Downregulation of Connexin40 and Increased Prevalence of Atrial Arrhythmias in Transgenic Mice with Cardiac Restricted Overexpression of Tumor Necrosis Factor

Sam E. Sawaya,1 Yadavendra S. Rajawat,1 Tapan G. Rami,1 Gabor Szalai,1 Robert L. Price,2 Natarajan Sivasubramanian,1 Douglas L. Mann,1 Dirar S. Khoury1

From the 1Section of Cardiology, Department of Medicine, Baylor College of Medicine, Houston, Texas, and 2Department of Cell and Developmental Biology and Anatomy, School of Medicine, University of South Carolina, Columbia, South Carolina.

Inflammation and Atrial Remodeling

Address for correspondence:
Dirar S. Khoury, PhD
The Methodist Hospital
6565 Fannin St, F764
Houston, TX 77030
Tel: (713) 441-1388
Fax: (713) 441-0188
E-mail: dkhoury@tmh.tmc.edu
ABSTRACT

Atrial arrhythmias, primarily atrial fibrillation, have been independently associated with structural remodeling and with inflammation. We hypothesized that sustained inflammatory signaling by tumor necrosis factor (TNF) would lead to alterations both in underlying atrial myocardial structure and in atrial electrical conduction. We performed ECG recording, intracardiac electrophysiology studies, epicardial mapping, and connexin immunohistochemical analyses on transgenic mice with targeted overexpression of TNF in the cardiac compartment (MHCsTNF) and on wild type (WT) control mice (age, 8-16 wk). Atrial and ventricular conduction abnormalities were always evident on ECG in MHCsTNF mice, including a shortened atrioventricular interval with a wide QRS duration secondary to junctional rhythm. Supraventricular arrhythmias were observed in 5 out of 8 MHCsTNF mice, whereas none of the mice demonstrated ventricular arrhythmias. No arrhythmias were observed in WT mice. Left ventricular conduction velocity during apical pacing was similar between the two mouse groups. Connexin40 was significantly downregulated in MHCsTNF mice. In contrast, connexin43 density was not significantly altered in MHCsTNF mice, but rather dispersed away from the intercalated disks. In conclusion, sustained inflammatory signaling contributed to atrial structural remodeling and downregulation of Cx40 that was associated with an increased prevalence of atrial arrhythmias.

Keywords: Atrial fibrillation, Electrophysiology, Heart failure, Inflammation
INTRODUCTION

The association of inflammation with some forms of atrial fibrillation (AF) has been demonstrated in animal models [17], in patients following cardiac surgery [5], and in non-postoperative patients [6]. Common to these findings is the observed elevation in C-reactive protein, which is an inflammatory marker that is driven by proinflammatory cytokines. Statins, known to reduce C-reactive protein levels, have recently been shown to reduce inducibility of AF in various canine models [29]. Recently, intraperitoneal lipopolysaccharide, a potent inflammatory mediator, has been shown to attenuate cell-to-cell communication in mouse aortic endothelial cells in part through reduction in connexin40 (Cx40) levels, the major connexin in the atria and specialized conduction system [31]. Persistence of atrial tachyarrhythmias has been related to structural changes in the atria, which eventually result in AF. The relationship between altered Cx40 expression and AF has been verified in experimental animal models [2] and in humans [10,15].

The proinflammatory cytokine TNF is upregulated in heart failure, in which there is an increased prevalence of AF. Previously we have generated a transgenic mouse model with targeted overexpression of TNF in the cardiac compartment (referred to as MHCsTNF), that progresses to a dilated cardiac phenotype by 12 weeks of age [32]. In the present study we hypothesized that sustained inflammatory signaling in MHCsTNF mice would lead to alterations both in underlying atrial myocardial structure and in atrial electrical conduction.
MATERIALS AND METHODS

Animals

The study was performed on hemizygous MHCsTNF mice generated in an ICR x C57 background [18,32] and on age-matched wild type (WT) littermate controls. All mice (age, 8-16 wk) were anesthetized by intraperitoneal injection (15 ml/kg) of a mixture of sodium pentobarbital (4.1 mg/ml), sodium chloride (6.6 mg/ml) and ethanol (0.18 ml/ml). The study protocol adhered to the PHS guidelines for the care and use of laboratory animals.

