Atrial Fibrosis and Conduction Slowing in the Left Atrial Appendage of Patients Undergoing Thoracoscopic Surgical Pulmonary Vein Isolation for Atrial Fibrillation

Sébastien P.J. Krul, MD; Wouter R. Berger, MD; Nicoline W. Smit, MSc; Shirley C.M. van Amersfoorth, MSc; Antoine H.G. Driessen, MD; Wim Jan van Boven, MD, PhD; Jan W.T. Fiolet, PhD; Antoni C.G. van Ginneken, PhD; Allard C. van der Wal, MD, PhD; Jacques M.T. de Bakker, PhD; Ruben Coronel, MD, PhD; Joris R. de Groot, MD, PhD

Background—Atrial fibrosis is an important component of the arrhythmogenic substrate in patients with atrial fibrillation (AF). We studied the effect of interstitial fibrosis on conduction velocity (CV) in the left atrial appendage of patients with AF.

Methods and Results—Thirty-five left atrial appendages were obtained during AF surgery. Preparations were superfused and stimulated at 100 beats per minute. Activation was recorded with optical mapping. Longitudinal CV (CV_L), transverse CV (CV_T), and activation times (>2 mm distance) were measured. Interstitial collagen was quantified and graded qualitatively. The presence of fibroblasts and myofibroblasts was assessed immunohistochemically. Mean CV_L was 0.55±0.22 m/s, mean CV_T was 0.25±0.15 m/s, and the mean activation time was 9.31±5.45 ms. The amount of fibrosis was unrelated to CV or patient characteristics. CV_L was higher in left atrial appendages with thick compared with thin interstitial collagen strands (0.77±0.22 versus 0.48±0.19 m/s; \(P=0.012\)), which were more frequently present in persistent patients with AF. CV_T was not significantly different (\(P=0.47\)), but activation time was 14.93±4.12 versus 7.95±4.12 ms in patients with thick versus thin interstitial collagen strands, respectively (\(P=0.004\)). Fibroblasts were abundantly present and were associated with the presence of thick interstitial collagen strands (\(P=0.008\)). Myofibroblasts were not detected in the left atrial appendage.

Conclusions—In patients with AF, thick interstitial collagen strands are associated with higher CV_L and increased activation time. Our observations demonstrate that the severity and structure of local interstitial fibrosis is associated with atrial conduction abnormalities, presenting an arrhythmogenic substrate for atrial re-entry. (Circ Arrhythm Electrophysiol. 2015;8:288-295. DOI: 10.1161/CIRCEP.114.001752.)

Key Words: action potential optical mapping ■ atrial appendage ■ atrial fibrillation ■ atrial remodeling ■ electrophysiology ■ fibrosis

In patients with atrial fibrillation (AF), an increased amount of fibrosis is found in the atria. Atrial fibrosis is an important component of the arrhythmogenic substrate in patients with AF. Fibrosis can be arrhythmogenic by increasing the extracellular matrix collagen content, separating atrial myocytes, and increasing the length of activation pathways, or by direct fibroblast-cardiomyocyte coupling resulting in an increased passive electric load to the cardiomyocytes. In particular, myofibroblasts—differentiated fibroblasts that develop during pathological stimuli—contribute to the pathological fibrotic remodeling and couple directly with cardiomyocytes have been described. The changes in fibrosis formation and (myo)fibroblast-cardiomyocyte interaction can facilitate re-entry after a premature beat emanating from the pulmonary veins. The effect of the quantity and the structural organization of fibrosis on atrial conduction abnormalities in man is unknown. Animal experiments show an increase in the heterogeneity of conduction as a result of increased interstitial fibrosis. We studied the amount and organization of interstitial fibrosis and investigated the effect of interstitial fibrosis on conduction characteristics in the left atrial appendages (LAAs) from patients with AF.

Methods

Thoracoscopic Surgery

Thirty-five LAAs were obtained from patients with AF during thoracoscopic surgery, as described before. The patient characteristics are

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Correspondence to Joris R. de Groot, MD, PhD, Department of Cardiology, Academic Medical Center, Meibergdreef 9, 1100 DD Amsterdam, The Netherlands. E-mail j.r.degroot@amc.uva.nl

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WHAT IS KNOWN

- Atrial fibrosis may contribute to atrial fibrillation by separation of cardiomyocytes through extracellular matrix deposition, which may result in longer activation pathways and re-entry.
- Myofibroblasts (differentiated fibroblasts) have been suggested facilitate arrhythmogenesis by direct electric coupling to cardiomyocytes and forming a passive electric load.

