Apoptosis of ventricular and atrial myocytes from pacing-induced canine heart failure

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Abstract

Objective: Rapid ventricular pacing in dogs results in a low output cardiomyopathic state similar to idiopathic dilated cardiomyopathy in man. Cell death by apoptosis may play an important role in the loss of cardiac function. This study investigates the molecular pathways involved in the regulation of apoptosis in dogs with pacing-induced heart failure. Methods: Apoptosis was identified by terminal transferase nick end-labelling (TUNEL) in the ventricles and atria of dog hearts affected by rapid-ventricular pacing. Western blots were used to determine expression of the components involved in the initiation (Fas, Fas-Ligand, FADD), regulation (Bcl-2, Bax) and execution (caspase-2 and caspase-3) of apoptosis. Results: Pacing-induced heart failure resulted in a significant increase in the number of ventricular and atrial myocyte nuclei undergoing apoptosis as measured by TUNEL. Compared to the samples from control hearts (n=6) the expression of Bcl-2, an inhibitor of apoptosis, was significantly reduced in ventricles from five dogs with pacing-induced heart failure. No change in the expression of the apoptotic inducer Bax was detected. Fas and FADD were significantly elevated in all paced ventricles, and Fas-L was only detected in the paced hearts. Both caspase-2 and caspase-3 were elevated following ventricular pacing. Conclusions: We have identified components of the signalling pathways along which apoptosis proceeds following the induction of heart failure in dogs. Apoptosis was also detected in the atria raising the possibility that, like human dilated cardiomyopathy, the molecular changes are global. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Apoptosis; Cardiomyopathy; Myocytes; Heart failure

1. Introduction

Experimental heart failure induced by rapid ventricular pacing in dogs results in a low output cardiomyopathic state. Myocardial remodelling and chamber dilatation occur to counteract the increased wall stress [1]. Along with these changes there is depressed ventricular contractility [2]. These alterations are similar to those observed in both human and naturally occurring canine dilated cardiomyopathy (DCM) [3]. Cardiomyopathies are associated with a progressive loss of myocytes [4] throughout the ventricular wall and papillary muscles [5]. At a subcellular level there is evidence for myofibrillar disarray and structural changes in the mitochondria [6].

Apoptosis has been observed in cardiomyopathic conditions including human DCM and ischaemic cardiomyopathy [7,8] and in pacing-induced canine heart failure [9,10]. Over the past few years, it has become clear that apoptosis is a feature of DCM and other forms of heart failure, although details of the molecular mechanisms involved in the induction and execution of the apoptotic process are not fully understood. Caspase (cystein proteases that cleave after Asp residues) activation is known to play a central role in the execution of apoptosis [11], but little is known of caspase involvement in the process of cardiomyocyte apoptosis.

Apoptosis is a tightly-controlled process requiring...
energy. Characteristic changes occur within apoptotic cells. These include a reduction in cell volume without rupturing the sarcolemma, misshapen nuclei, chromatin condensation due to the activation of an endogenous endonuclease, and changes to the cell surface and active protein synthesis [12]. Factors which lead to the induction of apoptosis in cardiomyocytes during heart failure may include mechanical stress [13], disruption to Ca\(^{2+}\) cycling leading to increased intracellular Ca\(^{2+}\) [14], or the production of superoxide anions resulting from mitochondrial damage [15].

In this study we provide the first evidence for elevated expression of caspases in failing canine left ventricle together with confirmatory evidence for the presence of apoptosis in these samples. We also report that the changes in the paced dog hearts appear to be global, affecting left atria as well as left ventricles. As the molecular details of apoptosis become clearer, they may reveal ways of delivering new therapies to the failing heart.

2. Methods

2.1. Animals

Experiments were performed using mongrel dogs of either sex, weighing 18–25 kg. This study examined the hearts of animals subjected to right ventricular pacing to induce heart failure. Non-paced dogs were confirmed as healthy by a veterinary clinician and were used as control animals. All experiments were approved by the Animal Care and Ethics Committee, The University of Sydney, approval number K21/8-94/2/1016.