Electrocardiographic and Intracardiac Electrophysiologic Evaluation

Intracardiac pacing and recording was performed in 8 MHCsTNF and 8 WT mice by inserting an 8-electrode catheter (1.1 F, EPR-801, Millar Instruments; or 2 F, ClBer, NuMED) through the jugular vein and advancing it into the right atrium and ventricle. Electrophysiology methods were employed as previously described [1]. Electrical stimulation was applied through the catheter electrodes using an external stimulator (S8800, AstroMed). Inducibility of arrhythmias was investigated by delivering up to two extra stimuli (S2 and S3) that were programmed at progressively shorter intervals following an 8-beat conditioning drive train (S1).

Electrocardiographic limb lead signals were continuously recorded during electrophysiology studies. In addition, to determine time-dependent effects of MHCsTNF gene expression, ECGs were recorded in MHCsTNF and in WT mice 1 d, 1 wk, and 3 wk after birth.
The ECG signals were filtered between 5 and 100 Hz, and intracardiac electrograms were filtered between 5 and 500 Hz, all sampled at 1 kHz (CardioLab, GE Healthcare). All ECG intervals were determined from lead II.

**Epicardial Activation Mapping**

Anesthetized mice were ventilated with room air using a small animal respirator (tidal volume, 0.5 cc; rate, 130 breaths/min, Columbus Instruments). The heart was then exposed following a midline thoracotomy. An array comprised of 36 electrodes (interelectrode spacing, 300 microns) and spanning an area of 1.5 mm × 1.5 mm was used for electrical mapping on the epicardium of the intact beating heart (Multichannel Systems). The array was placed on the anterior-basal aspect of the LV epicardium. A total of 28 bipolar electrograms were filtered between 5-500 kHz, sampled at 2 kHz, and simultaneously recorded (CardioLab).

Custom algorithms (Matlab, Mathworks) determined activation times, constructed isochrone maps that depicted activation sequences, and computed conduction velocities. Local activation time was determined as the time of absolute maximum value in the bipolar electrogram [28]. The gradient of activation time as a function of distance was computed at each data point relative to surrounding data points on a two-dimensional grid, and conduction velocity was calculated as the inverse of the gradient.

**Connexin Analysis**

Western Blot analysis was performed to assess Cx40 and Cx43. The frozen heart was pulverized and homogenized in a buffer containing (mmol/L) NaCl 50, Tris-HCl (pH 7.4)
20, EGTA 1, NaN₃ 5, b-mercaptoethanol 10, and phenylmethylsulfonyl fluoride 2 plus protease inhibitors as described previously [3]. Protein concentration in the samples was assessed by BCA method and 30 µg protein was loaded on each lane. The samples were run on a 10% SDS-polyacrylamide gel and Western blotting was performed using goat polyclonal antibody for Cx40 (0.02 µg/µL, cat #SC-20466, Santa Cruz Biotechnology) and rabbit polyclonal antibody for Cx43 (1.6 µg/ml, cat #71-0700, Zymed). The immunoblot was blocked in 5% non-fat milk for 2 hr at room temperature. The blots were then scanned (OfficeJet 5510, Hewlett-Packard) and quantified (Image J, NIH). Levels of Cx40 and Cx43 were normalized to total protein concentration in the samples, in so far as we were unable to determine a myocardial membrane protein that did not differ between the WT and MHCsTNF myocardium. Therefore, protein concentration was measured multiple times before each western blot analysis, and the average protein concentration was used for normalization.

