Contribution of non-cardiomyocyte apoptosis to cardiac remodelling that occurs in the transition from compensated hypertrophy to heart failure in spontaneously hypertensive rats

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ABSTRACT

Various alterations in molecular and cellular events have been considered as possibly contributing to the cardiac remodelling that occurs during the transition from compensated hypertrophy to heart failure. The aim of the present study is to clarify (1) whether cardiac apoptosis occurs during the transition from compensated hypertrophy to decompensated heart failure, and (2) whether expression of the genes encoding Bax (an apoptosis inducer) and Bcl-xL and Bcl-2 (apoptosis inhibitors) is altered during this transition. We used 12-month-old and 20-month-old male spontaneously hypertensive rats (SHR12 and SHR20 respectively) and age-matched Wistar–Kyoto rats (WKY12 and WKY20 respectively). These rats were killed after measurement of haemodynamic parameters by transthoracic echocardiography and use of a tipmanometer via the right carotid artery. The expression of bcl-2, bcl-xL and bax was analysed by Northern blotting. Samples were also fixed in 4% paraformaldehyde for in situ nick end-labelling (TUNEL) methods and immunohistochemistry. SHR12 had well compensated left ventricular hypertrophy with normal fractional shortening and normal end-systolic wall stress. In contrast, the hearts of SHR20 developed decompensated dilatation, with a decrease in fractional shortening and an increase in end-systolic wall stress. TUNEL-positive cells were seen exclusively in the hearts of SHR20. The major cell types that showed TUNEL-positive nuclei were non-cardiomyocytes. The expression of bax remained unchanged during the transition to heart failure. However, there was increased expression of bcl-xL in the failing stage, whereas the expression of bcl-2 remained unchanged. Immunohistochemical studies revealed that Bcl-xL protein was up-regulated in the hearts of SHR20. In conclusion, non-cardiomyocyte apoptosis may play a contributory role in the remodelling that occurs in the transition from compensatory hypertrophy to decompensated heart failure. In addition, it is suggested that enhanced expression of bcl-xL plays an important role in the preservation of cardiomyocytes during this transition.

INTRODUCTION

Hypertension results in left ventricular hypertrophy, which is a well recognized adaptation of the myocardium aimed at reducing systolic wall stress. In addition, in response to the long-lasting pressure overload, disproportionate non-myocyte cell proliferation or collagen gene expression results in pathological hypertrophy with

Key words: apoptosis, apoptosis-related gene, non-myocyte, remodelling, spontaneously hypertensive rat.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SHR, spontaneously hypertensive rats; SHR12 and SHR20, SHR aged 12 and 20 months respectively; TUNEL, in situ nick end-labelling; WKY, Wistar–Kyoto rats; WKY12 and WKY20, WKY aged 12 and 20 months respectively.

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myocardial failure [1]. The progression of myocardial failure is accompanied by left ventricular geometric changes.

Spontaneously hypertensive rats (SHR) are a genetic model of early hypertrophic adaptation to hypertension and subsequent transition to severe heart failure [2,3]. SHR show persistent hypertension when they are over 2 months of age, and initial cardiac hypertrophy is followed by a relatively long period of stable, compensated hypertrophy. Over 18 months of age, left ventricular pump dysfunction ensues in male SHR. This is followed by pathophysiological manifestations of overt heart failure [4]. This remodelling process involves an increase in myocardial mass accompanied by hypertrophy of individual myocytes, alterations in gene expression of sarcoplasmic reticulum proteins [5], and changes in both the quantity and the quality of the extracellular matrix [6].

Over the past few years, many reports on a role for apoptosis in cardiovascular pathological states have appeared. Apoptosis is increasingly recognized as a contributory mechanism in processes such as postnatal maturation [7], aging [8], ischaemia/reperfusion injury [9–12], vascular remodelling [13], heart failure [14] and hypoxia [15]. However, the role of cardiac apoptosis in the cardiac remodelling process during the transition from compensated hypertrophy to decompensated heart failure remains to be elucidated.

It is well known that Bcl-2 family members, the tumour suppressor p53, interleukin 1β-converting enzyme/CED (‘cell death abnormal’)-3 family proteases, the cytokine receptor Fas/APO-1 and related ‘death domain’ proteins are associated with the molecular mechanisms of apoptosis. Among these factors, there is increasing awareness that the Bcl-2 family plays a critical role in the regulation of apoptosis. Bcl-2 family members consist of two functional categories: those that inhibit apoptosis (Bcl-2, Bcl-xL) and those that induce apoptosis (Bax). The determining factor is the ratio of the levels of death-inhibiting Bcl-2 family members relative to those of death-promoting Bcl-2 family members [16].