2.2. Right ventricular pacing

Prior to pacing, dogs underwent a complete veterinary examination to assess their health. Operating procedures were performed as described by Allworth et al. [2]. Under anaesthesia, a left lateral thoracotomy was performed. The pericardium was incised and a miniature pressure transducer (Konigsberg P6.5) was inserted through a small incision in the apex of the left ventricle (LV). A unipolar pacing electrode was inserted, with the aid of a guide wire, through a small incision in the right internal jugular vein. It was passed via the right atrium to the apex of the right ventricle. A single channel multi-programmable telemetric pulse generator (Refllex Model 8220, Teletronic Pacing Systems) was connected to the lead and inserted in a subcutaneous pocket cranial to the first rib. After recovery for 1 week, ventricular pacing at 245 bpm was maintained for 3–4 weeks to induce heart failure. Animals were observed daily. Heart failure was defined by the presence of four of the following signs: 10% increase in body weight; increased resting heart rate; echocardiographic evidence of congestive heart failure (ejection fraction <40); anorexia; exercise intolerance; and lethargy. Once heart failure was established the pacing rate was reduced to 190 bpm for 2–3 days to stabilise the condition. This protocol for inducing canine heart failure over a period of weeks does not allow an investigation of the more acute changes, for example those occurring within hours of inducing failure.

Echocardiographic assessment, to measure cardiac structure and function, was performed before the initiation of pacing and when stable heart failure had been established. Images were obtained as described by Allworth et al. [2]. Intraluminal LV dimensions were measured from the trailing edge of the septal wall image to the leading edge of the LV free wall at end-diastole and end-systole. Using B-mode echocardiography, left atrial (LA) chamber size was measured in two dimensions from the point of coaptation of the mitral leaflets to the opposite wall, and in a plane perpendicular to that just described.

2.3. Collection of heart tissue

Both paced and control dogs were euthanased by intravenous sodium pentobarbitone. The heart was excised and washed in perfusion buffer (140 mM NaCl, 4 mM KCl, 2.5 mM MgCl\(_2\), 18 mM D-glucose, 3 mM HEPES, pH 7.3) at 4°C. Transmural sections of the anterior free wall of the LV and LA were immediately frozen in liquid nitrogen for biochemical analysis or fixed in 10% formalin for immunohistochemistry.

2.4. TUNEL labelling

Frozen myocardial tissue was cryosectioned at 10 μm and mounted on gelatinised microscope slides. Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) was performed following the method described by Gaverelli et al. [16]. Cryosections of myocardial tissue (10-μm thick) were fixed in 3% buffered formaldehyde. Sections were then treated with proteinase K (20 μg/ml), washed and incubated with the reaction solution containing the TdT enzyme and biotinylated dUTP. The reaction was stopped after 100 min and non-specific binding sites were blocked by incubation in BSA. The dUTP bound to the cut ends of DNA was labelled with the fluorescent dye Cy3-streptavidin. Cardiomyocytes were identified by double labelling the tissue with an antibody against β-MHC. This was visualised using FITC conjugated to the secondary antibody. Stained sections were examined using a confocal microscope (MRC-600, BioRad) and the number of TUNEL-positive nuclei was calculated per 10\(^6\) cardiomyocyte nuclei.

Sections incubated with 100 ng/ml DNase I (Worthington) in 10 mM Tris, pH 7.6, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\) for 30 min at 37°C prior to incubation with the TdT enzyme were used as positive control (data not shown).
Negative controls were performed by omitting the TdT enzyme (data not shown).

