Relationship between cardiomyocyte cell death and cardiac function during hypertensive cardiac remodelling in Dahl rats

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ABSTRACT

The exact mechanisms responsible for the progression of heart failure remain unclear. We investigated the in vivo relationship between the incidence of apoptotic cell death and left ventricular function serially from the beginning of hypertension to decompensated heart failure in Dahl salt-sensitive rats. Dahl salt-resistant and Dahl salt-sensitive rats were fed on a high-salt diet from 6 weeks of age. Systolic blood pressure was recorded by the tail-cuff method every week. Cardiac function in vivo was evaluated by echocardiography and cardiac catheterization. Cardiomyocyte apoptosis was detected by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) method. The gene expression of Bax, Bcl-2 and Bcl-xL was analysed by Northern blotting. The TUNEL method revealed that the incidence of cardiomyocyte apoptosis was significantly increased in the hearts of 18-week-old Dahl salt-sensitive rats (apoptotic index 1.3±0.1%). Northern blot analysis revealed that the Bcl-xL mRNA level increased gradually during the progression towards heart failure. In conclusion, these data suggest that cardiomyocyte apoptosis is a terminal event, and plays a role as an aggravating factor in the vicious cycle of heart failure.

INTRODUCTION

Heart failure is the most devastating clinical syndrome, and is characterized by poor prognosis and by its progressive nature. While recent clinical and experimental data have revealed that the inhibition of angiotensin-converting enzyme and angiotensin II receptor blockade improves left ventricular dysfunction and the prognosis of heart failure [1–4], the mechanisms responsible for the progression of heart failure remain unclear. Accumulating evidence suggests that apoptotic cell death plays an important pathophysiological role in various cardiovascular diseases [5–9]. Because of the loss soon after birth of the proliferative ability of cardiomyocytes, cell loss due to postnatal insults against the myocardium, such as ischaemia, will result in a decrease in the number of contractile units, and this will eventually cause pump failure directly. With regard to apoptosis in heart failure, Kubota et al. [10] have demonstrated that cardiac-specific overexpression of tumour necrosis...
factor-\( \alpha \) results in left ventricular systolic dysfunction associated with cardiomyocyte apoptosis. In addition, Li et al. [11] demonstrated an increase in cardiomyocyte apoptosis in the left ventricular myocardium in spontaneously hypertensive rats (SHRs) with decompensated heart failure. In a clinical study, Narula et al. [12] revealed the existence of cardiomyocyte apoptosis in left ventricular tissue obtained from patients with severe heart failure. These observations indicate that cardiomyocyte apoptosis occurs at least in the failing myocardium, and that this apoptosis may play a pathophysiological role in heart failure. However, the \textit{in vivo} relationship between cardiac function and the incidence of apoptosis remains incompletely understood. Therefore, in the present study, we investigated the \textit{in vivo} relationship between the incidence of apoptotic cell death and left ventricular function serially from the beginning of hypertension to decompensated heart failure using Dahl salt-sensitive (DS) rats.

**METHODS**

**Experimental animals**

Congestive heart failure due to hypertension was induced by the method of Inoko et al. [13,14]. Briefly, 50 male Dahl salt-resistant (DR) rats and 50 male DS rats aged 4 weeks were purchased from Japan SLC Inc. (Hamamatsu, Japan). Initially, rats were fed on a low-salt diet (0.3% NaCl) until they were 6 weeks old. After this, the rats were fed on high-salt diet (8% NaCl; MF rat diet, Oriental Yeast Industry, Chiba, Japan). The systemic blood pressure and body weight of each animal were measured once a week. Peak systolic pressure was recorded by the tail-cuff method (Model PS-100; Riken Kaihatsu, Yokohama, Japan). Serial echocardiographic documentation and left ventricular catheterization were performed as described below. The experimental protocol was in accordance with institutional guidelines.