The aim of the present study is to clarify: (1) whether cardiac apoptosis occurs during the transition from compensated hypertrophy to decompensated heart failure in the hearts of SHR, and (2) whether the levels of expression of the apoptosis inducer Bax and of the apoptosis inhibitors Bcl-2 and Bcl-xL are altered during the ventricular remodelling process.

METHODS

Animal preparation
All procedures were carried out in accordance with the institutional guidelines for animal research of Ehime University. SHR were provided by Charles River Japan. SHR and their normotensive controls, Wistar–Kyoto rats (WKY), were studied in the following manner. Male SHR and WKY were purchased as retired breeders at the age of 6–9 months and boarded at the animal facility at the Ehime University animal institute until the time of study. At the age of 12 months, at which time compensatory hypertrophy is observed, SHR in one group were killed (SHR12; n = 5). At the age of 20 months, all remaining animals were observed on a daily basis. When evidence of cardiac decompensation (such as decreased activity and laboured respiration) was apparent, the rats were killed (SHR20; n = 5). Age-matched WKY were killed as control animals (WKY12 and WKY20 respectively; n = 5 in each group).

Echocardiographic studies
To elucidate contractile function in the rats, transthoracic echocardiography was performed when the animals were aged 12 months (compensated hypertrophy) and 20 months (heart failure) using a model SSH-140A instrument, with a 7.5 MHz sector scan probe (Toshiba, Tokyo, Japan). Rats were lightly anaesthetized with 15 mg/kg pentobarbital intraperitoneally. Animals were then placed in the left lateral decubitus position. An M-mode ECG was determined at the papillary muscle level and recorded on a printer (model TP 8700; Toshiba) at a paper speed of 100 mm/s. Left ventricular dimensions were determined using the methods of the American Society of Echocardiography [17]. Haemodynamic measurements derived from echocardiography and catherization were obtained during five consecutive beats, and results were averaged. From these measurements, left ventricular fractional shortening (LVFS) was calculated using the following formula:

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

where EDD is left ventricular end-diastolic dimension, and ESD is left ventricular end-systolic dimension.

After ECG studies, animals were carefully anaesthetized with diethyl ether. Left and right ventricular systolic and diastolic pressures were measured using a 2F catheter-tip transducer (Millar Instruments, Houston, TX, U.S.A.), which was passed in a retrograde direction from the carotid artery to the left ventricle. Left ventricular systolic and diastolic pressures were recorded at high and low gain. End-systolic meridional wall stress (ESWS; in 10⁶ dyn/cm², where 1 dyn = 10⁻⁶ N) was calculated from pressure and ECG data using the following formula [18]:

\[ \text{ESWS} = 0.334LVP \times \text{ESD}/(\text{PWTs}(1 + \text{PWTs}/\text{ESD})) \]

where LVP is left ventricular pressure, ESD is left ventricular end-systolic dimension and PWTs is left ventricular posterior wall thickness at end-systole.

Histopathology
After haemodynamic evaluation, hearts collected from SHR12, SHR20, WKY12 and WKY20 were fixed in 4%
paraformaldehyde overnight at 4 °C, and then embedded in paraffin. In order to differentiate muscle fibres from extracellular matrix, sections were stained with Azan staining.

**In situ nick end-labelling (TUNEL)**

**In situ** detection of apoptosis was performed using the ApoTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, U.S.A.), with some modifications. In brief, hearts collected from SHR<sup>#</sup>, SHR<sup>##</sup>, WKY<sup>†</sup>, and WKY<sup>‡</sup> were incubated in 4% paraformaldehyde overnight at 4 °C, and were then embedded in paraffin. From each heart, 7 μm cross-sections were cut by a microtome and mounted on silan-coated slides. The slides were deparaffinized and rehydrated by serial changes of xylene and ethanol. Endogenous peroxidase was blocked by immersing the sections in PBS containing 3% H<sub>2</sub>O<sub>2</sub>. The sections were then incubated with 20 μg/ml proteinase K (Sigma, Deisenhofen, Germany) for 20 min. After washing in PBS, the sections were covered with equilibration buffer (ApopTag kit) for 5 s, and incubated with a mixture of terminal deoxynucleotidyl transferase and reaction buffer (ApopTag kit) containing digoxigenin-labelled dUTP at 37 °C for 1 h. Then the sections were washed in diluted working-strength stop/wash buffer (ApopTag kit) and incubated with an anti-digoxigenin antibody conjugated with peroxidase for 30 min, followed by three washes in PBS for 5 min, and finally washed in 0.05% diaminobenzidine (Wako Pure Chemical Industries, Osaka, Japan). Tissue sections from each specimen were examined microscopically at 400 × magnification. At least 300 non-cardiomyocytes were counted in a minimum of five high-power fields in the interstitial fibrotic regions. The percentage of apoptotic cells was determined using an apoptotic index (number of TUNEL-positive cells/total number of non-cardiomyocyte nuclei × 100).