2.5. Western blotting

Finely ground frozen tissue was homogenised in 1% SDS, then centrifuged at 20,000×g for 20 min at 4°C and the supernatant removed. Protein concentration was determined using a modified Bradford assay [17]. Protein samples containing 50–150 μg protein were loaded onto a 10 or 12% SDS–PAGE gel. Following electrophoresis proteins were electroblotted onto either PVDF or nitrocellulose membranes. Transfer was performed at 1.5 mA/cm² for 60 min. Membranes were blocked in 3% skim milk powder in PBS-Tween for 60 min, followed by incubation with the primary antibody diluted in blocking solution. Membranes were probed using: Bel-2 (Ab-2) (Calbiochem); Bax (N-20; Santa Cruz); and Fas, Fas-L, FADD, caspase-2 and caspase-3 (Transduction Laboratories). Blots were washed in PBS-Tween and incubated with secondary antibody (conjugated to either horseradish peroxidase (HRP) or biotin) diluted in the blocking solution. Following extensive washing in PBS-Tween solution, bound antibodies were detected using ECL chemiluminescence reagents (Amersham) or BLAST amplification of HRP labelling (NEN Life Science Products). Scanning laser densitometry using Molecular Dynamics ImageQuant software performed quantification of the density of antibody-positive bands (arbitrary units). Blots were stained with Coomassie blue to ensure equal protein loading in each lane.

2.6. Immunohistochemical labelling

Following standard histological processing and paraffin embedding, 5-μm sections of LV were prepared for immunohistochemical labelling. Endogenous peroxidase activity was inhibited by incubation with 0.3% H₂O₂ in methanol for 30 min. Sections were blocked with normal goat serum for 1 h to prevent non-specific binding followed by incubation with the primary antibody (Fas-L) for 1 h at room temperature. The secondary antibody (biotinylated anti-mouse IgM) was applied for 30 min. Sections were then incubated with ExtrAvidin (Sigma) for 45 min at 37°C. Staining was visualised using diaminobenzidine (DAB, Sigma), then washed and counterstained with toluidine blue, cleared, mounted and examined by light microscopy.

2.7. Statistical analysis

Unpaired two-tailed t-tests were performed to analyse the statistical differences between the data from control and paced hearts. Values of P<0.05 were considered significant.

3. Results

3.1. Clinical assessment

All paced animals (n=9) developed congestive heart failure within the 3–4-week pacing period. On average the paced dogs had a 25% increase in resting heart rate (with the pacemaker switched off) and a 10% increase of average body weight. Echocardiographic data from these dogs are summarized in Table 1 and are also consistent with myocardial failure. The average ejection fraction in paced dogs was reduced to ~50% of control values. Increases in LV and LA size were measured by LV end diastolic and systolic volumes and LA dimensions.

3.2. TUNEL

LV samples from nine paced dogs and six controls were examined for TUNEL labelling which was detected by confocal microscopy. Fig. 1 illustrates TUNEL-labelled (red) nuclei while cardiomyocytes were counter-stained (green) with an antibody to β-MHC. Labelled nuclei were scattered throughout the thickness of the LV wall (Fig. 1A) and were normally, single, isolated nuclei surrounded by apparently normal cells. Apoptosis was rarely detected in non-cardiomyocytes. The identity of these cells was not determined.

The number of TUNEL-positive nuclei was calculated per 10⁶ cardiomyocyte nuclei using a fluorescence microscope. There was a 21-fold elevation of the number of TUNEL-positive myocyte nuclei in paced hearts (1173±323 per 10⁶ cardiomyocyte nuclei, mean±S.E.M.) compared to control ventricles (55±23). This difference was significant (P<0.001).

LA samples from control (n=3) and paced (n=3) hearts were also examined. TUNEL-positive myocyte nuclei were observed scattered throughout the LA of paced dogs (Fig. 1B). The number of TUNEL-positive nuclei increased 5-fold in the LA of paced hearts (333±91 per 10⁶ cardiomyocyte nuclei, mean±S.E.M.) compared to control

