Alterations in adhesion junction precede gap junction remodelling during the development of heart failure in cardiomyopathic hamsters

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Aims
The intercalated disc (ID) contains two complexes, the adhesion junction (AJ) and the gap junction (GJ). We studied ID remodelling and its potential role in arrhythmogenesis and investigated the effects of olmesartan on ID remodelling during development of heart failure (HF) in UM-X7.1 cardiomyopathic hamsters.

Methods and results
The UM-X7.1 hamsters showed left ventricular (LV) hypertrophy by the age of 10–15 weeks and a moderate impairment in LV contractility at 20 weeks. At age 10–15 weeks, 10–20% of the hamsters died suddenly without HF, and ventricular tachycardia (VT)/ventricular fibrillation (VF) was induced in ~30% of hamsters. Electron microscopy showed that density linking cell-to-cell adhesion was irregular and unclearly defined, and filamentous structures attached to electron-dense components were arranged in disorder. Western blotting showed that the total cellular expression level of β-catenin was decreased, and expression of nuclear β-catenin, which functions as a T-cell factor/lymphocyte enhancer binding factor transcriptional activator, was also remarkably decreased. At age 20 weeks, LV connexin43 expression showed a remarkable decrease, and the VT/VF induction rate was ~90%. In UM-X7.1 hamsters, olmesartan improved abnormal ID ultrastructural changes, attenuated the decrease of total cellular and nuclear β-catenin expression, decreased VT/VF induction, and improved survival rate.

Conclusion
These results suggest that changes in AJ protein precede connexin43 GJ alterations, and ID remodelling might contribute to arrhythmogenesis during the development of HF. Angiotensin receptor blockade might be a new therapy for lethal ventricular arrhythmia by modulating both AJ and GJ remodelling.

Keywords
Intercalated disc • Arrhythmia • Heart failure • Cardiomyopathy • Adhesion junction

1. Introduction
Life-threatening ventricular tachyarrhythmias often occur in patients with heart failure even at a compensated stage. The underlying mechanisms are not well understood. The intercalated disc (ID) contains different junctional complexes [adhesion junctions (AJs; adherens junctions and desmosomes) and gap junctions (GJs)] that enable the myocardium to function as a syncytium.

In the heart, GJs provide the pathway for intercellular current flow, enabling co-ordinated action potential propagation and contraction. Gap junction channels are constructed from connexins (Cxs). In adult ventricular muscle, cell-to-cell coupling is provided predominantly by Cx43 channels and to a lesser extent by Cx45. It is well known that quantitative and qualitative alterations in Cx43 are common findings in diseased myocardium. Recently, some studies demonstrated that cardiac-specific changes
of Cx43 cause slow myocardium conduction velocity and induce unidirectional block, resulting in an arrhythmogenic substrate and sudden cardiac death. However, it is not well understood how GJs are regulated and maintained at the ID in diseased myocardium.

The AJs (adherens junctions and desmosomes) mediate intercellular coupling at IDs through linkage to the actin cytoskeleton and desmin filament, respectively. The adherens junction is made up of N-cadherin, and its cytoplasmic binding proteins, catenins or actinin. In the desmosome, desmoglein and desmocollin are found, and they interact with linker proteins, plakoglobin and desmoplakin. Adherens junction formation plays an important role in GJ assembly in cultured adult rat cardiomyocytes. Recent studies reported that Cx43 is directly targeted by microtubule plus-end-tracking proteins from the cell interior to N-cadherin and β-catenin, deletion of N-cadherin in the heart leads to dissolution of the ID and induces sudden cardiac death, and that there is a significant decrease of Cx43 in N-cadherin knockout hearts, causing ventricular conduction slowing. Moreover, recent studies have linked arrhythmogenic right ventricular cardiomyopathy, an inherited disease with sudden cardiac death, with mutation in proteins of the cardiac desmosome. It is estimated that the mutations linked to familial arrhythmogenic right ventricular cardiomyopathy are in the gene coding for desmoplakin protein, plakophilin (PKP)2.