Immunohistochemical staining was performed in the atria and ventricles in both MHCsTNF and WT mice using standard methods described previously [24, 26]. Samples were prepared from excised hearts that were immediately frozen in liquid nitrogen and stored at –70°C. The hearts were sliced to a thickness of 20 microns and stained with immunofluorescent goat anti-Connexin40 (1:100 dilution, cat #SC-20466, Santa Cruz Biotechnology) and rabbit anti-Connexin43 (1:100 dilution, cat #C-6219, Sigma-Aldrich), as well as a Cy-5 phalloidin stain for f-actin (1:250 dilution, Molecular Probes) and a DAPI stain for nuclei (1:25000 dilutionMolecular Probes). The slices were visualized using a Zeiss Confocal microscope.
**Statistical Analysis**

Continuous variables were expressed as mean ± standard error. Differences between MHCsTNF and WT mice were compared by the Student t-test.
RESULTS

Electrocardiography and Electrophysiology

The ECG parameters in MHCsTNF and WT mice are summarized in Table 1. Unlike normal atrioventricular (AV) activation in WT mice, the ECG in MHCsTNF mice consistently exhibited AV junctional rhythm, with either absent or negative P wave in Lead II, short PR interval, and wide QRS complex (Figure 1).

Representative intracardiac electrograms recorded from the right atrium of MHCsTNF and WT mice are shown in Figure 1. The electrograms confirmed a short PR interval in MHCsTNF mice, consistent with the presence of a junctional rhythm. Baseline electrophysiologic parameters are summarized in Table 1. Unlike WT mice, sinus node function was either suppressed or its impulses blocked in MHCsTNF mice, as evidenced by (1) development of a junctional rhythm at a slow rate at baseline and (2) regular appearance of a junctional rhythm at the conclusion of decremental atrial pacing in MHCsTNF mice, substantiating a long sinus node recovery time. Atrial conduction was also significantly slower in MHCsTNF compared to WT mice as evidenced by the widened P wave duration. Decremental atrial pacing resulted in progressively long AV nodal conduction times; however, the longest atrial pacing cycle causing Wenckebach AV conduction was prolonged in MHCsTNF mice, suggestive of abnormal AV nodal conduction. The ventricular refractory period was significantly longer in MHCsTNF mice than in WT mice, consistent with long QTc intervals observed on ECG in MHCsTNF mice. Supraventricular arrhythmias were observed in 5 out of 8 MHCsTNF mice. These arrhythmias included atrial tachycardia induced by atrial or ventricular electrical stimulation in 4 mice and lasting more than 4 beats, spontaneous...
atrial fibrillation in one mouse, and spontaneous atrial bigeminy in 3 mice (Figure 2). None of the MHCsTNF mice had spontaneous or inducible ventricular arrhythmias. No arrhythmias were observed in WT mice.

Figure 3 shows ECGs in MHCsTNF and WT mice. At 1 day of age, none of the MHCsTNF mice (0/4) had AV junctional rhythm phenotype. Further, at 1 wk of age only 3 out of 5 MHCsTNF mice had the phenotype. At 3 wk of age and older, all MHCsTNF mice (14/14) exhibited the junctional phenotype.

Left Ventricular Conduction Pattern

Epicardial electrical mapping revealed that the sequence of LV activation in MHCsTNF mice was reversed during intrinsic rhythm when compared to the sequence of LV activation in WT mice. That is, whereas early activation originated from LV apex in WT mice (Figure 4A), early LV activation originated from anterior basal region in MHCsTNF mice (Figure 4B). Furthermore, during intrinsic rhythm, apparent conduction velocity in MHCsTNF mice (0.64±0.03 m/s, n=4) was significantly slower than the apparent conduction velocity in WT mice (1.13±0.08 m/s, n=4); P<0.01. However, as summarized in Figure 4C, the conduction velocities in both mouse genotypes were similar during pacing at LV apex, and increased appropriately with increasing pacing cycle length.

Connexin Expression

We performed Western blot analysis for Cx40 and Cx43 to explore the potential reasons for the abnormalities in atrial and ventricular conduction in the MHCsTNF mice. The analysis (Figure 5) showed that the relative density of Cx40 in the atria was significantly
less in MHCsTNF compared to WT mice (33±12% vs. 100%, n=4, \( P=0.02 \)). Meanwhile, Western blot analysis showed that Cx43 existed in multiple phosphorylated isoforms. However, no significant differences were observed in Cx43 levels between WT and MHCsTNF mice (Figure 5). Immunohistochemical analysis showed that the distribution of Cx43 was dispersed throughout the sarcolemma and intercalated disks of cardiac myocytes in the MHCsTNF, whereas Cx43 was primarily located in the intercalated disks in the WT mice (Figure 6A and Figure 6B), respectively.