<table>
<thead>
<tr>
<th>Cardiac parameters</th>
<th>Control dogs (mean±S.E.M.)</th>
<th>Paced dogs (mean±S.E.M.)</th>
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<tbody>
<tr>
<td>LA dimension (mm)</td>
<td>28.2±1.4</td>
<td>37.9±1.6*</td>
</tr>
<tr>
<td>LV internal diastolic diameter (mm)</td>
<td>35.9±2.2</td>
<td>46.4±1.9*</td>
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<tr>
<td>LV internal systolic diameter (mm)</td>
<td>23.9±1.6</td>
<td>37.2±0.7*</td>
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<tr>
<td>Diastolic wall thickness (mm)</td>
<td>9.8±0.4</td>
<td>6.7±0.5*</td>
</tr>
<tr>
<td>Systolic wall thickness (mm)</td>
<td>13.8±0.2</td>
<td>9.8±0.8*</td>
</tr>
<tr>
<td>Diastolic septal thickness (mm)</td>
<td>9.3±0.8</td>
<td>6.5±0.6*</td>
</tr>
<tr>
<td>Systolic septal thickness (mm)</td>
<td>14.1±0.6</td>
<td>7.9±0.6*</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>69.3±2.1</td>
<td>36.2±2.5*</td>
</tr>
<tr>
<td>Ventricular fractional shortening (%)</td>
<td>33.4±1.1</td>
<td>19.9±1.7*</td>
</tr>
<tr>
<td>Number of dogs</td>
<td>8</td>
<td>9</td>
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* Indicates a statistically significant difference (P<0.05) between the two groups of dogs.
atria (70±27). This increase was statistically significant (P<0.05). The level of apoptosis in the control ventricles and atria was not statistically significant (P>0.05), however there was significantly more apoptosis in the paced ventricle compared to the paced atria (P<0.05).

3.3. Western blotting

LV extracts from control (n=6) and paced (n=5) dogs were examined by Western blotting to detect the expression of members of the Bcl-2 family, the Fas signalling pathway and the caspases.

3.3.1. Expression of Bcl-2 and related proteins

Bcl-2, a 29-kDa protein, was detected by Western blotting extracts from the LV of both control and paced hearts (Fig. 2A). The levels of Bcl-2 in the control hearts were much higher than in the paced dog where the 29-kDa band is just visible. A significant decrease (P<0.05) in Bcl-2 expression was observed in LV samples from paced hearts (403±218, mean AU±S.E.M.) compared to controls (2530±444 AU).

Fig. 2B demonstrates the presence of Bax in a Western blot. A 21-kDa protein was detected in both control and paced ventricular myocardium. The intensity of Bax-positive band following pacing (2011±301 AU, mean±S.E.M.) was not significantly (P=0.96) altered compared to the controls (1966±861 AU).

3.3.2. Expression of Fas and related proteins

Fas was detected as a 45-kDa band in LV from both the control and paced hearts, demonstrated in Fig. 3A. The expression of Fas was significantly (P<0.05) elevated in

Fig. 2. Western blots showing the presence of (A) Bcl-2, a 29-kDa protein and (B) Bax, a 21-kDa protein, in LV samples from three control and three paced dogs. Lanes were loaded with a nominal 150 μg of protein.
the paced ventricle (16.12±3.11 AU, mean±S.E.M.) compared to controls (4.74±1.59 AU).

A 37-kDa protein, Fas-L, was detected in Western blots of LV myocardium from paced hearts (lanes 3 and 4, Fig. 4) but was undetectable in samples from control dogs. We were unable to observe a Coomassie-stained band corresponding to the expected migration position of Fas-L in LV samples of control dogs (lanes 1 and 2) in either the blot membranes or in SDS–PAGE gels (data not shown). An extract of human endothelial cells was used as a positive control for the Fas-L antibody as seen in lane 5 in Fig. 4. Fas-L was determined to be located on the cardiomyocytes, by immunohistochemistry (Fig. 5).

Fig. 3B demonstrates a 24-kDa band, detected as FADD. Expression levels of FADD in the paced myocardium (27.66±2.13 AU) compared to controls (5.73±2.65 AU) represent a significant elevation of expression (P<0.05) following ventricular pacing.

3.3.3. Caspase expression

Caspase-2, a 48-kDa protein, was detected in Western blots of LV samples seen in Fig. 6A. Caspase-2 expressed at a significantly higher level (P<0.001) in the myocardium of the paced dogs (20.08±1.58 AU) compared to the controls (6.66±1.38 AU).

Caspase-3 was detected as a 32-kDa protein band and was found to be present in both the control and paced myocardium, as demonstrated in Fig. 6B. Caspase-3 was detected in extracts of LV from paced hearts (190.9±17.69 AU) and in control myocardium (14.39±2.93 AU). This represents a significant increase (P<0.001) in the paced heart.

