Regulation of apoptosis in the endocardial cushions of the developing chick heart

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ABSTRACT

During the early stages of heart development, there are two main foci of cell death: in the outflow tract (OT) endocardial cushions and in the atrioventricular (AV) endocardial cushions. These tissues contribute to the septa and valves of the mature heart, and receive cell populations from neural crest (NC) cell migration and epicardial cell invasion. We examined embryonic chick hearts for expression, in the cushions, of bcl-2 family members, caspase-9, and the caspase substrate poly (ADP)-ribose polymerase (PARP). Anti-apoptotic bcl-2 is expressed heavily in the OT and AV regions, throughout ED 4-7, with a decrease in levels at ED 4 in the OT and ED 5 in the AV cushions. Pro-apoptotic bax predominantly associated with the prongs of the NC-derived aorticopulmonary (AP) septum, but was expressed throughout the AV cushions. Pro-apoptotic bak also associated with the prongs of the AP septum in the OT, while protein levels were up-regulated at ED 4-5 in the OT and ED 4-6 in the AV cushions. Bid expression showed a similar time-course. We found the 10 kDa cleavage fragment of active caspase-9 from ED 4-8 in the OT and from ED 5-8 in the AV cushions, and the 24 kDa cleavage fragment of PARP throughout ED 3-8 in the OT and ED 7-8 in the AV cushions. Caspase-3 cleavage occurred throughout the time period examined. Using cushion cell cultures, we found that inhibitors of caspases-3 and -9 and a universal caspase inhibitor all significantly reduced apoptosis, as did retroviral over-expression of bcl-2 using an RCAS expression vector. Pre-migratory NC cells were fluorescently labeled in vivo with DiI. Subsequent nuclear staining of cushion cells with DAPI revealed the presence of apoptotic nuclei in the neural crest cells in the OT cushions, and in the prongs of the AP septum. These results demonstrate a developmentally-regulated role for the bcl-2 and the caspase families of molecules in the endocardial cushions of the developing heart, and lend support to the possibility that some of the dying cells in the cushions are neural crest-derived.

heart development; cell death; morphogenesis; Bcl-2; caspase
INTRODUCTION

Cardiac development is a complex morphogenetic process that involves the remodeling of a simple tubular structure to form a mature four chambered organ (4, 17, 38). Among the more complex morphogenetic events are the development of the endocardial cushions in the outflow tract (OT) and atrioventricular (AV) regions (reviewed by 27) and the invasion of the heart by neural crest (NC) cells to specific sites of remodeling (reviewed by 5, 10).

The endocardial cushions are primarily pads of mesenchymal tissue, which are induced to form by an epithelial-to-mesenchymal cell transformation from the endocardium, under the influence of myocardial stimulation (9, 26). The cushions contribute to valve development and septal alignment.

The OT is comprised of a linear myocardial tube containing paired longitudinal ridges of mesenchymal cushions that will ultimately fuse to separate the initially common lumen into the primitive pulmonary artery and aorta (46, 51). Fusion of the opposing cushions occurs via the caudal growth of the aorticopulmonary (AP) septum, which is primarily of neural crest origin, and the cranial growth of the conotruncal septum, with the outflow valves forming where these septa meet (18). The OT cushions also fuse with the anterior crest of the interventricular septum and assist in the correct alignment of the ventricular chambers with the major outflow vessels of the heart (17, 45).

The AV cushions initially form as two lateral outgrowths (superior and inferior) that fuse between the common atrium and single ventricle to divide the single lumen into the left and right AV orifices (17). Subsequent fusion and remodeling in the AV region provides the framework for the mitral and tricuspid valves (28, 49), and contributes to the correct septation of the
chambers through fusion with the inter-atrial and inter-ventricular septa (23, 48). Correct alignment and fusion of both sets of cushions is crucial, with many congenital heart defects resulting from improper development of these structures (6, 27).

