Heterogeneity of Myocardial Sleeve Morphology and Gap Junctions in Canine Superior Vena Cava

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Background—The myocardial sleeve of the superior vena cava (SVC) has been identified as a potential initiating focus in atrial fibrillation, but information on cell-to-cell linkage at this site is lacking.

Methods and Results—We examined the SVC in 8 dogs by immunoconfocal and electron microscopy. Cardiomyocytes outlined with vinculin and bearing striations positive for α-actinin are found in the proximal segment of the SVC. These cells, grouped in bundles of various orientations according to location, extend cephalically as far as 3 cm from the right atrium (RA)-SVC junction. Comparison between the junctional level and the level 2 cm distal shows that the myocardial layer in the latter is thinner and not as compact and is composed of longer cells (87.3±15.7 versus 71.6±14.4 μm, P<0.01). Gap junctions made of connexin43 (Cx43), Cx40, and Cx45 are aggregated mainly at the intercalated disks, and colocalization of connexins is a common feature throughout the myocardial sleeve. Areas of atypical expression exist, however, characterized by a center of abundant Cx43 labels surrounded by a periphery of scattered tiny Cx40-labeled spots. Although in the ventral subluminal compact myocardial layer, individual cells at both levels are surrounded by similar numbers of cells, the number of aggregation of labeled gap junctions at the distal level is less (2.3±0.6 versus 3.7±0.9, P<0.01). In addition, electron-microscopic examination demonstrates that the gap junctions at the distal level are smaller in size (0.37±0.30 versus 0.55±0.34 μm, P<0.01).

Conclusions—The myocardial sleeve in the canine SVC is a heterogeneous structure, which could potentially form a substrate for heterogeneity of electrical coupling. (Circulation. 2001;104:3152-3157.)

Key Words: junctions • proteins • veins

Recent evidence indicates that the proximal portions of the thoracic veins, including the superior vena cava (SVC), are potential initiating foci of atrial fibrillation.1-2 Although the existence of a myocardial sleeve and electrical activity in the SVC of mammals is well established,3-6 the structural basis of the arrhythmogenic substrate at this site is not known. Among factors capable of initiating and maintaining arrhythmia, anisotropy plays an essential role. Anisotropy is determined mainly by the spatial and geometric characteristics of the muscle cells and by the gap junctions linking individual cells.7

Gap junctions, made of connexin (Cx) subunits, are clusters of cell membrane aqueous channels linking the cytoplasmic compartments of adjacent cells, thereby providing low-resistance pathways for electrical coupling between cardiomyocytes. In mammals, cardiomyocytes express mainly Cx43, Cx40, and Cx45.8 Changes in expression patterns of these connexins have been demonstrated to be associated with a variety of cardiac pathologies and to contribute to the development of cardiac arrhythmia.9-13 The distribution of gap junctions and expression of connexins in the myocardial sleeve of the SVC, however, remains unclear.

In this study, we investigated the phenotypes of the myocardial sleeve, including its gap junctions and connexins, in canine SVC. Our results show that the proximal segment of the SVC is a complicated structure, with features that may potentially contribute to its arrhythmogenicity.

Methods

Samples and Tissue Processing

Eight adult mongrel dogs (20 to 25 kg) were anesthetized with sodium thiopentone (25 mg/kg IV), and the chest was opened through the fifth intercostal space. After heparinization (20 000 U), the samples, including the right atrial (RA) appendage, the whole length of the SVC (including the tributary azygos vein), and the inferior vena cava, were removed and the great veins cut into rings. All samples were stored under liquid nitrogen before cryosectioning. Selected samples were prepared for histological examination and thin-section electron microscopy by standard procedures.14 The work was performed according to the principles of laboratory animal care described by the Institutional Animal Care Committee. All dogs were treated according to the Guiding Principles for Research Involving Animals and Human Subjects.15

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**Immunohistochemistry**

**Anti-Connexin Antibodies and Other Cell Markers**

Affinity-purified rabbit polyclonal antisera against Cx40 [designated $\text{S}_{15}$C (R83)], Cx43 (C16), or Cx45 [Q15N(R402)], were used for the immunofluorescence detection of the gap-junctional proteins. Details of these antisera, including confirmation of their specificity, have been reported previously. In addition, a mouse monoclonal antibody for Cx43 was used (Chemicon). Cardiomyocytes were identified with mouse monoclonal antisera against α-actinin and vinculin (Sigma), smooth muscle cells by mouse monoclonal antisera against smooth muscle α-actin (Dako), and endothelial cells by rabbit polyclonal anti-von Willebrand factor (vWF) antibody (Dako).

