Introduction

Myocarditis and subsequent dilated cardiomyopathy (DCM) are major causes of heart failure in...
young patients. Myocarditis is characterized by infiltration of inflammatory cells into the myocardium, with consequent loss of myocytes and development of fibrosis and necrosis. In a significant fraction of patients, the loss of cardiomyocytes leads to ventricular remodeling, permanent ventricular wall dysfunction, DCM, heart failure, and/or arrhythmias. Myocarditis is induced by a variety of means, including genetic susceptibility, toxins, viruses, bacteria, and parasites. It is associated with arrhythmias and sudden death, and the treatment of ventricular arrhythmias is critical for improvement in patient prognosis.

Experimental autoimmune myocarditis (EAM) in the rat is a unique and useful model for understanding giant cell myocarditis and subsequent DCM. EAM rats are highly susceptible to ventricular arrhythmia and prolonged action potential duration (APD). Reduced expression of Ito-related molecules, including Kv4.2, Kv1.5, frequenin and KChIP2 is considered to play a key role in ventricular remodeling, and to cause the characteristic clinical findings of EAM. Cardiac inflammation – a hallmark of myocarditis – is known to increase oxidative stress. Niwano et al. demonstrated previously that the anti-oxidant N-acetylcysteine suppresses ventricular remodeling in EAM rats, suggesting that oxidative stress plays a role in remodeling and the development of myocarditis during the acute phase of myocarditis. It was recently reported that oxidative stress can activate 

Materials and methods

This study protocol was approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine and Cardiovascular Research Institute, and conforms to the guidelines of the American Heart Association.

Induction of experimental autoimmune myocarditis

Purified cardiac myosin (M0531, Sigma Aldrich, Schnelldorf, Germany) was emulsified in an equal volume of complete Freund’s adjuvant (BD biosciences, Heidelberg, Germany) supplemented with mycobacterium tuberculosis H37 Ra (Difco, Detroit, USA) at a concentration of 10 mg/mL. Six-week-old male Lewis rats were immunized by subcutaneous injection of 2 mg purified cardiac myosin in each rear footpad on days 1 and 8 (Myo group: n=15). Control rats received injections of 0.5 mL complete Freund’s adjuvant in the same manner (Control group: n=15). In a separate group of 15 rats, 6 mg steroid was administered simultaneously with cardiac myosin on days 1 and 8 (MyoS group: n=15). Ambulatory Holter monitoring was performed using a telemetric system (Telemetry Research, Auckland, New Zealand).

Histology and inflammatory cytokine assay

Following measurement of hemodynamic parameters, hearts were immediately excised and weighed:
heart weight to body weight ratios were calculated. Hearts were stained with hematoxylin and eosin, and Masson’s trichrome stain. Immunostaining was performed using a tumor necrosis factor-α (TNF-α) antibody to evaluate the degree of inflammation.

Blood was obtained from the abdominal aorta of each rat on day 21. An enzyme-linked immunosorbent assay (ELISA) was performed to determine serum levels of high-mobility group box protein 1 (HMGB1), interleukin 6 (IL-6), and TNF-α. Serum protein levels were quantified with kits for HMGB1 (IBL International, Hamburg, Germany), IL-6 (R&D System, Minneapolis, MN, USA) and TNF-α (R&D System, Minneapolis, MN, USA), according to manufacturer’s instructions.

Optical mapping

On the 21st day after the initial immunization, rats (250–300 g) were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). Chests were opened via median sternotomy and the hearts were rapidly excised and immersed in cold Tyrode’s solution (composition in mmol/L: 125 NaCl, 4.5 KCl, 0.25 MgCl₂, 24 NaHCO₃, 1.8 NaH₂PO₄, 1.8 CaCl₂, and 5.5 glucose). The ascending aorta was immediately cannulated and perfused with Tyrode’s solution prewarmed to 37°C and equilibrated with 95% O₂ and 5% CO₂ to maintain a pH of 7.4. Coronary perfusion pressure was maintained between 80 and 95 mmHg. Two widely spaced bipolar electrodes were used for continuous pseudo-ECG monitoring.