Another complex morphogenetic event in heart development is the invasion of populations of neural crest cells to sites of cardiac remodeling (46). These cells originate in the cranial folds of the neural tube between the otic placode and the caudal limit of the third somite (21, 46), and they migrate through the embryo to populate the pharyngeal arches, where they will ultimately contribute to the endothelial lining of the vessels. From the arches, some crest cells continue into the cardiac outflow tract, forming the condensed mesenchyme of the AP septum. This septum grows in a caudal direction, with well characterized lateral prongs of mesenchyme invading the truncal and conal cushions of the OT, thus facilitating their fusion (46). Below the level of the incipient semilunar valve, the prongs disperse, with only individual crest cells continuing their migration into the conal cushions and myocardium. Some crest cells, by way of subendocardial migration, reach the site of closure of the interventricular septum with the AV cushion and the, by now partially muscularised, conal cushions, which aids in correct separation of the heart chambers. Another population of neural crest cells reaches the AV cushions and regions of the prospective conduction system by way of the venous pole of the heart (30), where they are thought to play a role in the differentiation of these specialized tissues.

Intrinsic to normal cushion development are a number of phases of apoptotic cell death at specific sites and times of development. It has been shown that two main foci of cell death occur consecutively in the cushions of the OT and the AV regions (19, 29, 31, 43). Although the exact role of these episodes of cell death still remains unclear, it has been suggested that some of the apoptotic cells may be neural crest-derived (31).
Among the key players in the apoptotic process are the bcl-2 family of regulators (1, 41), and the
caspase family of effector enzymes (39). Anti-apoptotic members of the family, e.g. bcl-2 and
bcl-X\textsubscript{L}, which have up to four bcl-2-homology domains (BH1-4), are normally present on
membranes of mitochondria and the endoplasmic reticulum (33). Some pro-apoptotic members,
such as bax and bak, (possessing BH domains 1-3), reside mostly in the cytosol. Upon receiving
stimuli, e.g. increased pH, or dimerization, (14, 20), these molecules translocate to the outer
mitochondrial membrane (50) and are thought to facilitate the release of cytochrome c via
interaction with a voltage dependent anion channel (VDAC; 35). Other pro-apoptotic members,
e.g. bid and bim, (BH3 only), also normally reside in the cytosol, where, upon stimulation, they
translocate and insert into the mitochondrial membrane, resulting in cytochrome c release,
independent of the VDAC (36). Once in the cytosol, cytochrome c activates the effector family
of caspase enzymes (37). There can also be interaction between this mitochondria-dependent
pathway and a cell surface death-receptor pathway via the activation of the BH3-only molecule,
bid, also resulting in caspase activation.

Caspases are a family of cysteine proteases that have been divided into two groups. Initiator
caspases, e.g. caspases-8 and –9, when activated, bring about the downstream activation of the
second group of caspases, the executors, e.g. caspases-3, -6 and -7 (3). The latter cleave such
cellular proteins as poly(ADP)-ribose polymerase (PARP) and DNA fragmentation factor (DFF)
that results in the characteristic apoptotic demise of the cell.

Previous studies of apoptosis in heart development have relied on morphological assessment and
vital dye staining (29), or on labeling of the fragmenting DNA in the nuclei of late-stage
apoptotic cells with a nick end-labeling technique such as TUNEL (19, 31). In this paper, we
examine the mechanisms of cell death in the cushion cells, and show that the expression of pro-
and anti-apoptotic members of the bcl-2 family are developmentally regulated in the AV and OT cushions in such a way as to suggest their involvement in cushion cell death. In addition, we demonstrate active caspase-9 and PARP cleavage in the cushions, implicating the downstream mitochondrial-associated death pathway in these cells. Further, in vitro experiments on cultured cushion cells indicate that bcl-2 over-expression and exogenous caspase inhibitors can block the onset of apoptosis in these cells. Finally, fluorescent labeling of migratory neural crest cells and assessment of their nuclear morphology in the heart, lends support to the contention that some of the dying cells in the cushions may be neural crest in origin.