**Transthoracic echocardiography**

To study contractile function \textit{in vivo}, transthoracic echocardiography was performed using a 7.5 MHz sector scan probe (model SSH-140A; Toshiba, Tokyo, Japan) when the rats were 6, 8, 11, 14 and 18 weeks old. The rats were lightly anesthetized by intraperitoneal administration of 15 mg/kg pentobarbital. Then rats were placed in the left lateral decubitus position and a probe was placed on the chest wall. An M-mode echocardiogram at the level of the papillary muscle was recorded on a printer (model TP 8700; Toshiba) at a paper speed of 100 mm/s. Left ventricular dimensions were determined according to the recommendations of the American Society for Echocardiography [15]. Left ventricular endocardial fractional shortening was calculated as:

\[
\text{Fractional shortening} (\%) = \left( \frac{\text{EDD} - \text{ESD}}{\text{EDD}} \right) \times 100
\]

where EDD is end-diastolic dimension and ESD is end-systolic dimension. Meridional left ventricular end-systolic wall stress (MESWS; kdyn/cm\(^2\)) was calculated from pressure and echocardiographic data using the following formula [16]:

\[
\text{MESWS} = 0.334 \times \text{LVSP} \times \text{ESD}/(\text{PWT}_s(1 + \text{PWT}_s/\text{ESD}))
\]

where LVSP is left ventricular systolic pressure and PWT\(_s\) is posterior wall thickness at end-systole.

**Haemodynamic measurements**

The invasive measurement of carotid artery and left ventricular pressure was performed when the rats were 6, 8, 11, 14 and 18 weeks old. Data for 10 DS rats and 10 age-matched DR rats were recorded at each time point. Animals were anesthetized with sodium pentobarbital (50 mg/kg; intraperitoneally), and a catheter pressure transducer (model SPC-320; Millar, Houston, TX, U.S.A.) was inserted through the right carotid artery into the left ventricle. Haemodynamic parameters were recorded on a multi-channel recorder (model WS-682G; Nihon Kohden, Tokyo, Japan) at a paper speed of 100 mm/s. All haemodynamic parameters were averaged for five consecutive beats. After haemodynamic recordings had been obtained, the heart was excised rapidly. Samples were fixed in 4% paraformaldehyde overnight for TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) analysis. The remaining specimen was frozen immediately in liquid nitrogen, stored at \(-80\) °C, and later processed for Northern blot analysis. Age-matched DR rats were killed as control animals.

**Extraction of RNA and Northern blot analysis**

Total RNA from the left ventricle of each animal was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. The RNA concentration in the myocardium was determined by spectrophotometry by measuring the absorbance at 260 nm. The absorbance ratio (260/280 nm) was 1.8 for all samples. For each group, 20 \(\mu\)g portions of total RNA were fractionated on a 1.2% (w/v) agarose/formaldehyde gel at 5 V/cm and then transferred on to a nylon membrane (Hybond-N\(^+\); Amersham). Hybridization was performed using QuickHyb Hybridization Solution (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer’s instructions. The probes used for Northern blot analysis were rat Bax cDNA, rat Bcl-2...
cDNA and rat Bcl-xL cDNA. All cDNA probes were synthesized by the reverse transcriptase–PCR method. The oligonucleotides used as primers were as follows [17]: Bax, 5'-GACACCTGAGCTGACCTTG-3' (sense) and 5'-GAGGAAGCTCAGTGTCCAGC-3' (antisense); Bcl-2, 5'-ATGGCGCAAGCCGGAGA- AAC-3' (sense) and 5'-CATACTGGGGCAACAAGTGA-3' (antisense); Bcl-xL, 5'-ATGTCTCAGAGCACCAGGTA-3' (sense) and 5'-CATCTCAGTGCGAAAGTGA-3' (antisense). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, we used reverse transcriptase–PCR control Amplimer Sets (Clontech, Palo Alto, CA, U.S.A.). The resulting probes were labeled with dideoxy chain-termination method. 

Statistical analysis

All values are shown as means ± S.E.M. Differences at specific time points (between groups and within groups) were assessed using one-factor ANOVA with post hoc comparison. A P value of < 0.05 was considered significant.