Representative Cx40 immunostaining in the atria of WT and MHCsTNF mice are shown in Figure 7A and Figure 7B, respectively. As depicted, the amount of Cx40 was decreased and less uniformly distributed in the atria of MHCsTNF when compared to WT mice. Figure 7C and Figure 7D show connexin expression in the bundle branch and adjacent ventricular myocardium in WT and MHCsTNF mice, respectively. As shown, Cx40 in the bundle branch and Cx43 in the ventricular myocardium were decreased in MHCsTNF mice relative to WT mice.
DISCUSSION

Atrial arrhythmias, primarily AF, have been independently associated with inflammation and with structural remodeling caused by downregulation of Cx40. To determine whether sustained inflammatory signaling through targeted overexpression of TNF was sufficient to lead to alterations in underlying atrial myocardial structure, alterations in atrial electrical conduction, and atrial arrhythmias, we performed ECG screening, intracardiac electrophysiology studies, epicardial mapping, and connexin immunohistochemical analyses on MHCsTNF and WT control mice. The major new finding of this study was that sustained inflammatory signaling contributed to atrial structural remodeling and downregulation of Cx40 that was associated with an increased prevalence of atrial arrhythmias.

The study showed that MHCsTNF mice develop abnormalities of both atrial and ventricular conduction, including a shortened AV interval with a wide QRS duration secondary to junctional rhythm. Atrial arrhythmias were observed in the majority of MHCsTNF mice, whereas none of the mice demonstrated ventricular arrhythmias. The LV epicardial activation pattern was reversed and apparent conduction velocity slowed during intrinsic rhythm in MHCsTNF mice compared with WT mice, consistent with a bundle branch block pattern. However, both the activation pattern and conduction velocity during LV pacing (independent of the specialized conduction system) were similar between the two mouse groups suggesting that ventricular conduction velocity was not altered in the MHCsTNF mice. Connexin40, which is the major connexin in atrial myocytes and the specialized conduction system, was significantly downregulated
in MHCsTNF mice. In contrast, the major ventricular myocyte connexin, Cx43, was dispersed away from the intercalated disks in MHCsTNF mice, which did not appear to be functionally significant in our model.

Atrioventricular junctional rhythm, presenting within the first week after birth in some mice, is liable to be caused by TNF expression rather than heart failure. Specifically, the atrial electrical abnormalities observed in the MHCsTNF mice are most likely due to the observed abnormalities of Cx40. Indeed, studies in Cx40 deficient mice are consistent with the electrical phenotype observed in our MHCsTNF mice, including depressed sinus node activity, increased AV Wenckebach periodicity, increased inducibility of atrial tachyarrhythmias, and reduced conduction in the His-purkinje system with preserved conduction velocity in the ventricular myocardium [4,12,30,35,36]. Our results of atrial arrhythmias in a mouse model with overexpression of TNF confirm previous observations by others [27]. However, a novel finding of our study was the role of Cx40 in the setting of inflammation. Furthermore, while myocardial tissue fibrosis was evident in TNF mice aged 2 – 9 mo [27], it is unlikely that fibrosis played a major role in the pathogenesis of atrial arrhythmias in our model, insofar as fibrosis does not develop significantly in this model until 12 wk of age [32].

Ion channel currents are altered in heart failure; specifically it is thought that L-type calcium currents are upregulated and transient outward potassium currents are downregulated [14]. A recent study showed that action potential prolongation and abnormal calcium handling could contribute to the initiation of atrial and ventricular arrhythmias observed with TNF overexpression in mice aged 3 to 9 months [19]. The younger ages of the MHCsTNF mice that we studied (2 to 4 months) and absence of
spontaneous or inducible ventricular arrhythmias favor structural problems and
downregulation of Cx40 as the likely mechanism for the observed atrial arrhythmias in
the MHCsTNF mice, although we cannot exclude other ion channel mediated events.