**Secondary Antibody/Detection Systems**

Donkey anti-rabbit and anti-mouse immunoglobulins conjugated to either CY3, CY5, or FITC (Chemicon) were used. CY3-conjugated antibodies were used for single labeling and 1 CY3-conjugated plus 1 CY5-conjugated for double labeling. In experiments in which 1 connexin was visualized with the anti-CX3, simultaneous cardiomyocyte marking was detected with the anti-rabbit CY5. To visualize vWF in triple labeling, donkey anti-rabbit FITC was used.

**Immunolabeling of Connexins and Cell Markers**

For single labeling of one connexin type, cryosections of the samples were blocked in 0.5% BSA (15 minutes) and incubated with rabbit anti-Cx40 (1:200), anti-Cx43 (1:100), or anti-Cx45 (1:50) at 37°C for 2 hours. The samples were then treated with CY3-conjugated secondary antibody (1:500, room temperature, 1 hour). In experiments in which 2 of the 3 connexins were localized simultaneously in the same samples, incubation was with a mixture of the monoclonal anti-Cx43 (1:1000) plus either anti-Cx40 or anti-Cx45, followed by incubation with a mixture of the 2 corresponding species-specific secondary antibodies (CY3 and CY5; 1:500). When simultaneous marking of cardiomyocytes was carried out, single connexin-labeled samples were incubated with anti–α-actinin (1:200) followed by treatment with the 2 secondary antibodies. At this step, in selected experiments, the sections were further sequentially treated with the anti-vWF (1:200) plus anti-rabbit FITC (1:50). Single or double labeling of cell markers followed similar procedures. Finally, the sections were mounted. All experiments included RA sections as positive controls and omission of primary antibody as negative controls.

**Confocal Laser Scanning Microscopy**

Immunostained samples were examined by confocal laser scanning microscopy with a Leica TCS SP. The images from sections of multiple labeling were taken with either simultaneous or sequential multiple channel scanning. For determination of the size of and the spatial relationship between individual cardiomyocytes, in samples labeled with anti-vinculin, consecutive optical sections taken at 0.5-μm intervals through the full thickness of cardiac muscle, in which the long axis of the cells lies horizontal to the sections, were used. The number of surrounding contact cells was then counted by inspection of each optical section. Quantification of gap junction aggregations, defined as the number of aggregations of Cx43-labeled spots in linear or disk shape between the cells, was conducted in samples double-labeled for Cx43 and vinculin by use of similar principles. Cell size, including the long and short axis, was measured with QWIN image analysis software (Leica).

**Statistical Analysis**

Data, expressed as mean values (±SD), were compared statistically by ANOVA and t test. A value of $P<0.05$ is considered to be significant.

**Results**

**Distribution of Cardiac Muscle in SVC**

Sections stained with trichrome show that cardiac muscle cells grouped in bundles extend from the RA upward into the SVC up to 3 cm distant from the RA-SVC junction (Figure 1, A through C, in contrast to the abrupt disappearance of the muscle at the very proximal portion of the inferior vena cava. Observation of the cross sections of the SVC showed that the thickness of the cardiac muscle layer varies between different levels. In general, it is thicker in the proximal portions than the distal. At the same level of the SVC, however, the thickness of the cardiac muscle layer is unequal around the lumen, usually being thinner or even absent at the dorsal side (Figure 1, D through F). Similarly, assembly and orientation of cardiac muscle differ between the various levels as well as within the same level (Figure 1, D through E). Adjacent to the RA-SVC junction, the muscle bundles are compact in the subluminal zone and loose in the subadventitial zone. Beyond the junctional portion as the vein goes cephalically, the inner
compact zone gradually becomes loose, so that the distinction between the inner and the outer zones fades. In addition, at the same level, the transition is not identical at different sides (Figure 1, D and E). With regard to orientation, typically the muscle bundles in the inner zone are tangential to the lumen and perpendicular to the direction of the SVC, whereas in the outer zone, they lie parallel to the direction of the SVC or show diverse orientation (Figure 1, D and E). The space between cardiac muscle bundles is occupied primarily by adipose tissues (Figure 1, D and E). Myocardial sleeve is also found in the wall of the proximal portion of the azygos vein (up to 2 cm distal to its junction with the SVC) with a feature similar to that of the SVC (Figure 1B, inset).