We investigated ID remodelling and its potential role in the pathogenesis of ventricular arrhythmias, and studied the effects of the angiotensin II receptor blocker, olmesartan (OS), on ID alterations during development of heart failure in UM-X7.1 cardiomyopathic hamsters (UM-X7.1), Syrian ß-sarcoglycan-deficient hamsters and a derivative of the BIO14.6 strain. We used UM-X7.1 because of its characteristic features. The animals show left ventricular (LV) hyper trophy at age 10–15 weeks, moderate compensated LV contractile dysfunction at age 20 weeks, and serious decompensated heart failure at ages beyond ~24 weeks. In the present study, we investigated stage-dependent changes in ID protein expressions in UM-X7.1 at ages 6–20 weeks. Associated alterations in thepropensities for life-threatening arrhythmias were also investigated. The results reveal that changes in AJ protein precede GJ remodelling, and ID remodelling might contribute toventricular arrhythmogenesis during development of heart failure. Treatment with the angiotensin II receptor blocker, olmesartan, might be a new upstream therapy for ventricular arrhythmias by modulating ID alterations.

2. Methods

2.1 Animal model

UM-X7.1 hamsters and sex- and age-matched normal golden hamsters (control; Japan SLC Inc., Hamamatsu, Japan) were used as experimental animals. For olmesartan (kindly provided from Daiichi-Sankyo Co., Tokyo, Japan) treatment, 0.01% OS was administered in drinking water from age 6 weeks and thereafter. Our choice of this dose was based on a pilot study showing that the serum level of OS increased significantly without producing a decrease in blood pressure in the hamsters. All animal procedures were approved by the Yamaguchi University Experimental Animal Care and Use Committee. The investigation conformed with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

2.2 Echocardiography, histology and electron microscopy

Echocardiography was performed using an echocardiograph model HDI-5000, with a 15 MHz sector scan probe (Philips, Amsterdam, The Netherlands). For recording the echocardiogram, the animal was placed on its back under anaesthesia induced with sodium pentobarbital (15 mg/kg, i.m.) once every examination. This relatively small dose of sodium pentobarbital did not affect the level of blood pressure, heart rate, or the state of respiration in either type of hamster at any stage. The probe was gently placed in contact with the middle of the thorax through an ultrasonic transmission medium (Aquasonic 100; Parker Laboratories, Orange, NJ, USA). Left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and fractional shortening (FS) were calculated according to a standard formula, as applied in our previous reports.

The heart was removed from hamsters anaesthetized deeply with sodium pentobarbital (30 mg/kg, i.p.) and immersed in 0.9% NaCl. Before the thoracotomy was performed, we assessed the adequacy of anaesthesia by monitoring ECG and respiratory rate. Specimens for histological examination were obtained from heart cross slices cut mid-way, and 4-µm-thick sections were cut and stained. The connective tissue volume fraction was assessed with the aid of Azan staining and calculated as previously described. Cardiac tissue was quickly cut into 1 mm³ cubes, immersion fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) overnight at 4°C, and post-fixed in 1% buffered osmium tetroxide. The specimens were then dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections (90 nm), double stained with uranyl acetate and lead citrate, were examined under an electron microscope (H-800; Hitachi, Tokyo, Japan). Qualitative morphology of IDs was analysed by the methods previously described.

2.3 Immunoblotting and immunohistochemistry

For immunoblotting of total cellular fraction, LV tissues were lysed with lysis buffer (150 mmol/L NaCl, 100 mmol/L Na3VO4, 1.5 mmol/L MgCl2, 50 mmol/L NaF, 50 mmol/L HEPES, 1 mmol/L EGTA, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, and 10 µg/mL leupeptin, pH 7.5), and the tissue extracts were electrophoresed in sodium dodecyl sulfate–polyacrylamide gels as previously described. The nuclear fraction was prepared as previously described. The following primary antibodies were used: rabbit anti-Cx43 antibody (1:1000 dilution; Zymed, San Francisco, CA, USA), mouse anti-N-cadherin antibody (1:5000 dilution; Zymed), mouse anti-β-catenin antibody (1:500 dilution; Zymed), mouse anti-desmoglein1 + 2 antibody (1:200 dilution; PROGEN Biotechnik, Heidelberg, Germany), mouse anti-γ-catenin antibody (1:10 000 dilution; BD transduction Laboratories, Franklin Lakes, NJ, USA), mouse anti-plakophilin2 antibody (1:100 dilution; BIODSIGN international, Saco, ME, USA), mouse anti-GAPDH antibody (1:1000 dilution; Sigma, St Louis, MO, USA), and mouse anti-histone H1 antibody (1:1000 dilution; Leinco Technologies Inc., St Louis, MO, USA). For the second antibody, the following were used: anti-rabbit IgG horseradish peroxidase conjugate antibody (1:1000 dilution; Promega, Madison, WI, USA), and anti-mouse IgG horseradish peroxidase conjugate antibody (1:1000 dilution; GE Healthcare, Little Chalfont, UK). The amount of protein recognized by the antibodies was quantified by means of an ECL™ immunoblotting detection system (GE Healthcare).