WHAT THE STUDY ADDS

- The structure of atrial fibrosis, rather than the percentage, forms an arrhythogenic substrate and is associated with conduction block and re-entry in human atrial myocardium.
- Particularly, the presence of thick strand of interstitial fibrosis is associated with the occurrence of conduction block on premature stimulation.
- Fibroblasts were abundantly present in left atrial tissue of patients with atrial fibrillation, but no myofibroblasts could be detected.
- Our study suggests a limited role of myofibroblasts once the substrate of clinical, symptomatic atrial fibrillation has developed.

shown in Table. The study was in accordance with the declaration of Helsinki and approved by the Institutional Review Board. All patients gave written informed consent. The LAAs were removed using an endoscopic stapling device (Endo Gia stapler, Tyco Healthcare Group). The tissue samples were transported to the optical mapping setup in 100-mL cooled superfusion fluid (Na+, 155.5 mmol/L; K+, 4.7 mmol/L; Ca2+, 1.45 mmol/L; Mg2+, 0.6 mmol/L; Cl−, 136.6 mmol/L; HCO3−, 27 mmol/L; PO43−, 0.4 mmol/L; glucose, 11.1 mmol/L; and heparin, 1000 IE).

Optical Mapping

Preparations were submerged in a tissue bath. The superfusion fluid was kept at a temperature of 36.5°C to 37.5°C and oxygenized with a mixture of 95% O2 and 5% CO2 to maintain a pH of 7.4. All LAAs were stimulated at 100 beats per minute at twice diastolic threshold with a pulse width of 2 ms using an epicardial electrode. In 6 LAAs preparations, short-coupled premature stimuli were applied and optical mapping recordings were made of the shortest conducting S1–S2 interval. The preparation was equilibrated for ≥30 minutes. Di-4-ANEPPS (Tebu Bio) was used as a membrane potential-sensitive fluorescent dye. A contraction uncoupler 2 to 10 mmol/L 2,3-butanedione monoxime (DAM; Sigma-Aldrich, B0753) was added if motion artifacts precluded recording of fluorescent action potentials (n=7). A MiCAM Ultima camera (SciMedia USA Ltd) was used to record epicardial images of an area of 1 cm² with a resolution of 100×100 pixels and a sample time of 0.5 ms. Images were stored using MiCAM Ultima Experiment Manager. A custom-made analysis program based on MATLAB R2006b (The MathWorks, Inc) was used to construct epicardial activation maps. The occurrence of motion artifacts precluded analysis of the repolarization of the LAA.

Measurement of Conduction Velocity

Local activation times (ATs) were determined from the steepest upstrokes of the optical action potential at each pixel. Activation maps were constructed from local ATs. To assess conduction velocity (CV), a line was drawn in the activation map in the direction of the activation wavefront (Figure 1). Subsequently, ATs were plotted against the distance from the stimulation site. This relation allowed for identification of the latency close to the stimulation site and breakthrough of multiple wave fronts at larger distance of the stimulation site. CV was calculated from the slope of the linear portion of the relation between distance to the stimulation site and AT. Local longitudinal CV (CVL) and local transverse CV (CVT) were calculated from the line starting at the point of earliest activation along, which activation spread most rapidly, and the line perpendicular to that respectively, assuming that this represented fiber direction. In addition, to assess the influence of fibrotic barriers on gross transverse conduction delay in the LAA, the AT was measured along an arbitrarily chosen 2-mm line on the activation maps perpendicular to the fiber direction at the site of greatest transverse conduction slowing (Figure 1).

Collagen Quantification and Organization

After optical mapping, the LAAs were frozen at −80°C with liquid nitrogen. Slides were prepared from the recording area of the LAA. Picrosirius red staining was performed for visualization and quantification of interstitial collagen in 32 LAAs. Three to 5 photographs from each section of randomly selected areas were taken of nonoverlapping fields at ×10 magnification. The percentage of interstitial collagen of the total tissue (collagen and cardiomyocytes) was determined, after manual exclusion of epicardial, endocardial and perivascular fibrosis.11-12 Two independent observers (blinded to the origin of the sections) assessed the width of interstitial collagen strands (ICS) qualitatively (Figure 2). The width of the ICS was assessed and qualified as predominantly containing either thin (≤0.01 mm) or thick fibrotic strands (≥0.01 mm).

