected myocytes were not observed in longitudinal sections.

In transverse sections closer towards the left atrium, fibers on the endocardial side of the vessel wall showed a similar circumferential pattern. Again, sharp transitions in fiber orientation within the wall were sometimes observed, with fibers on the epicardial side oriented more parallel to the long axis of the vein (Fig. 3E). The same pattern, shown for another vein in Fig. 3F, was observed in sections still closer to the left atrium. At higher magnification (Fig. 3G), myocytes within the myocardial sleeve appeared to have normal cross-striation and a morphology comparable to normal atrial myocytes.

3.3. Connexin expression in PVs

To investigate cell-to-cell coupling between myocytes around the veno-atrial transition, expression of Cx40 and Cx43 was characterized using immunohistochemistry. As a reference, a representative sample of myocardium from the left atrial free wall is shown in Fig. 4G, H and I. With the contractile apparatus visualized using anti-desmin antibody, both Cx40 and Cx43 were predominantly present at end-to-end connections between myocytes, but also at smaller side-to-side connections (Fig. 4G and H). As expected, Cx40 and 43 were colocalized in intercalated discs (Fig. 4I).

Fig. 5. Ultrastructure of PV cardiomyocytes. (A) Thick section of the tissue specimen from a RI vein used for B–D. The sample was taken from the tip of the myocardial sleeve, but its orientation with respect to the axis of the vein is unknown. (B) Ultrastructure of a small group of myocytes, exhibiting well-organized sarcomeric structure and long intercalated discs (arrows), magnification 13,000×. Inset shows secretory granules at higher magnification (blue arrow). (C) Myofilament structure at higher magnification (38,750×). Blue arrow indicates the position of another secretory granule. (D) Example of an intercalated disc at an end-to-end connections between two myocytes, magnification 38,750×.
The first and second rows of Fig. 4 show connexin expression in myocardial sleeves at sites roughly corresponding to Fig. 3A and D, respectively, and to the regions indicated in Fig. 2A by the green and red lines, respectively. Both Cx40 (Fig. 4A and D) and Cx43 (Fig. 4B and E) were present throughout the length of the sleeve. However, the intensity of anti-Cx40 immunostaining was lower than in the left atrial free wall. By contrast, Cx43 immunoreactivity in the myocardial sleeve was the same as, or slightly higher than in the left atrial free wall.

At the endocardial side, surrounded by fibrous tissue, strands of smooth muscle cells were present, also stained by the anti-desmin antibody, but with a pattern distinct from that in cardiomyocytes (Fig. 4B and E). Direct contact or close apposition between myocardial tissue in the sleeve and the smooth muscle layer was not observed. Especially at the tip of the sleeve, large gap junctional plaques at end-to-end connections were visible in the en face view, in accordance with myocytes long axes perpendicular to the plane of sectioning and thus perpendicular to the long axis of the veins. These large en face plaques and smaller plaques at side-to-side connections between myocytes in the myocardial sleeve were comparable in size and structure to those in the rest of the left atrium.

3.4. Ultrastructure of myocytes at the veno-atrial transition

Using electron microscopy, myocardial tissue at the tip of the sleeve was similar in all veins examined. Fig. 5 shows results obtained from a right inferior PV. Light microscopy of a serial section confirmed that the specimen was taken from the extreme tip of the myocardial sleeve (Fig. 5A). At the ultrastructural level, myocytes at this location (Fig. 5B) were similar to myocytes in the atrial working myocardium, with secretory granules visible at higher magnification (panel B, inset). A highly developed contractile apparatus, comparable to that in normal atrial myocytes was apparent, without a clear T-tubular system (Fig. 5C). In addition, long intercalated discs with numerous gap junctional plaques were observed at end-to-end connections between myocytes (Fig. 5D), similar to those present in the myocardium of the left atrial appendage (not shown).

4. Discussion

In this study, we have identified the myocardial anatomy, histology, architecture and gap junction distribution of the canine PVs. Cellular size and morphology, ultrastructure and gap junction distribution of the myocardial sleeve within PVs are similar to those elsewhere in the atrial myocardium and distinctly different from those in specialized areas such as the SA and AV nodes. Our data suggest that fibers in the myocardial sleeve are predominantly oriented in a circumferential pattern around the lumen of the veins.

Numerous recent clinical studies have shown that AF is often caused by rapid focal activity in PVs [8–16], often in the absence of structural heart disease [7–11]. However, it is unclear why ectopic foci occur much more frequently in myocardial extensions of the PVs than in the rest of the atrial myocardium. Conceivably, groups of myocytes within the myocardial sleeve may exhibit pacemaker properties due to spontaneous or triggered automaticity [34,35]. Alternatively, a particular fiber organization of inherently normal atrial myocytes might make myocardial sleeves a fertile substrate for rapid reentry. In this context, it is notable that firing rates of foci in the PVs of up to 400 bpm far exceed maximal beating rates of nodal pacemakers [7–14].

Based on transient expression of the marker HNK-1 during development, it has been suggested that myocardial tissue in the PVs is related to nodal tissue in the SA and AV nodes [36]. However, it is unclear whether this ontological relationship is reflected in similarities in structure of adult tissue. In a recent study, a portion of myocytes isolated from canine PVs showed spontaneous activity [37]. Both quiescent and spontaneously active PV myocytes were rod-shaped and resembled normal atrial myocytes. By contrast, pacemaker myocytes in the SA and AV nodes are not rod-shaped and do not have a fully developed contractile apparatus [38]. On the electron microscopic level, nodal gap junctional plaques are sparse and small, consisting of only tens of channels [39]. Correspondingly, expression of connexin proteins within the SA and AV nodes is low [26,40–42]. In fact, theoretical studies indicate that low electrical coupling is a prerequisite for a node or ectopic focus to drive a larger area of working myocardium; at higher levels of cell-to-cell coupling, a focus, or spontaneously active myocytes in the myocardial sleeve, would in effect be silenced by the relatively low resting membrane potential of the surrounding working myocardium [26,43].