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The figure captions are as follows:

**Fig. 3.** Western blots of LV homogenates from three control and three paced hearts, detecting the presence of (A) Fas and (B) FADD. All lanes were loaded with 50 µg protein. A Jurkat cell extract was used as a positive control (+ve) for Fas labelling.

**Fig. 4.** Western blot analysis of Fas-L in canine LV. Lanes 1 and 2 contain LV extracts from two control dogs and lanes 3 and 4 were loaded with LV homogenates from two paced dogs. Lanes 1–4 were loaded with 150 µg of protein. Lane 5 contains 5 µg of human endothelial cell lysate (HE) which was used as a positive control.

**Fig. 5.** Immunohistochemical detection of Fas-L in (A) paced and (B) control ventricles. Labelling is indicated by the arrowheads. Similar to the Western blotting results Fas-L was not detected in the control ventricle. The bar indicates 20 µm.
Fig. 6. Examples of Western blots detecting (A) a 48-kDa band identified as caspase-2 and (B) a 32-kDa band identified as caspase-3 in LV samples from three different control and paced dogs. Extracts of human endothelial or Jurkat cells were used as a positive controls (+ve) for caspases-2 and -3, respectively. Each lane was nominally loaded with 50 μg.

4. Discussion

Congestive heart failure induced by rapid ventricular pacing in the dog results in a marked dilatation of the ventricles and atria. In the ventricles we observed increased numbers of TUNEL-positive cardiomyocyte nuclei, and altered expression levels of proteins involved in the induction (Fas, Fas-L, and FADD), regulation (Bcl-2 and Bax), and execution (caspases-2 and -3) of apoptosis.

An important new finding is the evidence for apoptosis in the atria of the paced dogs. There were significantly more TUNEL-positive myocyte nuclei in the LA of the paced dogs compared to the controls, although the degree of elevation in the LA (5-fold) was significantly less than in the LV (21-fold). These changes may be due to the pacing per se, but they may also result from other factors such as pressure overload and/or over-stretching of the heart chambers.

4.1. Pathways for apoptosis

Fig. 7 summarizes our interpretation of the nature of the interaction between the seven elements examined in this paper. Fas-L is associated with the cardiomyocyte sarcolema [18] and probably represents the first step in the initiation of apoptosis. The binding of Fas-L can activate Fas which is also present on the cardiomyocyte surface or it may be activated by different cell types such as activated T-cells, or by agonistic Fas-antibodies [19]. Both Fas and Fas-L expression were increased in ventricular tissue after pacing, suggesting that here Fas is activated by Fas-L. Fas has been independently implicated in cardiomyocyte apoptosis associated with ventricular pacing in the dog [9], as well as in hypoxia [20] and stretching [13].

Once activated, a Fas complex is assembled involving at least two other proteins: FADD (Fas activated death domain) and caspase-8. FADD contains a death domain in its C-terminus which interacts with the death domain on Fas [21]. FADD also contains a death effector domain which binds to a similar domain on caspase-8 (also called MACH, Mch5 or FLICE). The increased levels of FADD detected in ventricular extracts from the paced dog hearts suggest that Fas activation initiates apoptosis in these hearts.

The relationships between the inducers (FasL/Fas/ FADD complex), the cytoplasmic executors (caspase-2 and caspase-3) and the regulators (mitochondria-bound Bcl-2 and its ligand, Bax) of apoptosis may diverge as indicated in Fig. 7. The pathways clearly involve more components than those examined in this investigation. For example, apoptosis is known to involve the mitochondria [22], but this aspect of apoptosis was not investigated in the present study. Nevertheless, by identifying altered regulation of these elements we have established that both pathways are involved in failing cardiomyocytes.
4.2. The role of the Bcl-2 family

Bcl-2 blocks cell death following a variety of stimuli, for example it can block or restrict apoptosis induced by Fas [23]. Consequently, Bcl-2 expression is inversely proportional to the changing levels of apoptosis observed during cardiac development in the rat [24]. Bcl-2 is located on the outer mitochondrial membranes (Fig. 6), the nuclear envelopes, and the membrane of smooth endoplasmic reticulum [25]. It can form homodimers or it may dimerise with other members of the Bcl-2 family including Bcl-x and Bax. Our observation of decreased Bcl-2 levels in the myocardium from paced hearts confirms the recent findings by Leri et al. [10]. Up-regulation of p53 (a transcriptional regulator of Bcl-2 expression) has been detected in the paced dog model [10], suggesting a mechanism for the reduced levels of this protein.