**Immunohistochemistry**

For immunohistochemical analysis, the slides were deparaffinized and rehydrated by serial changes of xylene and ethanol. After washing in PBS, endogenous peroxidase was blocked by immersing the sections in PBS containing 3% H<sub>2</sub>O<sub>2</sub>, followed by washing in PBS. Non-specific antigens were blocked by incubation of the slides with 10% (v/v) normal goat serum for 30 min. The sections were then incubated for 1 h with the following primary antibodies: rabbit polyclonal anti-Bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), goat polyclonal anti-Bcl-2 antibody (Oncogene Science, Cambridge, MA, U.S.A.) and rabbit polyclonal anti-Bcl-xL antibody (Santa Cruz Biotechnology), at dilutions of 1:100, 1:200 and 1:200 respectively in PBS. After washing in PBS, immunostaining was performed with an avidin/biotin immunoperoxidase using a Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.), followed by visualization with diaminobenzidine. Tissue sections were lightly counterstained with haematoxylin, dehydrated and placed under coverslips.

**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 concentration of RNA in the myocardium was determined by spectrophotometry (absorbance at 260 nm). The absorbance ratio (260/280 nm) was > 1.8 in all samples. For each group, 20 μg of total RNA was fractionated on a 1.2% (w/v) agarose/formaldehyde gel at 5 V/cm and transferred on to a nylon membrane (Highbond-N°; Amersham). Hybridization was performed using Quick-Hyb 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 and rat Bcl-xL cDNA [19]. All cDNA probes were synthesized by the reverse transcriptase–PCR method. The oligonucleotides used as primers of Bax, Bcl-2 and Bcl-xL were as follows: Bax, 5′-GACACCT-GAGCTGACCTTGG-3′ (sense primer) and 5′-GAGGAAAGCTCAGTGTCCAGC-3′ (antisense primer); Bcl-2, 5′-ATGGCGCAAGGCGAGAAC-3′ (sense primer) and 5′-CATACTGGGCACAAAGTGA-3′ (antisense primer); Bcl-xL, 5′-ATGTCGACAGCA-CCGGGA-3′ (sense primer) and 5′-CCTCTCAGTGGGAAGTGA-3′ (antisense primer). For the cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), we used reverse transcriptase–PCR control Amplimer Sets (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer’s instructions. The resultant probes were subcloned into pBluescript II (Stratagene), and DNA sequences were determined by the dideoxy chain-termination method. 32P-labelled cDNA probes specific for rat Bax and Bcl-xL were prepared by the random primer method using a Prime-It II random primer labelling kit (Stratagene). The radioactive intensity of the resulting bands was quantified with a BAS-1000 instrument (Fuji Film Co., Kanagawa, Japan). Results were normalized to hybridization signals of GAPDH mRNA as an internal control. After washing, autoradiography was performed with Hyperfilm-MP® (Amersham) with an intensifying screen at −80 °C.

**Statistical analysis**

All data are presented as means ± S.E.M. of the individual values in rats from each group. Differences among groups were tested by one-way analysis of variance. Subsequent analysis for significant differences was performed with a multiple-comparison test (Scheffé’s methods). Significance was assumed at P < 0.05.
RESULTS

Haemodynamic data and transition from compensated hypertrophy to heart failure

Tables 1 and 2 show heart weight, body weight, blood pressure and haemodynamic data derived from echocardiography and cardiac catheterization. Examples of ECGs obtained from SHR and SHR are presented in Figure 1. Left ventricular weight as a proportion of body weight was significantly higher in SHR than in age-matched WKY at both 12 months and 20 months of age.

Peak left ventricular systolic pressure and positive and negative dP/dt values (maximum rate of increase in systolic pressure) were significantly decreased in SHR as compared with SHR (P < 0.05). Left ventricular end-diastolic pressure was greater, fractional shortening was less and end-systolic wall stress was higher in SHR than in other three groups.