For immunohistochemistry, the following primary antibodies were used: rabbit anti-Cx43 antibody (1:1000 dilution; Zymed), mouse anti-N-cadherin antibody (1:250 dilution; Zymed), and mouse anti-β-catenin antibody (1:100 dilution; Zymed). After quenching and blocking (10% goat serum), samples were incubated with the respective antibody overnight at room temperature. These primary antibodies were visualized by fluorescein isothiocyanate-conjugated anti-mouse IgG.
2.4 Reverse transcription and PCR (RT-PCR) amplification of Cx43 mRNA
Total RNA was isolated from frozen hamster hearts, using RNeasy Fibrous Tissue Mini kit (Qiagen, Hilden, Germany), and reverse transcribed with Omniscript Reverse Transcriptase (Qiagen) and oligo-(dT)15 primer (Takara Biotechnology, Shiga, Japan). Levels of RT-PCR product were measured using Quantitect SYBR Green Kit (Qiagen). The following primers were used for amplification of Cx43 and GAPDH: sense primer 5′-CCGGTGACAGAAACAATTCC-3′ and anti-sense primer 5′-GTCTGCAGGAAGTCAAAAG-3′ for Cx43; and sense primer 5′-TGAATCGGGGAAGTCAAAAG-3′ and anti-sense primer 5′-GCCAAAGTCAAAAAG-3′ for GAPDH, respectively.

2.5 Electrophoretic mobility shift assay
Electrophoretic mobility shift assays (EMSAs) were performed using Light-Shift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA). As the optimal T-cell factor/lymphocyte enhancer binding factor 5 (TCF/LEF) probe, we used a double-stranded nucleotide oligomer (CCC TTT GAT CTT ACC), based on the previous report.24 The nuclear fraction was prepared as previously described.23 The antibody used was mouse anti-β-catenin antibody (BD Transduction Laboratories, Franklin Lakes, NJ, USA). After electrophoresis, the gels were transferred onto the membrane (Biodyne B Nylon Membrane; Pall, Pensacola, FL, USA). The blotted bands were processed for Chemiluminescent Nucleic Acid Detection module (Pierce).

2.6 Induction of ventricular tachycardia/ventricular fibrillation
We used a Langendorff system to examine the ventricular tachycardias (VT)/ventricular fibrillation (VF) induction in the heart. Details of the system were described in our previous reports.11 In brief, isolated hamster hearts were continuously perfused on a Langendorff apparatus with modified Krebs–Ringer solution equilibrated with 95% O2 and 5% CO2 (37 °C, pH 7.4). We used a premature stimulation protocol to induce VT/VF. Following rapid stimulation at an S1–S1 interval of 150 ms, a single premature stimulation (S2) was applied at the centre of the anterior LV free wall, and the sequence was repeated with progressive shortening of the S1–S2 interval. Widely spaced bipolar electrograms were recorded between the apex of the LV and the high lateral wall of the RV to monitor the whole ventricular excitation.

2.7 Statistics
All data are presented means ± SEM. Comparisons between data were made by ANOVA. Frequency of VT/VF induction was compared by χ² analysis. The cumulative survival curve was plotted by the Kaplan—Meier method, and statistical difference was evaluated by a log-rank test. Differences were taken to be significant at P < 0.05. All data were analysed using PASW statistics18 software (SPSS Inc., Chicago, IL, USA).

3. Results
3.1 Somatic growth, cardiac growth and echocardiography
The body weight of UM-X7.1 was significantly smaller at age 10–15 and 20 weeks than that of golden hamsters; however, the value of the biventricular weight-to-body weight ratio was significantly higher in each UM-X7.1 group (Table 1, n = 7 in each group). In UM-X7.1 at age 10–15 weeks, olmesartan treatment significantly inhibited the increase of biventricular weight/body weight. Table 2 presents the changes in LV dimensions and FS in UM-X7.1 and golden hamster groups (n = 7 in each group). At age 20 weeks, LVEDD and LVESD were remarkably greater, and FS was significantly lower in UM-X7.1 than in golden hamsters. Olmesartan treatment did not significantly improve these parameters in UM-X7.1 at age 10–15 and 20 weeks.