As noted above, apparent ventricular conduction in MHCsTNF mice was
significantly slowed during intrinsic rhythm, as demonstrated by wide QRS on ECG and
by epicardial mapping. Meanwhile, the epicardial conduction velocity during LV pacing
(which bypassed the specialized conduction system) was similar between MHCsTNF
and WT mice. Interestingly, Cx40 knockout mice have been shown to exhibit slowing or
block in the bundle branches with a LV sequence of activation propagating from base to
apex [34], similar to what has been observed in the present study.

Conduction between ventricular myocytes depends on the excitability of
cardiomyocytes, the geometrical relationship between adjacent cardiomyocytes, and the
distribution of Cx43, which is primarily located in the intercalated disks of cardiac
myocytes, and has not been localized in the fibers of the specialized conduction system.
Previous studies have shown that reducing levels of Cx43 by 50% (a fraction similar to
that observed in human heart failure [16]) leads to a reduction in cardiomyocyte
conduction velocity [8,11], whereas other studies have not found this to be the case
[21,33]. Disruption of Cx43 has also been correlated with increased propensity for
ventricular tachyarrhythmias [25]. We did not find Cx43 levels to be downregulated;
however, by immunohistochemistry, we found Cx43 to be redistributed away from the
sarcolemma [23]. As noted above, the alterations in Cx43 expression in MHCsTNF mice
were probably not sufficient to cause a decrease in ventricular myocyte conduction
velocity or to provoke ventricular arrhythmias. However, these observations neither
exclude the formal possibility that greater disruptions of Cx43 might be functionally significant, nor preclude a potentially important role for Cx43 in transgenic models that do develop ventricular conduction disturbances.

Since many proteins were up- and down-regulated in MHCsTNF hearts [9,32], we were unable to find a good normalization control (calsequestrin levels - data not shown) for the membrane fractions of atrium and left ventricle that did not change between littermate controls and MHCsTNF animals. Hence, the total protein concentration was used to normalize the levels of Cx40 and Cx43. Therefore, the observed abnormalities in both atrial and ventricular conduction in MHCsTNF myocardium may be additionally due to the other proteins involved in this function whose levels might have changed in MHCsTNF hearts.

Previously we found that MHCsTNF mice die prematurely when compared to age matched littermate controls [13]. Progression of the observed atrioventricular conduction block in MHCsTNF mice may eventually advance into complete heart block and ultimately result in bradyarrhythmic death, although we cannot exclude other reasons for death such as metabolic and respiratory causes. Continuous telemetry in our conscious MHCsTNF mice remains to be performed; however, the mode of death in a different line of transgenic mice with targeted overexpression of TNF was previously noted to be secondary to bradyarrhythmias [19]. Interestingly, bradyarrhythmias were also the cause of death in mice with downregulated Cx40 [22]. Additionally, the major mode of cardiac arrest in patients with nonischemic heart failure has been found to be due to bradyarrhythmias [20], raising the interesting possibility that inflammation and Cx40 downregulation may play a contributory role. Finally, it is worth mentioning that
other cardiomyopathies that are associated with inflammation, including Lyme disease, viral myocarditis, Sjogren’s syndrome, and Chagas disease myocarditis all result in a higher proportion of patients with conduction system block.

We have generated several lines of mice that overexpress TNF in the cardiac compartment. The line of mice (MHCsTNF) generated in the present study has TNF protein and mRNA levels that are in excess of what one would expect to see in human heart failure. However, we have generated additional lines of mice (MHCsTNF2) that have levels of TNF proteins and mRNA that are similar to human heart failure [7]. The major difference between these two lines is that the time course for development of cardiac remodeling is greatly accelerated in the line of mice with higher TNF levels.