For optical recording, cardiac contractility was inhibited with 10–17 µmol/L blebbistatin. Hearts were stained with di-4-ANEPPS (Invitrogen, California, USA) and excited with quasi-monochromatic light (520 ± 30 nm) from two green LED lamps. Emitted light was collected by an image-intensified charge-coupled device camera (Dalsa Inc., Waterloo, Canada) with a 610–nm long pass filter. Data were gathered at 3.75–ms sampling intervals, acquiring simultaneously from 125 × 125 pixels, each 0.08 mm × 0.08 mm. The mapped area included parts of the right and left ventricular free walls.

Optical recordings were performed during steady-state and programmed stimulation. Programmed stimulation was induced with bipolar electrodes at the lateral side of the left ventricle (LV). Pacing was initiated at a cycle length (CL) of 300 ms, using stimuli of twice the pacing threshold, and was subsequently reduced in decrements of 10 ms until 2:1 capture was achieved. APD at 90% repolarization (APD₉₀) was measured at the base and apex of the LV. APD dispersion was defined as the difference between maximum and minimum APD. After the initial electrophysiological study, we attempted to induce ventricular tachycardia (VT) or ventricular fibrillation (VF) using a standard pacing technique (burst pacing at CLs down to 70 ms). All sustained (>30 s) and non-sustained VT or VF episodes were documented.

Optical mapping and VT induction studies were performed in 6 rats from each group.

Immunoblot analysis of Ca²⁺ handling proteins

Immunoblotting for CaMKII, ryanodine receptor type 2 (RyR2), phospholamban (PLB) and the phosphorylated form of each protein was performed using the following monoclonal antibodies: anti-CaMKII and anti-p-CaMKII (1:1,000; Santa Cruz Biotechnology); anti-RyR2 (1:1000; Abcam Reagents); anti-p-RyR2 (1:1000; Badrilla); and anti-p-PLB (1:1000; Santa Cruz Biotechnology). Targeted antigens were visualized by labeling with corresponding HRP-conjugated secondary IgG (1:5,000; Santa Cruz Biotech-
technology) followed by enhanced chemiluminescence assay (ECL Plus, Amersham, Piscataway, NJ). Blots were scanned, and band intensity was quantified using the Image J software.

Statistical analysis

Data are expressed as mean ± SEM. The Student’s t-test with Bonferroni correction was used to compare the means of two numeric values. The Pearson’s chi-square test was used to compare two categorical variables. Paired t-tests were used to compare the means of maximum slope of restitution curves between baseline and myocarditis. p values <0.05 were considered statistically significant.

Results

Arrhythmia and survival of EAM

For the control, Myo and MyoS groups, heart weights were 1.2 ± 0.1 g, 1.7 ± 0.1 g, and 1.3 ± 0.1 g, respectively (Figure 1A). Heart weight was signifi-
significantly greater in the Myo group than in the control (p=0.002) and MyoS (p=0.03) groups. Compared with controls, the ratio of heart weight to body weight was significantly increased in the Myo group (5.2 ± 0.5 vs. 7.1 ± 0.4, p<0.001), but not in the MyoS group (5.8 ± 0.3, p=0.31). Figure 1B shows histological findings 21 days after the initial immunization with cardiac myosin. While no inflammatory changes were observed in the control rats, there was infiltration of inflammatory cells in Myo rats, including giant cells mainly in the right ventricle and epicardial layer of LV. Severe interstitial fibrosis and increased TNF-α expression were also observed in Myo rats. Infiltration of inflammatory cells, fibrosis and increased TNF-α expression were attenuated in the MyoS group. Figure 1C shows the Kaplan–Meier survival curves for the three groups. While 5 out of 15 rats (33%) in the Myo group died suddenly at 17 ± 2 days after induction of myocarditis, no rats and one rats (6%) died in the control and MyoS groups, respectively. The Myo group had a lower cumulative survival rate than the control group (p=0.02). Figure 1D shows the various arrhythmias recorded by ambulatory Holter monitoring in the Myo group. VT, sinus pause and atrioventricular block were observed in 4 rats in the Myo group, 3 in the control group, and 3 in the MyoS group.
group. While arrhythmias were not observed in control rats, they were observed in 5 of the 9 surviving rats (56%) in the Myo group, and in none of the 15 rats in the MyoS group (p=0.03).