As shown in Figure 1 (Kaplan–Meier survival curve), UM-X7.1 died from congestive heart failure over age 25 weeks. Interestingly, at age 10–15 weeks, about 10–20% UM-X7.1 died suddenly. Based on these results, we focused on the ID remodelling during the hypertrophic stage (age 10–15 weeks) in UM-X7.1. Interestingly, olmesartan treatment at the dose used in the present study significantly improved survival rate in UM-X7.1 without affecting cardiac parameters of LVEDD, LVESD, and FS.

3.2 Histology and electron microscopy
The connective tissue volume fraction of UM-X7.1 was significantly higher than that of golden hamsters at age 10–15 weeks (4.8 ± 0.8 vs. 1.0 ± 0.1%, P < 0.01) and at age 20 weeks (6.1 ± 0.7 vs. 1.6 ± 0.4%, P < 0.01; Figure 2A; n = 7 in each group). The treatment with olmesartan did not significantly attenuate the increase of connective tissue volume fraction of UM-X7.1.

Electron microscopy revealed that the IDs between cardiomyocytes of the UM-X7.1 were more highly convoluted and appeared more electron dense compared with those of hearts of golden hamsters (Figure 2B). The high electron density was due to abnormally developed desmosomes with great width, which consisted of long segments or an accumulation of repetitive short ones. These features were already apparent at age 6 weeks in UM-X7.1, although not significant, and tended to become more marked in the older animals. Myofibrillar derangement and loss (myofibrilysis) were also notable at the adherens junction of the IDs in UM-X7.1, consistent with the previous report.25

In UM-X7.1 at age 10–15 weeks, the overall electron density of the AJs was weakened in the hearts of the olmesartan-treated animals (Figure 2B). The finding of abnormally developed adherens junctions in the untreated UM-X7.1 heart was apparently attenuated by olmesartan treatment (Figure 2C); 1.1 ± 0.1 in golden hamsters, 1.9 ± 0.1 in UM-X7.1, and 1.5 ± 0.2, P < 0.05 in UM-X7.1 with olmesartan at 10–15 weeks; and 1.2 ± 0.2 in golden hamsters, 1.9 ± 0.2 in UM-X7.1, and 1.5 ± 0.1, P < 0.05 in UM-X7.1 with olmesartan at 25 weeks.

These results showed that in UM-X7.1 abnormal electron microscopic findings were observed at an early stage of development, and olmesartan attenuated the abnormal adherens junction density.

3.3 Intercalated disc protein expression
To quantify ID protein expression, western blot analysis was performed. There was no significant difference in N-cadherin expression...
Table 1: Pathological findings in hamsters

<table>
<thead>
<tr>
<th>Group</th>
<th>10–15 weeks</th>
<th>20 weeks</th>
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<tr>
<td>Olmesartan</td>
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<tr>
<td>Body weight (g)</td>
<td>25.8 ± 0.2</td>
<td>24.6 ± 0.2</td>
</tr>
<tr>
<td>Biventricular weight (mg)</td>
<td>32.1 ± 0.1</td>
<td>31.1 ± 0.1</td>
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<tr>
<td>Biventricular weight/body weight</td>
<td>4.2 ± 0.0</td>
<td>3.8 ± 0.0</td>
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Values are means ± SEM, 

- **P** < 0.01 vs. corresponding 6 week value of each group.
- †† **P** < 0.01 vs. age-matched olmesartan (−) hamster group.

between UM-X7.1 and golden hamsters at each age (Figure 3, I, A; n = 7 in each group). β-Catenin expression in UM-X7.1 at age 6, 10–15, and 20 weeks, as compared with golden hamsters, was significantly reduced by 40, 42, and 43%, respectively (Figure 3, I, B; n = 7 in each group). The total content of Cx43 in UM-X7.1 hearts was decreased at age 10–15 weeks compared with golden hamster hearts at the same age (0.72 ± 0.04 vs. 0.98 ± 0.05, P < 0.01) and at age 20 weeks (0.28 ± 0.03 vs. 1.04 ± 0.13, P < 0.01; Figure 3, I, C; n = 7 in each group). Olmesartan treatment attenuated the decrease of β-catenin and Cx43 in UM-X7.1 at age 20 weeks (β-catenin 0.55 ± 0.05 without olmesartan vs. 0.99 ± 0.05 with olmesartan, P < 0.01; and Cx43 0.28 ± 0.03 without olmesartan vs. 0.53 ± 0.03 with olmesartan, P < 0.05; Figure 3, I, B and C). In golden hamsters, olmesartan did not affect the expression level of β-catenin and Cx43 proteins (Figure 3, I). Regarding the desmosomal protein expressions, there were no significant differences in expressions of desmoglein, plakophilin (PKP)2, and γ-catenin (Figure 3, II) compared with golden hamsters.