MATERIALS AND METHODS

Chick Embryos

Fertilized White Leghorn hens' eggs were incubated at 37°C for 4-8 days and the resulting embryos were staged according to Hamburger and Hamilton (16). The embryos were removed and washed in Tyrode's saline. For immunocytochemistry, the heads were removed and the embryos immersed in 4% paraformaldehyde in 0.1 M phosphate-buffered-saline (PBS) at 4°C, for 4-15 h, depending on the stage. After fixing, the embryos were washed in PBS and stored at 4°C. For Western blotting and cell culture, the hearts were removed to Tyrode's on ice. Using electrolytically sharpened tungsten needles, the AV endocardial cushions and the entire OT were dissected free from the heart without the use of digesting enzymes.

Immunocytochemistry

After fixing, the embryos were washed in PBS, dehydrated through a graded series of ethanol, and cleared in Hemo-De (Fisher Scientific). The embryos were then embedded in paraffin wax, sectioned at 8 μm and mounted on glass slides. Sections were cleared in Hemo-De (2x10 min),
rehydrated in graded ethanol and washed in double distilled water (DDW). To quench endogenous peroxidase, sections were treated with 0.3% H$_2$O$_2$ in DDW for 30 min, followed by washing in DDW. Sections were blocked in 10% goat serum with 0.5% Tween 20 (Fisher Scientific) for 1 h at room temperature. Excess solution was removed and sections were incubated with primary antibodies diluted in 1% goat serum at 4°C overnight. The primary antibodies used were; bcl-2 (N-19, 1:200), bax (I-19, 1:200) and bak (G-23 1:100), all from Santa Cruz. Sections were washed 3H5 min in PBS, and then incubated with a biotinylated secondary antibody (goat-anti-rabbit IgG, Vector Laboratories), at a dilution of 1:200 in 1% goat serum, for 1 h at room temperature. Following another wash, 3H5 min in PBS, sections were incubated with the Vectastain ABC reagent (Vector Laboratories Inc.) according to manufacturers instructions for 1 h at room temperature. Following another wash, sections were stained using 3,3’ diaminobenzidine (Sigma) with ammonium nickel sulphate. Sections were washed in PBS, dehydrated through graded ethanol, cleared in Hemo-De and mounted with permount (Fisher Scientific Inc.). Negative controls consisted of preincubating the primary antibody with the suppliers blocking peptide and resulted in absence of staining in all cases.

*Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting*

The dissected AV endocardial cushions, the outflow tract, and cultured cells were homogenized in protease inhibitor buffer, containing 15 μg/ml aprotinin, 1 μg/ml leupeptin, 5 μg/ml pepstatin, and 1.74 mg/ml phenylmethylsulphonyl fluoride (PMSF), and the protein concentration was determined using the Bio-Rad protein assay. Samples were loaded at a concentration of 10-15 μg per lane and run on a 10% polyacrylamide gel for 45 min. The separated proteins were transferred to nitrocellulose membranes, at 100 V for 2 h. The membranes were stained initially with Ponceau S to ensure even loading of lanes and a successful transfer. The membranes were
then subjected to blocking in 5% skimmed milk in TTBS for 1 h at room temperature. The membranes were probed with the following primary antibodies in 5% skimmed milk in TTBS, overnight at 4EC: Bcl-2 B46620 monoclonal (1:200), Transduction Laboratories; Bax B-9 monoclonal (1:50), Santa Cruz Biotechnologies Inc.; Bak Ab-2 monoclonal (1:50), Oncogene Research Products; Bid goat polyclonal (1:50), R&D Systems Inc.; PARP A-20 goat polyclonal (1:50), Santa Cruz Biotechnologies Inc.; caspase-3 rabbit polyclonal (1:500), Stress-Gen Biotechnologies Corp.; activated caspase-9 AAP-109 rabbit polyclonal (1:500), StressGen Biotechnologies Corp. The membranes were washed for 3-5 min in 5% skimmed milk and probed with biotinylated secondary antibodies at dilutions of 1:1000-1:2000 for 1.5 h. After another washing step, the membranes were incubated with the Vectastain ABC kit for 1.5 h, washed again and developed using ECL reagent (Amersham). The gels shown represent the result of a minimum of three repetitions of each experiment on different samples.