At the ultrastructural level, we show that myocytes in the PVs strongly resembled normal atrial myocytes, with a fully developed contractile apparatus (also evidenced by their anti-desmin staining pattern), secretory granules [44,45] and without a clear T-tubular system [46]. In addition, large gap junctions were observed at end-to-end connections between these myocytes. The existence of large gap junctions within the myocardial sleeve was supported by abundant expression of connexin43, at levels comparable to those in the atrial and ventricular working myocardium. Levels of Cx40 in veins were lower than in the left atrium in general, suggesting that conduction is relatively more dependent on Cx43 in PVs. Low expression of Cx40 in atrial myocytes is not unique to the canine PV region. For example, the crista terminalis in the rabbit has high Cx43 and low Cx40 levels [26,47]. We have not studied the distribution of two other connexins expressed
in the adult mammalian heart, Cx37 which is expressed by endothelial cells [48,49], and Cx45, expressed at low levels throughout in atrial working myocardium and in nodal tissue [50–52]. Apart from the low expression level of Cx40 and the possible contribution of other connexins, the abundant expression of Cx43 strongly suggests that myocytes in the sleeve are electrically well-coupled.

With either light or electron microscopy, we did not observe any P-cells, or ‘pale cells’, largely devoid of myofibrils, which have been described in nodal tissue [53,54]. Overall, the myocardial tissue in the PVs appeared to consist of myocytes that were indistinguishable from normal atrial myocytes with the techniques employed in this study, and did not have an appearance and gap junction distribution consistent with nodal tissue.

Although the gross anatomy of the pulmonary area in the dog is more complex than that in the human, the shape and dimensions of myocardial extensions into the veins are comparable [21]. Several anatomical studies have shown that on the endocardial side, macroscopic fiber orientation forms a spiralling pattern around the PV ostia [19,20]. On a microscopic level, circumferential layers were observed inside the sleeve in studies on human PVs [20,55], although more non-uniform patterns were found in another study [21]. Here, we have investigated the microscopic fiber orientation within myocardial sleeves in more detail. We report that fibers with a circumferential or spiralling pattern extended to the tip of these sleeves on the endocardial side. Fibers with a non-circumferential orientation, which were frequently observed on the epicardial side, did not extend as far into the veins. It is conceivable that the striking tissue geometry could provide a substrate for rapid circular reentry within the myocardial sleeve. In fact, high resolution optical mapping of conduction at the veno-atrial transition in our laboratory has indicated that this region can exhibit a zone of slow conduction and reentry, even in normal dog hearts. (Rishi Arora MD, Luis Scott MD, unpublished observations, 2001). As in human veins, the smooth muscle layer on the luminal side of veins was distinct from the myocardial sleeve [55].

In a recent study, Yeh et al. have described the tissue structure of the myocardial sleeve of the canine superior vena cava (SVC) [56]. These authors describe an arrangement of fibers in the SVC similar to that observed by us for the PVs, with circumferentially oriented fibers at the subluminal side of the sleeve and fibers parallel to the axis of the SVC at the subadventitial side. However, in contrast to PV sleeves, the thickness of the SVC sleeve was not constant around the lumen of the vein, and myocardial tissue was sometimes absent at the dorsal side of the vessel wall. In addition, myocytes within the sleeve of the SVC appear to be more loosely packed than the compact fiber arrangement throughout most of the PV sleeve in this study. Although Cx40, 43 and 45 were typically present throughout the sleeve of the SVC, Yeh et al. also described ‘atypical areas’, where a group of myocytes abundantly expressing Cx43 was surrounded by an area of diffuse Cx40 expression. We have not observed comparable ‘atypical areas’ in PV sleeves, which had a homogenous expression of low levels of Cx40 and high levels of Cx43.

Several animal models have been used to study atrial fibrillation. Structural as well as electrophysiological alterations have been reported for chronic rapid atrial pacing [57,58], and congestive heart failure models [31,59]. Pathological changes were observed throughout both atria in the rapid atrial pacing model and throughout the left atrium in the congestive heart failure model and are likely to include PV area. A recent study showed that in a canine model of rapid atrial pacing, dominant frequency was higher in the PVs than in the rest of the atrium, indicating that PVs were a source of rapid activations [60]. Isolated myocytes from PVs after chronic rapid atrial pacing showed altered electrophysiological characteristics [37]. In this and other models, pathological changes may combine with the preexisting tissue structure of the myocardial sleeve to form a fertile substrate for focal AF. The similarity of canine PV morphology to that of human PVs and the availability of canine models of heart disease makes the dog heart an interesting model for studying PV physiology and specific pathophysiological alterations.

5. Conclusions

The myocardial sleeve in canine PVs consists of myocytes similar to normal atrial myocytes, with abundant expression of the gap junction protein Cx43. These myocytes were organized in circumferential strands, spiralling around the lumen of the veins. This tissue geometry may contribute to the substrate for focal atrial tachycardias in the myocardial sleeve of pulmonary veins.

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