Histopathology

Figure 2 illustrates the structural characteristics of foci of fibrosis observed in WKY (Figure 2A), SHR (Figure

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<tr>
<th>Table 1</th>
<th>Heart weight, body weight and blood pressure data</th>
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<tr>
<td></td>
<td>LV, left ventricle; RV, right ventricle; BW, body weight; BP, blood pressure. Values are means ± S.E.M.; *P &lt; 0.05 compared with age-matched WKY.</td>
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<tr>
<td>Group</td>
<td>LV (g)</td>
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<tr>
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<tr>
<td>WKY12</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>SHR12</td>
<td>1.42 ± 0.05*</td>
</tr>
<tr>
<td>WKY20</td>
<td>1.13 ± 0.04</td>
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<tr>
<td>SHR20</td>
<td>1.35 ± 0.02*</td>
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<th>Table 2</th>
<th>Haemodynamic parameters of WKY and SHR</th>
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<td></td>
<td>EDD, end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening; EDP, end-diastolic pressure; dP/dt, maximum rate of change in systolic pressure; ESWS, end-systolic wall stress (1 dyn = 10⁻⁵ N). Values are means ± S.E.M.; *P &lt; 0.05 compared with age-matched WKY group.</td>
</tr>
<tr>
<td>Group</td>
<td>EDD (mm)</td>
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</tr>
<tr>
<td>WKY12</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>SHR12</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>WKY20</td>
<td>6.2 ± 0.1</td>
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<tr>
<td>SHR20</td>
<td>9.8 ± 0.3*</td>
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Figure 1 Examples of echocardiograms (short-axial and M-mode) from SHR and SHR

LV, left ventricle.
2B) and SHR\textsubscript{20} (Figure 2C). Histological analysis demonstrated a marked increase in connective tissue in SHR\textsubscript{20} relative to SHR\textsubscript{12} and both WKY groups. Fibres in WKY\textsubscript{20} were relatively uniform in cross-sectional area, and only a small amount of connective tissue was observed. On the other hand, SHR\textsubscript{12} showed an increase in connective tissue compared with WKY\textsubscript{20}.

**Localization of apoptotic cells**

Histological analysis demonstrated that the majority of apoptotic cells were observed in the left ventricle of SHR\textsubscript{20} manifesting heart failure. In addition, TUNEL-positive cells were localized in the fibrotic interstitium, as shown in Figure 3. The apoptotic index in SHR\textsubscript{20} was $0.6 \pm 0.1\%$. On the other hand, TUNEL-positive nuclei were not detected in the hearts of the SHR\textsubscript{12} and WKY groups.

**Immunohistochemical results**

Figure 4 shows representative photomicrographs of Bax, Bcl-2 and Bcl-xL protein immunoreactivity in WKY and SHR hearts. The expression of Bax remained unchanged in the hearts of SHR throughout the study period. Also, expression of Bcl-2 did not change between the hypertrophy stage and the failing stage. On the other hand, the expression of Bcl-xL in SHR\textsubscript{20} hearts was clearly increased compared with that in SHR\textsubscript{12} hearts. Furthermore, the cell type that showed immunoreactivity was the cardiomyocyte, and there was no immunoreactivity for Bcl-xL in cells localized in the interstitial fibrotic area (Figure 5).

**Alterations of apoptosis-related gene expression**

Levels of Bax and Bcl-xL mRNAs were analysed in RNA samples isolated from the hearts of WKY\textsubscript{12}, WKY\textsubscript{20}, SHR\textsubscript{12} and SHR\textsubscript{20} by Northern blotting with $^{32}$P-labelled...
cDNA probes specific for Bax Bcl-2 and Bcl-xL mRNAs. As shown in Figure 6, levels of Bax and Bcl-2 mRNAs remained constant during the transition from compensated hypertrophy to heart failure. On the other hand, the level of Bcl-xL mRNA was markedly increased in the hearts of SHR_20 (Figure 7).

**DISCUSSION**

SHR_12 displayed well compensated left ventricular hypertrophy, defined as normal fractional shortening and normal end-systolic wall stress. In contrast, hearts from SHR_20 showed decompensated dilatation, with a decrease in fractional shortening and an increase in end-systolic wall stress. The results of our study revealed that TUNEL-positive cells were seen exclusively in the hearts of SHR_20. In addition, the TUNEL-positive nuclei were derived not from cardiomyocytes but from non-cardiomyocytes localized in the fibrotic interstitium. There were no TUNEL-positive cardiomyocytes or non-cardiomyocytes in the hearts of SHR_12. Furthermore, the
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Figure 6 Northern blot analysis of Bax and Bcl-2 expression
Results were normalized to hybridization signals of GAPDH mRNA. The levels of Bax and Bcl-2 mRNAs remained unchanged during the transition from compensated hypertrophy to heart failure (n.s, not significant).