**Dissociated Primary Cell Culture**

Primary cell cultures were made of dissociated dissected AV cushions. Briefly, AV cushions were dissected to ice cold Tyrode's solution, from Hamburger and Hamilton (HH) stage 24 hearts (2 dozen/experiment) using electrolytically sharpened tungsten needles. Cells were then dissociated in 0.2% trypsin/EDTA (Sigma) in calcium/magnesium-free Tyrode's for 10 min at 37EC. Trypsinization was stopped with the addition of 1 ml medium 199 with 10% foetal bovine serum (FBS; Gibco/BRL). Cells were spun in a bench-top centrifuge for 5 min and the supernatant discarded. The cells were washed in 1 ml medium 199 without serum and re-centrifuged for 3 min. Cells were then re-suspended in 100 µl complete medium (medium 199 with 10% FBS and 1:1000 gentamycin), counted using a hemocytometer slide, and re-suspended in complete medium, at a final volume of 100 µl/coverslip. Coverslips were prepared by coating
with 1mg/ml type I rat tail collagen (Gibco/BRL; dissolved in 0.5% acetic acid, re-suspended 1:20 with 60% EtOH, and NaOH added to a final concentration of 15 mM) and exposed to ultraviolet light overnight. Once seeded, cultures were incubated overnight at 37°C in a 5% CO₂ incubator. Cultures were given fresh complete medium every second day for 4-6 days. Serum starved cultures were treated in the same way, with FBS absent from the medium.

**Retroviral Infections**

Retroviral vectors were produced as described by Logan and Francis-West (25). Briefly, pRCASBP(B) and pRCASBP(B)/bcl-2 (supplied by Dr. S.H. Hughes, Frederick Cancer Research and Development Centre, Maryland) were transfected into primary cultures of line 0 chick embryo fibroblast (CEF’s), that had been produced from specific pathogen free eggs (Hyvac, Adel, Iowa). Cultures were expanded for 7-10 days. For viral collection, on the second-last day of culture, when cells were reaching confluency, medium was replaced at half volume with medium plus 2% serum overnight. The supernatant was collected and concentrated by ultracentrifugation at 25,000 rpm at 4°C for 2.5 h. The supernatant was carefully removed and the remaining pellet resuspended in 100 µl Optimem (Gibco/BRL), which was then frozen in aliquots at -70°C. Controls included staining transfected CEF’s for both the human bcl-2 transcript and the p19 viral coat protein, as well as immunoblotting for bcl-2. Viral titer was obtained by serial dilution and infection of CEFs for 48 h. Cells were immunostained for the viral coat protein and the number infectious virions calculated as 5.0×10⁷ per ml. Dissociated primary cushion cultures were prepared as described and grown under serum starved conditions. Cells were infected with virus after the first overnight incubation. The initial 100 µl medium was removed and replaced with fresh 100 µl complete medium containing 5 µl concentrated virus
with 8 µg/ml polybrene. No other virus was added during the subsequent medium changes, but fresh polybrene was included in each medium change.

**Immunocytochemistry on Cultures**

Primary cell cultures were prepared as described above. For mitochondrial labeling, MitoTracker Red7 CMXRos (Molecular Probes Inc.) was used, by addition of 5 µl of 10 µM MitoTracker7 to 1 ml of medium. Cultures were incubated in this reagent for 45 min at 37°C, then washed in warm Tyrode's solution and fixed with 4% buffered paraformaldehyde for 45 min at room temperature. Cultures were washed 3H5 min in PBS, and treated with blocking solution of 10% goat serum in PBS with 0.5% Tween 20 for 30 min at room temperature. Primary antibodies used were as described for immunohistochemistry above; for bcl-2 at a concentration of 1:200, and the monoclonal AMV-3C2 anti-viral coat protein antibody supernatant (University of Iowa Developmental Studies Hybridoma Bank) overnight at 4°C in 1% goat serum with 0.5% Tween 20. Cells were then washed 3H5 min in PBS. Biotinylated secondary antibodies were used at a concentration of 1:200 for 1 h at room temperature, and then washed three times in PBS. Cells were then fluorescently labeled with streptavidin-FITC (Calbiochem) at a concentration of 1:200 for 1 h at room temperature in the dark. The cultures were washed again 3H5 min in PBS, and treated with DAPI for 4 min at room temperature. Cells were washed 3H5 min in PBS and mounted on slides with Vectashield mounting medium (Vector Labs). Specimens were examined using a Zeiss LSM510 confocal microscope equipped with argon, helium/neon and ultraviolet lasers.
**TUNEL Labeling**