The expression of Cx43 mRNA in UM-X7.1 hearts tended to decrease at age 10–15 weeks (0.73 ± 0.06 vs. 0.99 ± 0.05 in golden hamsters) and was significantly decreased at age 20 weeks (0.55 ± 0.07, P < 0.05 vs. 1.02 ± 0.07 in golden hamsters; Figure 3, III; n = 7 in each group). Olmesartan treatment attenuated the decrease of Cx43 mRNA in UM-X7.1 (0.73 ± 0.06 without olmesartan vs. 1.23 ± 0.10 with olmesartan, P < 0.05 at age 10–15 weeks; and 0.55 ± 0.07 without olmesartan vs. 1.21 ± 0.27 with olmesartan, P < 0.01 at age 20 weeks). In golden hamsters, olmesartan did not affect the expression level of Cx43 mRNA.

Quantitative immunofluorescence microscopy revealed that in UM-X7.1 at age 20 weeks, the N-cadherin positive area at IDs significantly increased (by 65%) compared with age-matched golden hamsters, and olmesartan did not significantly affect the increase of N-cadherin (Figure 4A; n = 7 in each group). In UM-X7.1, although the expression of β-catenin at IDs did not show any significant differences at any age, olmesartan tended, although not significantly, to increase its expression at age 10–15 weeks (Figure 4B). For Cx43, the positive area at IDs in UM-X7.1 hearts was decreased at age 10–15 weeks (1.0 ± 0.2, P < 0.01 vs. 1.3 ± 0.1 in golden hamsters at age 10–15 weeks) and at age 20 weeks (0.6 ± 0.2, P < 0.01 vs. 1.4 ± 0.1 in golden hamsters at age 20 weeks; Figure 4C; n = 7 in each group). Olmesartan treatment attenuated the decrease of the Cx43 positive area at IDs in UM-X7.1 at age 20 weeks (0.6 ± 0.2 without olmesartan vs. 0.8 ± 0.1 with olmesartan, P < 0.05; Figure 4C).

To clarify the dislocation of β-catenin in cardiomyocytes, we investigated the β-catenin expression of the nuclear fraction. The nuclear β-catenin functions as a TCF/LEF transcriptional activator, and Cx43 is known to be one of the TCF/LEF target genes. Interestingly, nuclear β-catenin expression was remarkably decreased in UM-X7.1 at age 10–15 weeks (0.51 ± 0.05, P < 0.05) compared with golden hamsters (0.79 ± 0.05; Figure 5B; n = 7 in each group). Olmesartan treatment significantly inhibited the decrease of nuclear β-catenin expression in UM-X7.1 (1.01 ± 0.11 P < 0.01 vs. the group without olmesartan). In addition, olmesartan also significantly increased nuclear β-catenin expression in golden hamsters (1.22 ± 0.09, P < 0.01 vs. 0.79 ± 0.05 in golden hamsters without olmesartan).

These results indicated that in UM-X7.1, a decrease of total cellular β-catenin expression precedes Cx43 Gj remodelling, followed by a marked decrease of nuclear β-catenin expression. Olmesartan

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increased β-catenin expression in both IDs and the nuclear fraction of UM-X7.1 cardiomyocytes at age 10–15 weeks.

3.4 Electrophoretic mobility shift assay

β-Catenin interacts with members of the TCF/LEF classes of transcriptional factors to activate genes involved in development.26 Interestingly, Cx43 is known to be one of the TCF/LEF target genes. We investigated the possibility that the decrease of β-catenin expression in the nuclear fraction is associated with a decrease of TCF/LEF-dependent transcription. The EMSA data of nuclear extract with the TCF/LEF-binding site oligomer are presented in lane 2 of Figure 6. The TCF/LEF DNA–β-catenin binding complex was super-shifted when anti-β-catenin antibody was added (Figure 6, lane 4). In UM-X7.1, the super-shifted complexes band was decreased compared with golden hamsters (Figure 6, lane 6), and olmesartan attenuated the decrease of the super-shifted band in UM-X7.1 (Figure 6, lane 8). These results indicate that in UM-X7.1 at 10–15 weeks, the TCF/LEF DNA–β-catenin binding complex in the nucleus was reduced, resulting in the decrease in the expression of Cx43 gene/Cx43protein.