**Inflammatory markers in EAM**

Serum levels of HMGB1, IL-6 and TNF-α are presented in Supplementary Table 1. Compared with controls, serum levels of HMGB1, IL-6 and TNF-α in the Myo group were increased 1.1-fold (p<0.001), 2.1-fold (p<0.001), and 4.0-fold (p<0.001), respectively. Conversely, no significant change in expression levels was observed in the MyoS group.

**Increased APD and APD dispersion in EAM**

Optical mapping was performed in 6 rats from each group. Figure 2A shows the action potential traces obtained from the base of the LV during pacing CLs of 300 ms and 200 ms in Langendorff-perfused rat hearts. Mean APD in the Myo group was relatively prolonged compared to the control and MyoS groups. A comparison of mean APD in all
three groups is presented in Figure 2B. Mean APD$_{90}$ at a pacing CL of 300 ms was significantly longer in the Myo than in the control group (152 ± 52 ms vs. 98 ± 7 ms, p=0.03). APD$_{90}$ was not prolonged in the MyoS group (89 ± 7 ms, p=1.0), however. Mean APD$_{90}$ was also longer in Myo rats than control and MyoS rats at pacing CLs of 200 and 160 ms. Figure 2C shows corresponding activation and repolarization maps. The conduction time of both ventricles was prolonged in the Myo group compared with control and MyoS groups. APD dispersion was similarly increased in the Myo group (23.6 ± 3.6 ms) compared to controls (5.8 ± 2.0 ms, p<0.001), though this increase was attenuated in the MyoS group (10.5 ± 1.7 ms, p<0.001). Figure 2D shows the optical action potentials of early afterdepolarization (EAD) in Myo rats during sinus rhythm.

APD alternans and ventricular arrhythmia in EAM

APD alternans were evaluated in all 3 groups (Figure 3). Discordant alternans were observed during a pacing CL of 80 ms in control rats (Figure 3A), 150 ms in Myo rats (Figure 3B) and 110 ms in MyoS rats (Figure 3C). Compared with controls, the pacing CL required to induce discordant alternans in Myo rats was increased from 80 ± 9 ms to 178 ± 22 ms (p<0.001);
no significant difference was observed between control and MyoS rats (114 ± 5 ms, p=0.06). These findings suggest that spatially discordant alternans were more easily induced in the Myo group, than in other groups. Conduction block was also more frequently observed at longer pacing CLs in the Myo group (107 ± 10 ms) than in the control group (73 ± 6 ms, p<0.001), which was not significantly different from the MyoS group (84 ± 10 ms, p=0.24).

Ventricular arrhythmias were evaluated in 6 rats from each group. For the control, Myo and MyoS groups, triggered activity was observed in 1 (17%), 5 (83%) and 2 (33%) rats, respectively. Of the 6 rats in each group, ventricular arrhythmias were induced in 0, 6 (100%) and 1 (27%), respectively. The Myo group exhibited triggered activity (p=0.02) and ventricular arrhythmia (p=0.03) more frequently than the control group. Conversely, the MyoS group showed no significant difference in triggered activity (p=0.55) or ventricular arrhythmia (p=0.57) compared with controls.

**Increased APD restitution slope and dominant frequency in EAM**

Figure 4 shows a typical example of an APD resti—
The restitution curve measured at the LV base and apex. For the control, Myo, and MyoS groups, the maximum APD restitution slopes were $0.23 \pm 0.09$, $0.70 \pm 0.10$ and $0.28 \pm 0.04$ at the LV base, respectively. Slopes at the LV apex were $0.26 \pm 0.07$, $1.19 \pm 0.11$ and $0.24 \pm 0.09$, respectively. The APD restitution slopes in the Myo group were steeper than controls at both the LV base (p<0.001) and apex (p<0.001). A significant increase relative to control was not observed in the MyoS group, however (p=1.00).

The dominant frequency (DF) maps of VF episodes were evaluated in the 3 groups. DF was 6 Hz in the control group (Figure 5A), 8 Hz in the Myo group (Figure 5B) and 5 Hz in the MyoS group (Figure 5C). Compared with the control group, mean DF was significantly higher in the Myo group ($8.1 \pm 1.0$ vs. $6.3 \pm 0.3$ Hz, p=0.006) but not in the MyoS group ($5.8 \pm 0.5$ Hz, p=0.82).