Staining was performed on fixed primary cultures. The cells were washed in PBS after fixing and pretreated with TdT buffer (30 mM Trizma base, 140 mM sodium cacodylate, 1 mM cobalt chloride; pH 7.2) for 5 min. Cultures were then incubated with the reaction mixture for 1.5 h at 37°C in a humid chamber, using the kit from Roche Molecular Biochemicals at a volume of 20 µl per culture; (100 µl volume: DDW, 81 µl; TdT buffer, 6.5 µl; cobalt chloride, 3.26 µl; biotin-16-dUTP stock (1 nmol/µl), 1.86 µl; dUTP, 5.5 µl; and TdT (10 units/µl), 2 µl). Cultures were then washed 3H5 min in PBS and blocked with 3% skimmed milk in PBS with 0.5% Tween 20 for 30 min. Cultures were then washed 3H5 min in PBS and the fluorochrome Streptavidin-FITC was added used at a concentration of 1:200, for 1 h at room temperature. Cultures were again washed 3H5 min in PBS and were treated with DAPI in PBS for 4 min. Following more washes, the coverslips were mounted on slides with Vectashield and imaged digitally. The total number of TUNEL-positive cells was counted in each culture, and were analyzed statistically using ANOVA and Tukey's multiple comparison post-test, on five replicates of each treatment.

When TUNEL labelling was combined with immunocytochemistry for bax, the TUNEL procedure was carried out first, followed by the immunocytochemistry, and the two secondary antibodies were applied simultaneously in mixed solution.

**Caspase Inhibitors**

Primary dissociated cushion cultures were prepared as described and serum starved to induce apoptosis. The cultures were then treated with specific peptide caspase inhibitors to prevent cell death. The following peptide caspase inhibitors were used: caspase-3 inhibitor II (Z-DEVD-FMK); caspase-9 inhibitor I (Z-LEHD-FMK), both from Calbiochem. A universal caspase...
inhibitor (BOC-Asp(OME)-FMK) (Enzyme Systems Products Inc., Livermore, CA) was also used to inhibit all caspases. All inhibitors were dissolved in DMSO at a concentration of 50 mM, aliquoted and stored at -20°C. Caspase inhibitors were added to fresh medium199 without serum, which was added to cultures every other day, at a final concentration of 50 µM. After 4-5 days, cells were washed in PBS and fixed in 4% paraformaldehyde in PBS and stained with TUNEL and DAPI.

**DiI Labeling of Neural Crest Cells**

In order to label premigratory neural crest cells, *in ovo* microinjection of the fluorescent lipophilic dye, DiI (Molecular probes, Inc.) was used (34). The dye was prepared by dissolving 3 mg of DiI in 0.1 ml of 100% ethanol. This was then diluted with 1.1 ml of 3% BSA in PBS (2). Eggs were windowed and the dye was injected into the lumen of the neural tube at HH stage 9-11, using a Picospritzer (General Valve Corp.). Sub-blastodermal injection of India ink was used as a contrast agent. Following injection, the eggs were sealed with Scotch tape and were reincubated for 3-4 days. After this time, the embryos were dissected and the heads removed from the body. Specimens were then fixed in 4% paraformaldehyde in 0.1 M PBS overnight at 4°C and then were embedded in OCT compound. The specimens were then frozen and sectioned at 10 µm. The frozen sections were stained with DAPI for 4 minutes at RT and mounted with Vectashield mounting medium. The slides were examined using a Zeiss LSM510 confocal microscope equipped with argon, helium/neon and ultraviolet lasers.
RESULTS