3.5 Vulnerability to VT/VF

Vulnerability to VT/VF was investigated by a premature stimulation method. Following 19 basic rapid stimulations (S1–S1 ¼ 150 ms), a single premature stimulus (S2) was applied with progressive shortening of the S1–S2 interval (Table 3). In golden hamsters (n = 12 at each age), no arrhythmias were induced. In UM-X7.1, in contrast, VT/VF was induced in four of 14 hamsters at age 10–15 weeks (29%, P < 0.05 vs. golden at age 10–15 weeks) and in 12 of 14 hamsters at age 20 weeks (86%, P < 0.01 vs. UM-X7.1 at age 10–15 weeks). In UM-X7.1 at age 10–15 weeks, although olmesartan treatment tended to attenuate the decrease of VT/VF induction (18%) compared with the untreated group (29%), this was not statistically significant. Also in olmesartan-treated UM-X7.1 at age 10–15 weeks, VT/VF induction showed no significant difference between UM-X7.1 at age 6 weeks (P = 0.06) or corresponding golden hamsters (P = 0.06). Olmesartan remarkably inhibited the vulnerability to VT/VF in UM-X7.1 at age 20 weeks. The lower panel of Table 3 shows representative ECGs of hamsters of each group.

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/92/1/95/540712/821189540712) Kaplan–Meier survival curves for golden and UM-X7.1 hamsters. small-dotted line, golden hamster (n = 60); large-dotted line, golden hamster with olmesartan (OS; n = 30); black line, UM-X7.1 (n = 108); and grey line, UM-X7.1 with OS (n = 101).

### Table 2 Echo geometry findings and cardiac function

<table>
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<tr>
<th></th>
<th>Golden hamster</th>
<th>UM-X7.1</th>
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<tr>
<td></td>
<td>6 weeks (–)</td>
<td>10–15 weeks (–)</td>
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<tr>
<td></td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.5 ± 0.1</td>
<td>4.7 ± 0.0</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.8 ± 0.1</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>FS (%)</td>
<td>37 ± 1</td>
<td>40 ± 1</td>
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</table>

Values are means ± SEM. LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening. *P < 0.05, **P < 0.01 vs. corresponding golden hamster. †P < 0.05, ††P < 0.01 vs. corresponding 6 week value of each group.
4. Discussion

The novel finding of this study is that UM-X7.1 at a hypertrophic stage of 10–15 weeks show a remarkable reduction in the amount of β-catenin protein in both the total cellular and the nuclear fractions without a decrease of the expression at IDs. The decrease of total cellular β-catenin precedes Cx43 GJ remodelling and might enhance the propensity to VT/VF. The angiotensin II receptor blocker, olmesartan, affected ID protein remodelling, decreased VT/VF induction, and improved survival rate in UM-X7.1.

4.1 Intercalated disc remodelling and arrhythmogenesis

Alterations in electrical coupling through GJs of cardiomyocytes play an important role in arrhythmogenesis in many forms of heart disease. Abnormal mechanical coupling through adhesion junctions (adherens junctions and desmosomes) occurs in experimental animals, and diseased human myocardium, and also plays a critical role as an arrhythmogenic substrate. However, the relationship between GJs and AJs of cardiomyocytes is not completely understood. Observational investigations of the dynamics of GJs and AJs in cultured cells during postnatal mammalian heart development have suggested that GJs are not necessary for the establishment of cell AJs at the ID. In addition, Gutstein et al. reported that despite the absence of GJs in conditional knockout hearts, the adherens junction and desmosomal distribution and morphology at the ID remained intact. They concluded the GJ is not necessary for the organization of the cell AJs and associated proteins in the cardiac ID.

The importance of maintaining cell junction integrity was recently highlighted by the finding that a mutation in the plakoglobin gene leads to arrhythmogenic right ventricular cardiomyopathy. Oxford et al. reported that loss of PKP2 expression led to a decrease in total Cx43 content and a decrease in dye coupling between cardiomyocytes. They showed the notion of a molecular cross-talk...
between desmosomal and GJ protein. There are some reports suggesting that perturbation of the cadherin–catenin complex in heart disease may be an underlying cause, leading to the establishment of the arrhythmogenic substrate by destabilizing GJs. Based on these reports, AJs play a critical role in the regulation and maintenance of GJs.