Fibroblast Histology

Fibroblasts were identified with antivimentin antibody (1:2000, DAKO, M0725) and an indirect peroxidase (3-aminoo-9-ethylcarbazole peroxidase) was used as the substrate in 27 LAAs. Sections were faintly counterstained with additional hematoxylin staining and 3 to 5 photographs of different, randomly selected areas were taken of nonoverlapping fields at ≥x10 magnification. The density of fibroblasts in the tissue was semiquantitatively assessed optically by 2 independent observers, blinded for section origins, based on
the density of fibroblasts scattered throughout the tissue area (excluding microvessels). Two groups were identified (intermediate, ±<30% of tissue area and many, ±>30% of tissue area). All immunohistochemical staining included the use of positive and negative controls with omission of the primary antibody and stained using the same techniques.

Myofibroblast Histology
α-smooth muscle actin (α-SMA) antibody’s (1:800, Sigma, A2547) was used for staining of myofibroblasts in 27 LAAs. Because α-SMA stains pericytes and vascular smooth muscle tissue as well, an anti-CD31 antibody staining (1:500, DAKO, M0823) was performed to stain endothelial cells. Cell nuclei were stained with Sytox green

Figure 1. A, Illustration of an activation map with a line drawn in direction of the activation wavefront. In the graph (B) the activation times (ATs) are plotted against the distance from the stimulus site. The latency (L) and potential breakthrough (Br) of multiple wave fronts are identified. The slope of the linear portion of the relation between distance and AT (a) is used to calculate the conduction velocity (CV). C, Typical example of an activation map (1 cm²) of left atrial appendage (LAA). The isochronal lines are 2 ms apart and color scale is in ms; red represents the earliest and purple the latest activation. The graph (D) shows the ATs along the solid and striped lines in the activation map, which are drawn perpendicular to the isochronal lines for longitudinal and transverse conduction, respectively. CVL indicates longitudinal CV, CVT, transverse CV; and S, stimulation.

Figure 2. A, Example of the picrosirius red staining of a left atrial appendage (LAA) with thick interstitial collagen fibers (ICS). The red color represents collagen and the yellow/orange staining represents cardiomyocytes. B, An LAA with thin ICS.
The combination of costaining of α-SMA and CD31 indicates microvasculature. Isolated α-SMA positive cells in the interstitium were considered to be myofibroblasts. Three to 5 photographs of different areas were taken of nonoverlapping fields at ×20 and ×40 magnification in each LAA to identify interstitial myofibroblasts. All immunohistochemical staining included the use of positive and negative controls with omission of the primary antibody and stained using the same techniques.

Statistics
Data are presented as mean±SD for normally distributed continuous variables or median and empirical limits for non-normally distributed variables. Categorical variables are presented in numbers with percentages. Differences were determined using an independent Student t test for normally distributed data or a Mann–Whitney U test for non-normally distributed data. To assess correlation in normally distributed data, the Pearson was used and in case of nonparametric data Spearman’s was used. P<0.05 was considered significant. Statistical analyses were performed using IBM SPSS Statistics version 20.

Results
Conduction Velocity
Mean CVL was 0.55±0.22 m/s and mean CVT was 0.25±0.15 m/s. Median anisotropy was 3.1 (1.05–13.4). CVL and CVT were not significantly different in patients with paroxysmal or persistent AF (CVL: P=0.25 and CVT: P=0.51). Other patient characteristics were not associated with CVL or CVT. In particular, the use of sodium channel blocking drugs flecainide and amiodarone were not related to CVL (P=0.94) or CVT (P=0.14). Mean AT was 9.31±5.45 ms. AT was not different between patients with paroxysmal or persistent AF (P=0.15). Patient characteristics, including the use of sodium channel blocking drugs flecainide and amiodarone were not associated with AT (P=0.76). A total of 7 LAAs required DAM to reduce motion artifacts. CVL (P=0.47), CVT (P=0.28), and AT (P=0.62) between the LAAs exposed to DAM and other LAAs were similar.