**Increased p-CaMKII, ryanodine and p-phospholamban in EAM**

Figure 6 shows a Western blot of Ca$^{2+}$ handling proteins. Compared with controls, p-CaMKII, RyR2, p-RyR2 and p-PLB were increased 2.5-fold (p<0.001), 2.9-fold (p<0.001), 5.1-fold (p<0.001) and 4.0-fold (p<0.001) in the Myo group (Figure 6). These increases are not evident in the MyoS group. Asterisks denote p<0.05.

*Figure 6. Western blot of Ca$^{2+}$ handling proteins. (A) Western blot analysis. (B) Quantification of band intensity showing increased expression of p-CaMKII, RyR2, p-RyR2 and p-PLB in EAM. These increases are not evident in the MyoS group. Asterisks denote p<0.05.*
and 2.3-fold (p<0.001), respectively, in the Myo group. This significant increase was not observed in the MyoS group, CaMKII expression was not different between the 3 groups.

Discussion

The salient findings of this study were four-fold. Firstly, EAM rats were found to exhibit fatal arrhythmia and decreased survival relative to controls. Secondly, the EAM model was characterized by prolonged APD with increased APD dispersion, easily inducible spatially discordant alternans, steeper APD restitution slopes and increased ventricular arrhythmia. Thirdly, increased activity of calcium handling proteins, including p-CaMKII, p-RyR and p-PLB, is induced in the EAM model. Finally, EAM-related arrhythmia and the activation of calcium handling proteins were attenuated by pretreatment with an anti-inflammatory steroid. Our results indicate that EAM can induce arrhythmia via CaMKII activation under conditions of inflammation and oxidative stress.

Ventricular arrhythmias and decreased survival in EAM rats

Ventricular arrhythmia is a significant cause of death, along with heart failure, in acute myocarditis. The myosin-induced EAM rat is one of the animal models used to study the events that occur in human giant cell myocarditis. The EAM model comprises an acute inflammatory phase evoked 2 weeks after myosin injection, and a subsequent recovery phase initiated around the 25th day after injection, followed by a dilated cardiomyopathy-like phase associated with chronic heart failure. In this study, 9 out of 15 EAM rats (60%) exhibited cardiac events, including sudden death and arrhythmia. These results suggest that the low survival rate associated with the EAM model is likely related to the development of arrhythmia. Interestingly, in addition to VT, sinus dysfunction and atrioventricular block were also commonly observed in the EAM model.

Increased repolarization gradient and CaM–KII activation in EAM

APD was prolonged in myosin-induced EAM. This prolongation may be explained by an initial reduction in Ito-related currents, following downregulation of Kv4.2, Kv1.5, frequenin and KChIP2. The role of Ca2+ handling proteins in EAM has not yet been established, however. Our results indicate that the activation of Ca2+ handling proteins may play an important role in EAM-induced arrhythmia. We observed increased expression of CaMKII and phosphorylated CaMKII following induction of myocarditis, suggesting that CaMKII activation may facilitate APD prolongation, representing a novel arrhythmogenic mechanism of EAM.

EAM–associated inflammation increases oxidative stress. In addition to activation by elevated intracellular Ca2+ levels (following β-adrenergic receptor stimulation) CaMKII activity is also known to be enhanced under pro-oxidant conditions. Oxidation of paired regulatory domain methionine residues sustains CaMKII activity in the absence of Ca2+/CaM. H2O2-induced afterdepolarizations depend on both impaired Ito inactivation, to reduce repolarization reserve, and enhanced Ica,L, to reverse repolarization, both of which are facilitated by CaMKII activation.