We have previously reported, during the development of heart failure in UM-X7.1 at age 20 weeks (compensated heart failure stage), that alterations of Cx43 GJ expression and phosphorylation created serious arrhythmogenic substrate through an inhibition of cell-to-cell coupling. In our previous paper, we examined Cx43 protein expression using UM-X7.1 at age 10 weeks, and Cx43 protein expression level was not decreased. However, in the present experiments we used hamsters at age 10–15 weeks, with a sudden cardiac death rate of 10–20%, and the Cx43 expression was significantly decreased. We explain the discrepancy of data as follows: (i) Cx43 expression might start to decrease at age 10 weeks and thereafter; and (ii) because we used mixed ages of 10–15 weeks in these experiments, we found a significant decrease in Cx43 expression.

Figure 3 1. Quantitative analysis of N-cadherin (A), β-catenin (B), and Cx43 protein levels (C) in left ventricular (LV) tissues (n = 7 in each group). Upper panels show representative data. The amount of each protein was normalized to the value of GAPDH protein. All data were normalized to the value of an age 6 week golden hamster, which was set to a value of 1. Filled bars, golden hamsters; open bars, UM-X7.1; shaded bars, golden hamster with olmesartan (OS); hatched bars, UM-X7.1 with OS. *P < 0.05, **P < 0.01 vs. corresponding golden hamster, †P < 0.05, ††P < 0.01 vs. corresponding 6 week value of each group. NS, not significant. II. Quantitative analysis of desmoglein (A), plakophilin (PKP)2 (B), and γ-catenin protein levels (C) in LV tissues (n = 7 in each group). Upper panels show representative data. The amount of each protein was normalized to the value of GAPDH protein. All data were normalized to the value of an age 6 week golden hamster, which was set to a value of 1. Filled bars, golden hamsters; open bars, UM-X7.1; NS, not significant. III. Quantitative analysis of Cx43 mRNA in LV tissues (n = 7 in each group). The amount of each mRNA was normalized to the value of GAPDH. All data were normalized to the value of an age 6 week golden hamster, which was set to a value of 1. Filled bars, golden hamsters; open bars, UM-X7.1; shaded bars, golden hamster with olmesartan (OS); hatched bars, UM-X7.1 with OS. *P < 0.05 vs. corresponding golden hamster, †P < 0.05, ††P < 0.01 vs. corresponding 6 week value of each group. NS, not significant.

Adhesion junction remodelling in cardiomyopathic heart

Figure 3
4.2 Alterations in β-catenin expression and Cx43 gap junction remodelling

In addition to mechanical characteristics, the cadherin–catenin complex plays an important role in signal transduction, activating rapid and localized alterations in adhesion in response to extracellular signals.\(^34\) In particular, β-catenin contributes to the adhesion processes associated with cadherin, while it participates in the Wnt/Wingless signalling cascade when freely present in the cytosol.\(^35–37\) In physiological conditions, Wnt signalling that stabilizes cytosolic β-catenin regulates the level of β-catenin. In the absence of Wnt signals, β-catenin is phosphorylated by glycogen synthase kinase (GSK)-3β, ubiquinated and degraded in proteosomes.\(^38,39\) The UM-X7.1 cardiomyocytes might be able to reorganize intercellular contacts in order to compensate for the looser cell-to-extracellular matrix adhesion caused by the lack of δ-sarcoglycan. Concerning the catenin system, we suppose that in our UM-X7.1 model, although the total cellular β-catenin expression in cardiomyocytes was significantly decreased, β-catenin might continue to be accumulated at IDs to compensate for the cell-to-extracellular matrix adhesion, resulting in the decrease of nuclear β-catenin expression.

Recently, reduced β-catenin-dependent transcription via TCF/LEF transcriptional factors was suggested to be the molecular mechanism behind arrhythmogenic right ventricular cardiomyopathy caused by the loss of desmoplakin.\(^40\) Interestingly, Cx43 is known to be one of the TCF/LEF target genes. The response of the Cx43 promoter to β-catenin-dependent transactivation suggests that a decrease of nuclear β-catenin expression affects Cx43 gene expression in cardiomyocytes. In our UM-X7.1 hearts, we suppose that the β-catenin dislocated to IDs to maintain the cell-to-extracellular matrix, and then its nuclear expression was decreased, resulting in the decrease of Cx43 expression. Indeed, we found that the TCF/LEF DNA–β-catenin binding complex in the nucleus was remarkably decreased, and the expression of Cx43 protein was attenuated in UM-X7.1. Also, we previously reported a decrease of Cx43 mRNA in UM-X7.1.\(^11\) These results suggest that in UM-X7.1, alterations in β-catenin precede Cx43 GJ remodelling, and that ID remodelling results, at least in part, in an important arrhythmogenic substrate.