Conclusions
Sustained inflammatory signaling, provoked by overexpression of TNF in MHCsTNF mice, induced both atrial structural remodeling by downregulation of Cx40 and increased prevalence of atrial arrhythmias

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Disclosures
The authors have no conflicts to disclose.
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FIGURE LEGENDS

Fig. 1. Intrinsic ECG (leads I, II, and III) and intracardiac electrograms in a WT mouse (left panel) and in a MHCsTNF mouse (right panel). RA, right atrial electrogram; and RV, right ventricular electrogram.

Fig. 2. Examples of supraventricular arrhythmias observed in MHCsTNF mice. The arrhythmias include spontaneous bigeminy (A), atrial tachycardia induced by ventricular extrastimulation (B and C), atrial tachycardia induced by atrial extrastimulation (D), and spontaneous atrial fibrillation (E). I, ECG lead I; LRA, intracardiac low right atrial electrogram; and HRA, intracardiac high right atrial electrogram.

Fig. 3. Electrocardiograms and PCRs of WT and MHCsTNF mice 1 d (left), 1 wk (middle), and 3 wk (right) after birth. A different group of mice was studied at each time interval. Mice demonstrating AV junctional rhythm are marked by an asterisk.

Fig. 4. Representative maps of LV epicardial activation sequences during intrinsic rhythm in WT mice (A) and in MHCsTNF mice (B). Arrows reflect conduction velocities. The maps are displayed such that the top aspect corresponds with the base and the left aspect with the left anterior descending coronary artery. Panel C summarizes LV epicardial conduction velocities in WT and MHCsTNF mice while pacing at LV apex.
Fig. 5. Western blots for Cx40 and Cx43 in WT and MHCsTNF mice. NP, non-phosphorylated isoform; P1, least phosphorylated isoform; P2, most phosphorylated isoform.

Fig. 6. Sample immunofluorescence data from ventricular myocardium in WT (A) and MHCsTNF (B) mice stained for Cx43 (red) both at 40X magnification. Nuclei are stained blue. Scale bar = 100 microns.

Fig. 7. Sample immunofluorescence data from atrial (A and B) and bundle branch (C and D) regions in WT and MHCsTNF mice stained for Cx40 (green) and Cx43 (red) all at 40X magnification. Nuclei are stained blue. Scale bar = 100 microns.
### Table 1. Electrocardiographic and Electrophysiologic Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MHCsTNF (n=8)</th>
<th>Wild Type (n=8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR</td>
<td>182±13</td>
<td>126±10</td>
<td>0.010</td>
</tr>
<tr>
<td>P †</td>
<td>13±1</td>
<td>10±1</td>
<td>0.050</td>
</tr>
<tr>
<td>PR †</td>
<td>11±1</td>
<td>31±1</td>
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<tr>
<td>QRS</td>
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<td>9.0±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>QTc ‡</td>
<td>73±3</td>
<td>46±2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>cSNRT</td>
<td>&gt;73±11</td>
<td>39±5</td>
<td>&lt;0.020</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>AERP</td>
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<td>35±2</td>
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</tr>
<tr>
<td>VERP</td>
<td>51±3</td>
<td>34±2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE. †P wave duration was approximated in all mice from bipolar electrograms recorded in high right atrium because a clear P wave could not be consistently observed on surface ECG in MHCsTNF mice. ‡PR interval was estimated in MHCsTNF mice from bipolar electrograms recorded in low right atrium during junctional rhythm. QT was defined from onset of QRS to return of T wave to baseline and corrected according to: QTc=QT/RR\(^{1/3}\). §Not available for MHCsTNF mice because the atrial refractory period was longer than that of the atrioventricular node. cSNRT, sinus node recovery time following atrial pacing at cycle length of 100 ms (corrected for intrinsic cycle length); AVW, longest atrial pacing cycle length resulting in Wenkebach atrioventricular conduction; AVERP, atrioventricular nodal effective refractory period; AERP, atrial effective refractory period; and VERP, ventricular effective refractory period.
Fig. 1. Intrinsic ECG (leads I, II, and III) and intracardiac electrograms in a WT mouse (left panel) and in a MHCsTNF mouse (right panel). RA, right atrial electrogram; and RV, right ventricular electrogram.
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