RESULTS

Development of hypertension and left ventricular hypertrophy, and transition from hypertrophy to heart failure

Table 1 shows the serial changes in systolic blood pressure and the ratio of left ventricular weight/body weight in DS and DR rats between the ages of 6 and 18 weeks. In DS rats, blood pressure increased progressively, reaching a value of 250 mmHg by the age of 11 weeks, which then remained constant until 14 weeks. In contrast, the blood pressure of DR rats remained unchanged. The left ventricular weight/body weight ratio increased gradually with the progression of left ventricular hypertrophy in DS rats. On the other hand, it remained unchanged in DR rats.

Haemodynamic parameters

Table 1 shows the serial changes in left ventricular dimensions and relative wall thickness in DR rats. EDD and ESD increased gradually with age. However, fractional shortening and relative wall thickness remained unchanged between 8 and 18 weeks. In DS rats, EDD at 11 weeks was smaller, and that at 18 weeks was greater, than in DR rats. ESD at 8 and 11 weeks was smaller in DS rats than in DR rats; after 14 weeks, ESD in DS rats increased progressively. Fractional shortening at 8 and 11 weeks was higher in DS rats than in DR rats, but decreased progressively after 14 weeks in the former group. Relative wall thickness in DS rats increased until 11 weeks, but then decreased gradually after 14 weeks.
Table 1  Haemodynamic parameters during salt loading of DR and DS rats
SBP, systolic blood pressure; FS, fractional shortening; RWT, relative wall thickness; PWTd, posterior wall thickness at end-diastole; LVW, left ventricular weight; BW, body weight. Values are means ± S.E.M. Significance of differences: *P < 0.05, **P < 0.001 compared with age-matched DR rats.

<table>
<thead>
<tr>
<th>Rats</th>
<th>Age (weeks)</th>
<th>SBP (mmHg)</th>
<th>EDD (mm)</th>
<th>ESD (mm)</th>
<th>FS (%)</th>
<th>RWT (2PWTd/EDD)</th>
<th>LVW/BW (mg/g)</th>
<th>MESWS (kdyn/cm²)</th>
</tr>
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<tr>
<td>DR</td>
<td>6</td>
<td>140 ± 2</td>
<td>6.87 ± 0.04</td>
<td>3.43 ± 0.08</td>
<td>50.1 ± 1.2</td>
<td>0.39 ± 0.003</td>
<td>0.0028 ± 0.0002</td>
<td>41 ± 2</td>
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<td></td>
<td>8</td>
<td>143 ± 2</td>
<td>7.28 ± 0.09</td>
<td>3.74 ± 0.11</td>
<td>48.7 ± 1.0</td>
<td>0.43 ± 0.007</td>
<td>0.0024 ± 0.0001</td>
<td>37 ± 3</td>
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<tr>
<td></td>
<td>11</td>
<td>150 ± 3</td>
<td>7.34 ± 0.05</td>
<td>3.73 ± 0.07</td>
<td>49.4 ± 0.7</td>
<td>0.48 ± 0.007</td>
<td>0.0023 ± 0.0002</td>
<td>38 ± 2</td>
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<td>14</td>
<td>152 ± 4</td>
<td>7.85 ± 0.13</td>
<td>3.87 ± 0.16</td>
<td>50.8 ± 1.5</td>
<td>0.45 ± 0.007</td>
<td>0.0023 ± 0.0001</td>
<td>35 ± 4</td>
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<td>18</td>
<td>154 ± 7</td>
<td>8.10 ± 0.13</td>
<td>4.22 ± 0.18</td>
<td>48.2 ± 1.4</td>
<td>0.46 ± 0.011</td>
<td>0.0022 ± 0.0002</td>
<td>42 ± 4</td>
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<tr>
<td>DS</td>
<td>6</td>
<td>145 ± 2</td>
<td>6.76 ± 0.09</td>
<td>3.48 ± 0.11</td>
<td>48.5 ± 1.2</td>
<td>0.40 ± 0.006</td>
<td>0.0026 ± 0.0002</td>
<td>43 ± 3</td>
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<td></td>
<td>8</td>
<td>187 ± 4**</td>
<td>7.24 ± 0.11</td>
<td>2.97 ± 0.11**</td>
<td>58.7 ± 1.1**</td>
<td>0.49 ± 0.014**</td>
<td>0.0031 ± 0.0002**</td>
<td>27 ± 2</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>247 ± 8**</td>
<td>6.89 ± 0.12**</td>
<td>3.01 ± 0.16**</td>
<td>56.5 ± 1.3**</td>
<td>0.70 ± 0.016**</td>
<td>0.0036 ± 0.0003**</td>
<td>25 ± 4</td>
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<td>247 ± 13**</td>
<td>7.96 ± 0.20</td>
<td>4.19 ± 0.31</td>
<td>49.0 ± 3.6</td>
<td>0.61 ± 0.024**</td>
<td>0.0045 ± 0.0002**</td>
<td>52 ± 10*</td>
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<td>18</td>
<td>220 ± 21**</td>
<td>9.00 ± 0.37*</td>
<td>6.70 ± 0.60**</td>
<td>25.7 ± 4.5**</td>
<td>0.56 ± 0.051*</td>
<td>0.0048 ± 0.0004**</td>
<td>110 ± 19**</td>
</tr>
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</table>