In the 6 LAAs with premature stimulation (shortest S1–S2 interval; mean 250 ms [200–260 ms]), CVL was 0.44 m/s [0.33–0.56 m/s] at baseline and 0.30 m/s [0.15–0.40 m/s; P=0.046] at shortest S1–S2 interval. CVT was 0.24 m/s (0.46–0.06 m/s) at baseline and 0.16 m/s (0.05–0.25 m/s; P=0.12) at shortest S1–S2 interval. AT increased significantly from 4.73 ms (3.33–6.90 ms) to 8.08 ms (4.35–13.33 ms; P=0.028). Activation maps of the shortest captured S1–S2 interval showed prolonged ATs and clear lines of conduction block, that were absent during stimulation at basic cycle length. Figure 3 shows 2 representative activation maps from the
same LAA. In this LAA, the CV_L was 0.46 m/s and CV_T was 0.27 m/s at baseline. Note that at the short-coupled premature stimuli the activation pattern shows a zig-zag pattern, calculated CV_L was 0.15 m/s and CV_T was 0.13 m/s, whereas the apparent CV along line C is 0.06 m/s. However, if we follow the activation wavefront (line D), circumventing the area of conduction block, apparent CV along the activation wavefront is 0.23 m/s.

**Interstitial Fibrosis**

In 4 LAAs, severe artifacts precluded the semiquantitative analysis of interstitial collagen. In the remaining 28, the collagen content was 16.8±7.5%. There was no significant difference in the percentage of collagen between patients with paroxysmal and persistent AF (P=0.22). CV_L and CV_T were not associated with the amount of collagen (CV_L: P=0.098 and CV_T: P=0.91). However, a larger degree of transverse conduction delay was observed in patients with a high amount of collagen (AT: P=0.015). Other clinical characteristics of the patients, including left atrial size were not associated with the interstitial collagen content.

After qualitative assessment, 24 LAAs contained mainly thin ICS, whereas 8 LAAs had a mainly thick ICS. Thick strands were more often found in patients with a high degree of interstitial collagen content (mean 14.0% versus 21.1%; P=0.027). In addition, 7 of the 8 patients with thick ICS had persistent AF. CV_L was higher in patients with thick ICS of 0.77±0.22 m/s compared with thin ICS of 0.48±0.19 m/s (P=0.012). CV_T was not significantly different between samples with thick ICS and thin ICS (0.24±0.14 m/s compared with 0.22±0.16 m/s; P=0.47; Figure 4). However, AT was significantly higher in patients with thick ICS compared with patients with thin ICS (14.93±6.93 versus 7.95±4.12 ms; P=0.004).

**Fibroblast Histology**

Fibroblasts were abundantly present (intermediate, n=10 and many, n=17). A high density of fibroblasts was associated with a high CV_L as shown in Figure 5 (intermediate, 0.44±0.11 m/s versus many, 0.71±0.24 m/s; P=0.007). No differences were observed in CV_T (intermediate, 0.21±0.09 m/s versus many,

Figure 4. Influence of the quantity and quality of fibrosis on conduction velocity (CV) and activation time (AT). A. No correlation between the amount of fibrosis and longitudinal CV (CV_L) and transverse CV (CV_T), but correlation with AT (P=0.015). B. Qualitative analysis reveals that a higher CV_L is observed in samples with thick interstitial collagen fibers (ICSs) between cardiomyocytes (P=0.012). No influence of width of interstitial fibrosis is found on CV_T. A longer AT is observed in the patients with thick ICS (P=0.004).
studies. However, no single patient characteristic was associated with the amount of fibrosis in the LAA in our data. In our patients, the organization of interstitial collagen rather than the amount was associated with conduction changes.\textsuperscript{9} The contribution of fibrosis to the arrhythmogenic mechanism has been extensively studied in monolayers of cultured myocytes, animal models, and in human ventricular myocardium.\textsuperscript{4,9,13,14} In our experiments, we observed the induction of activation delay, caused by propagation of the activation front around inexcitable barriers of collagen. In these LAAs, CV was only modestly affected, possibly because of increased transverse fiber separation.\textsuperscript{4,15} This may result in a heterogeneity of conduction, such as the occurrence of unidirectional conduction block and re-entry, facilitating both the induction and the maintenance of AF.\textsuperscript{16} At baseline, pacing some LAAs showed a high anisotropy and activation delay. High anisotropy might facilitate the development of AF because of ectopic foci.\textsuperscript{17} In the presence of short-coupled premature stimuli, such as during AF, lines of conduction block developed, and the activation wavefront propagated around this area of block (Figure 3).