Immunolabeling shows that cardiac muscle cells in the SVC and azygos vein bear striations positive for α-actinin and are outlined with vinculin, identical to the staining patterns of working cardiomyocytes seen in the RA (Figure 2, A through C). In addition, the cardiac muscle is negative for smooth muscle α-actin (Figure 2D). The spatial relationship between individual cardiomyocytes is clearly seen with the α-actinin labeling, which shows that in compact zones, individual cells are in contact with several others, whereas in loose zones, the interconnection is simple, as illustrated in the inset of Figure 2A, where 2 groups of intimately interconnecting cells are linked merely by a single cell.

**Gap Junction Distribution and Connexin Expression**

Sections from different levels of the SVC and the azygos vein double-labeled for α-actinin plus each of the 3 connexins show the presence of all 3 connexins in the cardiac muscle layer. Although all 3 connexins are clustered mainly in intercalated disks, their expression patterns are distinctive (Figures 2 through 4). For Cx43, in general, the expression is abundant, regardless of the ventral or dorsal side (Figure 2); however, the signal is diffuse or peculiarly distributed in some small regions consisting of <100 muscle cells and rather rare in the surrounding regions (Figure 3, A and B). Exclusion of the possibility that the atypical Cx43 label belongs to the adjacent vascular wall is confirmed by triple labeling of Cx43, vWF (a marker specific for endothelium), and α-actinin, which shows that the atypical Cx43 labels are colocalized with α-actinin but not with vWF (Figure 3C). Such areas of atypical expression are encountered more frequently in the proximal portions of the SVC, usually in areas of the outer loose zone, where the cardiac muscle is surrounded by elastic fibers, clearly seen under UV illumination (Figure 3, A and B). These atypical areas are rarely seen beyond 1 cm distal to the RA-SVC junction. For Cx40 and Cx45, apart from the areas in which atypical expression of Cx43 is found, the distribution is more or less homogeneous throughout the myocardial sleeve (Figure 4), although the intensity of signals of Cx40 and Cx45 is weaker at the dorsal side. By contrast, in the azygos vein, Cx40 and Cx43 have similar expression patterns, whereas Cx45 is less abundant, compared with the corresponding connexins in the adjacent SVC (Figures 2B and 4, B and D, insets).

Double-labeling experiments show that in areas of typical expression pattern where gap junctions are clustered in disk shapes, colocalization of Cx43 with Cx40 and of Cx43 with Cx45 is by far the predominant feature (Figure 5, A and B). In areas in which the expression is atypical, however, gap junctions containing Cx40 are rare in the center, where gap junctions containing Cx43 are abundant, but diffusely distributed in the periphery, where gap junctions containing Cx43 are deficient (Figure 5C). By contrast, Cx45 is virtually invisible throughout the whole atypical expression area.

With the identification of intercalated disks by anti-connexin antisera and cell outlines by anti-vinculin antibody, the morphology of individual cells as well as the interconnection between them can be analyzed (Figure 2, A and C). Comparison between the cells in the RA appendage, those at the RA-SVC junction, and those at the level 2 cm distal to the junction demonstrates that the cells at the junctional level are shortest (junction versus RA and junction versus SVC, $P<0.01$), whereas cells in all 3 locations are similar in width. With regard to the surrounding contact cardiomyocytes and aggregations of gap junctions in linear or disk shape, for each cardiomyocyte of the 3 locations, although the numbers of contact cardiomyocytes are similar, cells in the distal SVC.
contain fewer gap junction aggregations than those of the other 2 locations (junction versus SVC, \( P < 0.01 \)). The data are summarized in the Table.

**Electron-Microscopic Examination**

Consistent with the histological findings, at different levels of the SVC cardiac muscle, cells are packed with striated myofibrils, and the spatial relationship between individual cells varies. Even in the compact zone seen under the light microscope, the extracellular space between adjacent cardiomyocytes is generally wider at the distal level than the proximal level (Figure 6, A and B). Gap junctions are found exclusively at intercalated disks (Figure 6C). Although gap junctions in linear and circular aggregates are both present in samples of different levels, the junctions seen at the distal levels are smaller. In addition, the junctions seen in the SVC are smaller than those of the RA appendage. Comparison between samples taken from the RA-SVC junction, the zone at the level 2 cm distal to the junction, and the RA appendage demonstrates that the average size of individual junctions at the level 2 cm distal to the junction is the smallest (\( P < 0.01 \), Table).