Figure 1  Northern blot analysis of Bax, Bcl-xL and Bcl-2 expression in the hearts of DS and DR rats

Alterations in apoptosis-related gene expression
Representative autoradiograms of Northern blots of Bax, Bcl-2 and Bcl-xL are shown in Figure 1, and Figure 2 shows the quantitative analysis of Bax, Bcl-2 and Bcl-xL mRNAs. Bax and Bcl-2 mRNA levels remained constant during the progression from compensated hypertrophy to heart failure (Figure 1). On the other hand, the Bcl-xL mRNA level was 2.2-fold higher in 14-week-old DS rats than in age-matched DR rats; at 18 weeks, this difference was 3.6-fold. There was a significant correlation between the level of expression of Bcl-xL and MESWS in DS rats ($r = 0.642, P = 0.0021$).

Incidence of cardiomyocyte apoptosis after salt loading
Apoptosis was observed mostly in the cardiomyocytes, where muscle striations were noted. Figure 3 demon-

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Apoptotic cell death in Dahl salt-sensitive rats

Figure 3 Incidence of cardiomyocyte apoptosis in the hearts of DS and DR rats

strates the incidence of apoptosis at 6, 8, 11, 14 and 18 weeks. The incidence of cardiomyocyte apoptosis was significantly increased only in the hearts of 18-week-old DS rats (apoptotic index 1.3 ± 0.1 %). With regard to the localization of the cardiomyocyte apoptosis observed in 18-week-old DS rats, apoptotic cardiomyocytes were confined to the subendocardial region.

DISCUSSION

In the present study, we demonstrated for the first time that, in the progression from the development of hypertension to heart failure in DS rats, cardiomyocyte apoptosis was observed only at the failing stage characterized by increased ventricular dimensions and elevated end-diastolic pressure. Northern blot analysis revealed that the gene expression of Bcl-xL, an anti-apoptotic factor, increased gradually with the progression of hypertrophy, and showed a marked elevation at the stage of heart failure. In contrast with Bcl-xL, the gene expression of Bax and Bcl-2 remained unchanged. Therefore alterations in the gene expression of apoptosis-related factors shifted towards myocardial protection during the transition from compensated hypertrophy to heart failure. Despite the enhanced expression of the anti-apoptotic gene, cardiomyocyte apoptosis was marked at the stage of heart failure.

Cheng and colleagues [18] demonstrated experimentally that overstretch of rabbit papillary muscle induced cardiomyocyte apoptosis associated with the expression of Fas, the apoptotic death-promoting factor. They also demonstrated that the incidence of cardiomyocyte apoptosis was correlated with the tensile force loaded on to the isolated papillary myocardium in vitro. Teiger and colleagues [19] reported that acute ascending aortic banding induced cardiomyocyte apoptosis. In addition, consistent with previous reports, we demonstrated that wall stress plays an important role in the induction of cardiomyocyte apoptosis after pulmonary arterial banding [20]. Taken this evidence into consideration, it can be proposed that mechanical factors, including wall stress, may play an important role in cardiomyocyte apoptosis in vivo. The augmentation of wall stress may result in cardiomyocyte overstretch, which might in turn induce cardiomyocyte apoptosis. In the present study, cardiomyocyte apoptosis was increased significantly in DS rats aged 18 weeks. The incidence of apoptosis was not increased in left ventricular tissue from 14-week-old DS rats, even though the MESWS was already elevated in these animals. Therefore our results suggest that cardiomyocyte apoptosis does not correlate directly with wall stress in vivo. However, it remains unknown whether cell death due to apoptosis induces the left ventricular enlargement, or whether the marked increase in wall stress due to left ventricular enlargement accelerates the apoptosis. In our previous study [21], the apoptotic cells were localized in the interstitial region of left ventricles in the failing stage in SHRs.