CV is also determined by excitability and coupling, mediated by the voltage-gated sodium channels and connexins, respectively.\textsuperscript{18} Therefore, we cannot fully exclude that changes in CV between patients might be related to changed function or expression of the voltage-gated sodium channels.

Figure 5. A, Section of a left atrial appendage is shown with intermediate fibroblast staining. B, Section with a high number of fibroblasts. C, High number of fibroblasts was associated with a higher longitudinal CV ($CV_L$; $P=0.007$), whereas no difference is found in transverse CV ($CV_T$). CV indicates conduction velocity.

Figure 6. High contrast pictures of the combined fluorescence of Sytox Green (cell nuclei, blue), CD31 (endothelial cells, red), and $\alpha$-SMA ($\alpha$-smooth muscle actin; myofibroblasts, pericytes and vascular smooth muscle tissue, green) at $\times 20$ (A) and $\times 40$ (B) magnification. Contrast is increased to visualize the background staining of the cardiomyocytes with CD31 (red) and $\alpha$-SMA (green). Both the transverse and the longitudinal myocardial fibers are visible. The yellow and red/green costaining reveals the microvasculature. No isolated $\alpha$-SMA staining, indicating the presence of myofibroblasts, could be found.
The Role of Fibroblasts in the Substrate of AF

A high density of fibroblasts in the LAA was associated with thick ICS, suggesting a local increase of extracellular matrix deposition. The increased extracellular matrix results in a separation between cardiomyocytes and subsequently in an increase in CV. In in-silico models, fibroblasts have been described to act as passive electric load and to depolarize cardiomyocyte resting membrane potential through gap junctional coupling with myocytes. However, only the myofibroblast, a differentiated form of the fibroblast, connects to cardiomyocytes in in-vitro cell layer models. This connection can result in discontinuous slow conduction and induces spontaneous electric activity. Our observations argue against such a mechanism in the patients that we studied because CV was increased in the presence of a high density of fibroblasts, which is not expected if reduced direct coupling is present. Alternatively, fibroblasts might exert their influences on CV by a paracrine mechanism. In cell-cultures, a significant paracrine effect was found on CV, resulting in reduced CV. However, our data showed an increase in CV in the presence of many fibroblasts, thus it is unlikely that the paracrine influences on CV have a major role in these patients.

Clinical Implications

The major finding of our study is that in patients with AF, an arrhythmogenic substrate for atrial re-entry is present, caused by the deposition of collagen. It may be conceivable that thick collagen strands anchor rotors that sustain human AF. Identification of the patients with severely affected fibrotic atria might help to guide therapeutic decision-making. Recent studies using noninvasive characterization of the fibrotic phenotype using MRI have shown an inverse relation between the success of catheter ablation for AF and the amount of fibrosis present in the atrium. Although present imaging techniques allow the detection of large areas of fibrosis, future developments might increase the resolution and enable the detection of smaller fibrotic strands.

Conclusions

In patients undergoing thoracoscopic surgery for AF, the structural local interstitial components of fibrosis modulate conduction in the LAA. Patients with persistent AF had more thick interstitial fibrotic strands, which was associated with an increased longitudinal CV, consistent with a separation of myocardial fibers. Local transverse conduction was not affected by these fibrotic strands, but activation delay was present because of areas of activation block. Slowing of conduction and increased areas of conduction block were observed after short-coupled stimuli, but were absent under baseline stimulation. Fibroblasts were abundantly present in the human LAA and were associated with thick interstitial fibrotic strands and increased longitudinal conduction. However, myofibroblasts were absent in the human LAA. Our observations demonstrate that the severity and structure of local interstitial fibrosis is associated with atrial conduction abnormalities, presenting an arrhythmogenic substrate for atrial re-entry.
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