Consistent with elevated p–CaMKII, p–RyR2 and p–PLB levels were also increased in EAM. The RyR, or calcium release channel, on the sarcoplasmic reticulum is the major source of calcium required for excitation–contraction coupling in cardiac muscle,
Hyperphosphorylation of RyR2 results in defective function due to increased sensitivity to Ca\(^{2+}\)–induced activation.\(^\text{21}\)

Until now, it has not been clear whether APD prolongation in myocardial cells is homogeneous or heterogeneous in nature, because previous studies have only evaluated a single site in the heart using a macroscopic electrophysiological technique. In this study, using optical mapping, we observed heterogeneous APD prolongation and increased APD dispersion in EAM. Although APD prolongation is the main mechanism of long QT syndrome, enhanced dispersion of repolarization is critical for the induction of fatal arrhythmia.\(^\text{22}\) Transmural and apicobasal dispersion of repolarization was shown to be responsible for the initiation of reentrant activation in long QT syndrome patients. It was reported that numerous mammalian species, including humans, exhibit apex–base differences in cardiac repolarization.\(^\text{23}\) Increased spatial dispersion of repolarization across the anterior epicardial surface was also demonstrated to represent the electrical basis for spontaneous malignant arrhythmias in long QT type 2 rabbits.\(^\text{24}\)

**Mechanisms of ventricular arrhythmia in EAM**

Spatially discordant alternans were easily induced in EAM. These cause an increase in the spatial dispersion of repolarization, and are thought to result in T-wave alternans.\(^\text{25}\) T-wave alternans are precursors of cardiac electrical instability, and consequently sudden cardiac death.\(^\text{35}\) Spatially discordant alternans can be explained by the increased steepness of APD restitution slopes in EAM. A steep slope of electrical restitution ≥1 is especially associated with VF.\(^\text{28}\) Furthermore, the increased prevalence of discordant alternans may also have been the result of fibrosis and reduced gap junction conductance in EAM; these have previously been shown to lower the threshold for spatially discordant alternans.\(^\text{30–33}\) Finally, the development of discordant alternans may also be related to altered expression and activity of Ca\(^{2+}\) handling proteins. The net effects of EAM remodeling promote Ca\(^{2+}\) alternans via phosphorylation of RyRs and CaMKII signaling to increase their Ca\(^{2+}\) sensitivity (increasing both gain and leak).\(^\text{32–33}\)

**Attenuation of EAM-related arrhythmia by anti-inflammatory therapy**

In this model, the overexpression of inflammatory cytokines, such as HMGB–1, TNF–α and IL–6 can induce myocardial damage and possibly cause ventricular remodeling.\(^\text{6,34}\) Because these inflammatory cytokines are strong inducers of nitric oxide and reactive oxygen species, they may promote cardiac injury and electrical remodeling through precipitation of hyper–oxidative conditions, Niwano et al.\(^\text{9}\) previously reported that the anti–oxidant N–acetylcysteine suppressed ventricular remodeling in EAM rats. Concordantly, we have demonstrated that anti–inflammatory steroid therapy suppresses infiltration of inflammatory cells and EAM–induced electrical remodeling. Moreover, steroid pretreatment was associated with improved survival. This indicates the importance of inflammation and oxidative stress in remodeling and the progression of myocarditis.

**Study limitations**

In this study, we induced myocarditis by injection of cardiac myosin. Therefore, the arrhythmogenic
mechanisms delineated may not be representative of those underlying myocarditis caused by viral infection or other etiologies. Nonetheless, autoimmunization to myosin may represent a final common pathway of myocarditis. Moreover, inflammation and oxidative stress are frequently observed in diseased hearts.

Secondly, electrophysiological tests and optical mapping were performed on the 14th day after initial immunization. To identify individual differences after induction of myocarditis, it would be useful to perform optical mapping at different time points after immunization. Because an electrophysiological test could not be performed in rats that succumbed to sudden death, we could not provide direct evidence that APD dispersion and discordant alternans were related to mortality.

Finally, ventricular fibrillation was recorded during sudden death in rats with myocarditis. However, because Holter monitoring was performed in only 5 rats that died suddenly, we cannot rule out the possibility that the AV block was responsible for sudden death.

Conclusion

EAM–induced arrhythmia is associated with increased repolarization dispersion and spatially discordant alternans. These electrical changes may be related to altered Ca\(^{2+}\) handling protein activity, including phosphorylation of CaMKII and RyR2. These arrhythmogenic effects are associated with increased inflammation and oxidative stress, and in this study were attenuated by anti–inflammatory therapy.

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Disclosures

None.

References