In this study, olmesartan improved abnormal ID ultrastructural changes, attenuated the decrease of total cellular and nuclear β-catenin expression, decreased VT/VF induction, and improved survival rate. The anti-arrhythmic effect of olmesartan in UM-X7.1 might not be caused by the improvement of heart failure or the attenuation of fibrosis, because there was no significant improvement of cardiac function or connective tissue volume fraction with this dose of olmesartan. Olmesartan, at least in part, might affect the ID remodelling by attenuating the decrease in nuclear β-catenin expression and by modulating qualitative and/or quantitative Cx43 expression. In UM-X7.1 at age 10–15 weeks, although olmesartan treatment did not significantly attenuate the decrease of Cx43 protein expression compared with untreated UM-X7.1, olmesartan started to inhibit arrhythmogenicity compared with golden hamsters or UM-X7.1 at age 6 weeks. We suppose that olmesartan might effect qualitative alterations, e.g. phosphorylation in Cx43 protein, and further study will be needed.
Figure 5  Quantitative analysis of nuclear β-catenin protein levels in LV tissues (n = 7 in each group). (A) Localization of anti-histone H1 antibody, which was used as a specific marker of the nuclear fraction, in different protein fractions of hamster ventricles. The different protein fractions (50 µg per lane) were subjected to immunoblotting. A strong signal was observed in the nuclear fraction. C, cytosolic; M, total membrane; T, total fraction; N, nuclear fraction. (B) Upper panels show representative data. All data were normalized to the value of an age 6 week golden hamster, which was set to a value of 1. Filled bars, golden hamsters; open bars, UM-X7.1; shaded bars, golden hamster with olmesartan (OS); hatched bars, UM-X7.1 with OS. *P < 0.05 vs. corresponding golden hamster, †P < 0.05 vs. corresponding 6 week value of each group. NS, not significant.

Figure 6  Electrophoretic mobility shift assay (EMSA). The EMSA data of nuclear extract with the TCF/LEF-binding site oligomer are presented in lane 2. The TCF/LEF DNA–β-catenin binding complex was super-shifted (dashed box) when anti-β-catenin antibody was added (lane 4). In UM-X7.1, the super-shifted complexes band was decreased compared with golden hamsters (lane 6), and olmesartan attenuated the decrease of the super-shifted band in UM-X7.1 (lane 8). Dashed box, super-shifted complex.
Although we cannot establish the precise mechanisms of olmesartan on regulation of nuclear β-catenin expression, here we discuss the possible mechanisms of olmesartan on the attenuation the decrease in nuclear β-catenin. Angiotensin II is associated with activation of kinases and phosphatases. Connexin amounts, distribution and function can be modulated by their phosphorylation state.31 Kasi et al. reported that activation of the renin–angiotensin system causes Cx43 downregulation with conduction defects in mice with cardiac-restricted overexpression of angiotensin-converting enzyme.42 They reported that renin–angiotensin system activation can affect Cx transcriptional and post-transcriptional regulation. As previously described, renin–angiotensin system activation can affect Cx transcriptional and post-transcriptional regulation. As previously described, β-catenin is phosphorylated by GSK-3β, ubiquinated and degraded in proteosomes.38,39 If the activated renin–angiotensin system affects GSK-3β to increase β-catenin phosphorylation, these results might cause the degradation of β-catenin. Then, the angiotensin II receptor blocker, olmesartan, might be useful to inhibit the activation of GSK-3β and the degradation of β-catenin. Itoh et al. reported that in rabbit volume-overloaded hearts, an angiotensin II receptor blocker attenuated the decrease of β-catenin mRNA expression.43 In contrast, Bikkavilli et al. reported that p38 mitogen-activated protein kinase (MAPK), downstream of angiotensin II–MAPKs, regulates Wnt–β-catenin signalling by inactivation of GSK-3β.44 The nature of signalling intermediates between angiotensin II–MAPK–GSK-3β–β-catenin in several conditions awaits further study.

4.3 Conclusion

We identified a mechanism for ventricular arrhythmias resulting from alterations in cardiac β-catenin, which is required to maintain Cx43 GJ protein. Furthermore, our UM-X7.1 model suggests that changes in AJ protein precede Cx43 GJ alterations, ID remodelling might contribute to ventricular arrhythmogenesis during the development of heart failure, and angiotensin receptor blockers and/or angiotensin-converting enzyme inhibitors might be a new upstream therapy for lethal arrhythmia by modulating ID remodelling.

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References