In the present study, the gene expression of the anti-apoptotic factor Bcl-xL was progressively enhanced throughout the remodeling process. It is well known that the Bcl-2 family plays a critical role in the regulation of cardiomyocyte apoptosis [22]. Bcl-2 family members consist of two functional categories: those that inhibit apoptosis (Bcl-2 and Bcl-xL) and those that induce apoptosis (Bax, Bad). The overall effect of the Bcl-2 family depends on the relative levels of death-inhibiting family members to those of death-promoting family members [23]. Therefore a high level of Bcl-xL relative to Bax promotes cell survival, while an excess of Bax relative to Bcl-xL promotes cell death. Recent data from our laboratory indicated that an angiotensin II type 1 receptor antagonist, candesartan, increased myocardial apoptosis in ischaemic/reperfused rat hearts via Bax overexpression [24]. Bcl-xL forms heterodimers with Bax and inhibits apoptosis. In addition to the inhibition of apoptosis via heterodimerization with Bax, Hu et al. [25] revealed that Bcl-xL inhibits the apoptotic pathway by a direct inhibitory effect on the activation of caspase. Accumulating evidence indicates that Bcl-xL plays a cardioprotective role in the survival of cardiomyocytes against apoptotic insults in both experimental and clinical situations [26–29]. We showed previously that levels of both mRNA and protein for Bcl-xL were up-regulated in cardiomyocytes in the failing hearts of SHRs [21]. In
addition, we also demonstrated the possibility that up-regulation of the anti-apoptotic factor Bcl-xL might prevent cardiomyocyte apoptosis at the stage of heart failure. These data suggest that Bcl-xL plays an important role in the regulation of the anti-apoptotic pathway in the heart. Our present data also demonstrated that the gene expression of Bcl-xL was gradually increased in DS rats. Our previous data demonstrated that Bcl-xL was up-regulated at both the mRNA and protein levels at the stage of heart failure [21]. Therefore it is suggested that the level of Bcl-xL is increased both transcriptionally and translationally in hypertensive heart failure models.

In the present study, despite this increase in anti-apoptosis-related gene expression in the progression from compensated hypertrophy to heart failure, cardiomyocyte apoptosis was increased in the failing myocardium of DS rats aged 18 weeks. In contrast, apoptotic cell death was not increased before 18 weeks of age, even in the presence of elevated wall stress. In view of these observations, it is unlikely that apoptotic cell death contributes significantly to the transition from compensated hypertrophy to heart failure in this model. Li et al. [11] demonstrated that increased numbers of apoptotic cardiomyocytes were present in the failing hearts of SHRs as compared with the non-failing hearts of SHRs and age-matched Wistar-Kyoto rats. However, there were no data concerning the serial relationship between cardiomyocyte apoptosis and cardiac function in vivo in SHRs. Our results demonstrated that cardiomyocyte apoptosis occurred at only the terminal stage of heart failure, characterized by a markedly increased left ventricular dimension. Our previous study [21] using SHRs demonstrated that non-myocyte apoptosis was observed in the interstitial region of the failing ventricles. The pattern of apoptosis varies with different models and different stages of hypertension. In the present study, the increase in tensile force against the myocardium associated with left ventricular eccentric dilatation may be a cause of cardiomyocyte death. It is suggested that cardiomyocyte apoptosis is a terminal event, and plays an important role as an aggravating factor in the vicious cycle of heart failure.

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Received 24 May 2001/20 August 2001; accepted 8 November 2001

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