**Discussion**

The present study demonstrates that in the canine heart, the majority of cells of the myocardial sleeve in the proximal segment of the SVC possess phenotypic features identical to those of working cardiomyocytes of the RA appendage, as demonstrated by both the immunohistochemical and ultrastructural findings. In the myocardial sleeve, however, differences exist between and within the levels of the sleeve, including variation of the size and assembly of individual cardiomyocytes and the distribution of gap junctions and expression of their component connexins. All these findings indicate that the proximal segment of the SVC is a contractile structure of complex architecture, implying that functionally,
the SVC does not merely act as a passive conduit for conducting systemic blood flow back to the RA but also is electrically and mechanically integrated with the heart.

It should be noted that variation of the extent of myocardial sleeve in the SVC and the tributary azygos vein exists among species, as well as within the same species.3–6 In addition, in the presence of persistent left-side SVC after birth, it remains unknown whether the distributions of myocardial sleeve at both the side veins are similar. Furthermore, the findings may differ in diseased hearts. Therefore, additional studies are necessary to better understand such variations in humans and their pathogenic significance. Nevertheless, from the structural heterogeneity of the SVC shown in the present study, the electrophysiological properties would be predicted to vary between different locations of the venous wall. Previous studies have shown that in cardiac muscle, the propagation of action potential and the accompanying anisotropy is determined primarily by the inherent electrical characteristics of the cell membrane as well as the interrelationship between the

cells, including the orientation of muscle fibers and the linkage by gap junctions.16 Apart from cell membrane activities, the zone-specific and level-specific orientation and assembly of cardiomyocytes observed here indicate that the

Figure 5. Double labeling of 2 connexins. A and B, Extensive overlapping of Cx40 with Cx43 and of Cx45 with Cx43, respectively. By contrast, in C, clustered Cx43 labels do not overlap with surrounding diffuse, tiny Cx40 labels. Features as described in Figure 2. Bar=20 μm.

Figure 6. Thin-section electron micrographs demonstrating spatial relationship between individual cardiomyocytes and their gap junctions at different levels of SVC. A, Ventral subluminal compact zone of RA-SVC junction: limited extracellular space between cells; B, same zone 2 cm cephalic to junction: space is wider. Arrows in A and B, extracellular space. C, RA-SVC junction, shows gap junction (arrow) at intercalated disk. Insets in C, area near B, show gap junctions (arrows) in linear and circular shapes. A and B are same magnification. Bars=10 μm in B and 400 nm in C.
anisotropy could vary substantially from area to area. Such anisotropy could be further enhanced by the variation of gap junction distribution and connexin expression observed in the venous wall.

One interesting finding of the present study is the existence of atypical connexin expression areas, featuring a center rich in Cx43 labeling surrounded by a periphery of scattered tiny Cx40 labels. The pattern of connexin expression in these areas is similar to that reported for the sinoatrial node of the mouse heart.  

<table>
<thead>
<tr>
<th>Cell length, μm</th>
<th>RA Appendage</th>
<th>Junction</th>
<th>SVC 2 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>83.6±11.4</td>
<td></td>
<td></td>
<td>87.3±15.7</td>
</tr>
<tr>
<td>Cell width, μm</td>
<td>12.6±2.6</td>
<td>14.8±2.9</td>
<td>13.6±2.9</td>
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<tr>
<td>Contact cells, n</td>
<td>6.0±1.3</td>
<td>6.6±1.1</td>
<td>6.6±1.0</td>
</tr>
<tr>
<td>Gap junction aggregation, n</td>
<td>3.0±0.8</td>
<td>3.7±0.9</td>
<td>2.3±0.6‡</td>
</tr>
<tr>
<td>Gap junction size, μm</td>
<td>0.72±0.46</td>
<td>0.55±0.34</td>
<td>0.37±0.30†</td>
</tr>
</tbody>
</table>

*Data are based on examination of samples taken from the RA appendage, the ventral subluminal compact zone next to the RA-SVC junction, and the same zone at the level 2 cm cephalic to the junction (SVC 2 cm). Each value comes from analysis of 30 randomly selected gap junctions per sample for gap junction size and from 30 randomly selected cells per animal for the remaining measurements.  

†P<0.01 between the marked value and the corresponding other values.  
‡P<0.01 between the marked value and the corresponding value of the junction.

expression could potentially combine to create a substrate for heterogeneity of electrical coupling within the proximal portion of the vessel.

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References

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