Bax is a promoter of apoptosis [26]. However, no significant change in Bax expression was detected in the paced dog ventricle. This result is at variance with Leri et al. [10], who observed a 52% increase in Bax expression following pacing. This difference may be due to the pacing regimes used in the two studies. We used 245 bpm for 3–4 weeks followed by a lower rate (190 bpm) for 2–3 days. In contrast, Leri et al. paced their dogs at 210 bpm for 3 weeks followed by 240 bpm for 1 week [10]. Thus, it is possible that Bax expression in our dog hearts may have recovered during the 2–3 days they were paced at the lower rate whereas the fast pacing rates employed by Leri et al. may have induced an elevated expression of Bax. It is also difficult to compare the degree of failure of the animals in the studies as different parameters were examined. For example, Leri et al. assessed failure based on pressure measurements whereas we examined ventricular dimensions. However, our data are consistent with the report [27] of no change in Bax expression in the LV of human DCM. In the presence of Bcl-2, Bcl-2-Bax heterodimers predominate, promoting cell survival. However, when Bcl-2 levels are reduced (as observed in the paced heart), Bax (which is unchanged in the paced LV) can form homodimers which promote cell death.

4.3. Cysteine proteases in apoptosis

The caspases belong to a family of cysteine proteases, which cleave adjacent to an aspartic acid residue [28]. The induction of the Fas complex results in caspase-8 activation, which itself is capable of activating all ten caspases [29] suggesting it is at the top of the caspase cascade. We investigated the expression of caspases-2 and -3 and found that both were elevated in the paced dog LV samples. Over-expression of caspase-2 (ICL-1) results in apoptosis in several cell systems [30] and it is cleaved early in the apoptotic process. However no specific protein substrates for caspase-2 have been identified.

Caspase-3 (apopain, Yama or CPP32) is one of the key players in apoptosis. It is responsible for the cleavage of numerous proteins, including the DNA repair enzymes poly(ADP-ribose) polymerase and DNA-dependent protein kinase [31]. Caspase-3 has also been demonstrated to cleave and activate the caspase activated DNase (CAD) leading to the DNA laddering feature of apoptosis [32]. Another cellular target of caspase-3 is actin [33], cleavage of which leads to the disruption of the cytoskeleton and loss of its function as a DNase I inhibitor. There is a reciprocal interaction between Bcl-2 and caspase-3. Bcl-2 inhibits caspase-3 [34], but Bcl-2 is itself a target for caspase-3, rendering it inactive [35]. Caspase-3 cleavage of Bcl-2 may explain the reduction of Bcl-2 observed in the paced heart.

4.4. Other factors involved in apoptosis

Changing levels of the components in the apoptosis signaling pathway probably do not represent a definitive picture. Cardiotrophin (CT-1) is a potent factor involved in structural remodeling of cardiomyocytes in canine pacing-induced heart failure. Jougasaki et al. reported [36] that CT-1 is up-regulated in both the atria and ventricles of paced dogs but the degree of up-regulation is greater in the atria than the ventricles. CT-1 inhibits cytokine-induced cardiomyocyte apoptosis and although the relationship between CT-1 and apoptosis is yet to be clarified, it represents a potential for future treatments of heart failure using drugs which target CT-1 [37].

4.5. Concluding remarks

Taken together, the above data suggest that pacing-induced heart failure results in: (i) elevated levels of inducers of apoptosis (Fas, Fas-L and FADD); (ii) down-regulation of an inhibitor (Bcl-2) of apoptosis; (iii) increased expression of cysteine proteases (caspase-2 and caspase-3); and (iv) increased numbers of TUNEL-positive nuclei in failing LV and LA of paced dogs. Thus, at least two major pathways are activated in failing hearts that effect intracellular suicide.

Acknowledgements

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