Figure 7 Northern blot analysis of Bcl-xL expression
Results were normalized to hybridization signals of GAPDH mRNA. The level of Bcl-xL mRNA was significantly increased in the hearts of SHR20 (P < 0.05).

expression of Bax remained unchanged throughout the study. However, there was a markedly increased expression of Bcl-xL in the heart failure stage, whereas expression of Bcl-2 remained unchanged. In view of these findings, it is suggested that apoptosis in the myocardial interstitium plays an important role during the transition from compensated hypertrophy to heart failure. In addition, the up-regulation of Bcl-xL may preserve cardiomyocytes from apoptosis during long-lasting pressure overload in vivo.

Cardiomyocytes and non-cardiomyocytes in the heart are interconnected by a complex of connective tissue and extracellular matrix. The myocardial collagen matrix has been proposed to contribute to maintaining myocyte alignment, co-ordinating myocardial contraction, and maintaining left ventricular geometry. Thus the extracellular matrix is an important determinant of the structural characteristics of the myocardium. Weber and Brilla [20] reported that, in pressure-overload hypertrophy, the interstitial fibrosis associated with a disproportionate rise in collagen content had a detrimental influence on the diastolic and systolic stiffness of the myocardium. This pathological increase in the extracellular matrix may alter the mechanical properties of the myocardium, and also restrict the delivery of nutrients to cardiomyocytes.

In the present study, histological analysis demonstrated a marked increase in connective tissue on progression of left ventricular hypertrophy, as observed in previous studies [4,21]. In addition, the TUNEL-positive cells were non-cardiomyocytes localized in the cardiac interstitium. While it is well known that angiotensin II type 1 receptors induce cardiomyocyte hypertrophy and fibroblast proliferation, the physiological role of angiotensin type 2 receptors remains unclear. Yamada et al. [22] reported that angiotensin type 2 receptors mediated programmed cell death in vitro. On the other hand, in an in vivo study using cardiomyopathic hamsters, Ohkubo et al. [23] reported that angiotensin type 2 receptors were re-expressed by cardiac fibroblasts at the stage of heart failure, which was associated with the most marked interstitial fibrosis. These authors also demonstrated that stimulation of angiotensin type 2 receptors inhibited fibroblast proliferation and the production of fibrillar collagen in the extracellular matrix. In addition, they showed that long-term treatment with an angiotensin type 1 receptor antagonist prevented the progression of interstitial fibrosis, whereas an angiotensin type 2 receptor antagonist increased the extent of interstitial fibrotic regions. In view of these findings, it is likely that one fraction of fibroblasts in the interstitial region not only ceased to produce collagen matrix, but also died as a result of programmed cell death at the stage of heart failure. Our findings suggest that apoptosis of interstitial cells is closely related to the increase in connective tissue in the myocardium. The decrease in interstitial cellularity due to apoptosis may lead to the decrease in collagen matrix production, which augments the delivery of nutrients to cardiomyocytes.

With regard to the apoptosis observed in the hearts of SHR, Hamet et al. [24] reported that cardiomyocyte apoptosis occurred in the left ventricle as target organ damage of hypertension. In addition, Diez et al. [25]
reported that apoptosis of vascular smooth muscle of SHR was regulated via alterations in Bax and Bcl-2 protein expression. They also demonstrated overexpression of Bax protein and enhanced apoptosis in the left ventricles of 30-week-old SHR [26]. Furthermore, the investigation of Teiger et al. [27] revealed that cardiomyocyte apoptosis first appeared in the early stage after aortic banding. In our study, non-myocyte apoptosis was observed in the decompensated failing stage. Taking this evidence into consideration, it is suggested that the pattern of apoptosis varies with different stages and different models of hypertension.

In conclusion, the present study has demonstrated enhanced gene and protein expression of the apoptosis inhibitor Bcl-xL in the hearts of SHR, whereas the expression of Bcl-2 and Bax remained unchanged. In addition, the cell type that showed enhanced expression of Bcl-xL was the cardiomyocyte during heart failure. The combination of this up-regulation in cardiomyocytes and interstitial cell apoptosis may play an important role in protecting cardiomyocytes against a long-lasting haemodynamic overload.

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