*Immunocytochemistry of bcl-2 Family Molecules*

Sections of the developing heart, containing the endocardial cushions, were stained for various members of the bcl-2 family at times when peak cell death is observed in the cushions (19). At ED 4, in the distal OT (Figures 1A and 1B) bcl-2 protein was absent from the condensed mesenchyme of the AP septum, but was present in individual endocardial cushion cells in the loose mesenchyme. In the more proximal OT cushions (Figures 1C and 1D), the protein was expressed in the majority of cushion cells. A similar pattern was seen in the AV cushions at ED 6 and 4 (Figures 1E and 1F, respectively), with high bcl-2 expression in most cushion cells. In these, and in all the following immunocytochemical results, negative controls in which the antibody was pre-absorbed with antigen showed no staining.

Heart sections were also stained for bax, a pro-apoptotic family member. At ED 4, in the distal OT (Figure 2A), bax protein was not abundant in the core of the condensed mesenchyme of the AP septum, but was more highly immunoreactive in some of the adjacent cushion and myocardial cells, and at the edges of the condensed mass (arrow). Bax was also seen in the fusing cushions of the distal OT, adjacent to the AP septum (Figure 2B). In the more proximal OT cushions (Figures 2C and 2D), bax expression appeared to associate strongly with the prongs of the AP septum that protrude into the endocardial cushions, but was also present in individual cells in the surrounding cushions and in the myocardium. In the AV cushions (Figure 2E) bax immunoreactivity appeared to be scattered widely throughout the cells of the central cushion mass at ED 6, when peak cell death is occurring. TUNEL/bax double labelling (Figure 2F) showed that bax was expressed in the cytoplasm of all apoptotic cells.
Immunoreactivity for another pro-apoptotic family member, bak, was also examined in sections at times when peak cell death is occurring. In the OT, bak appeared to localize intensely to the prongs of the AP septum (Figures 3A and 3B), and was also present in some parts of the myocardium and the loose mesenchyme of the cushions. Bak also appeared to have a distribution in the AV cushions (Figure 3C), corresponding to regions that normally show extensive apoptosis.

Western Blotting for Bcl-2 Family, Caspase-9, Caspase-3 and PARP

The antibody to bcl-2 revealed the 26 kDa band of bcl-2 throughout the time course examined in both the AV and OT cushions, from ED 4-7 (Figure 4A). A decrease in protein expression was consistently seen at day 5 in the AV region, and when repeated blots were densitometrically measured and analyzed, this decrease was found to be significant (p<0.05; data not shown). This decrease corresponds with, or slightly precedes, the time of peak cell death in these regions that we showed previously. In the OT, expression at 26 kDa consistently decreased at ED4. The antibody also consistently recognized a ~23 kDa band, which corresponds to another bcl-2 isoform of uncertain significance (40). The positive controls, using Jurkat cells, showed a 26 kDa band as expected, and often the 23 kDa form, but at much lower levels than in the embryonic tissue, suggesting the possible involvement of this isoform in developing tissue.

In similar samples, a cross reactive monoclonal antibody for the pro-apoptotic molecule, bax, revealed constitutive expression of the 23 kDa protein in both the AV and OT regions (Figure 4B), in similar levels to the whole embryo positive controls, throughout the time course examined. Bax is known to be constitutively present in many tissues, and becomes pro-apoptotic upon activation.
The pro-apoptotic protein bak was also present. The 28 kDa protein was found to be consistently up-regulated in the AV cushions at days 4-6 and at days 4-5 in the OT region (Figure 4C). These times correspond to peak cell death times in both regions, implicating bak as a pro-apoptotic signal in this tissue.

The 18kDa protein, bid, indicative of the death-receptor pathway, was also expressed in these tissues during this embryonic period (Figure 4D). In the AV cushions, bid was most highly expressed at days 6 and 7, while in the OT cushions it appeared throughout ED 4 – 7.

The characteristic demise of the apoptotic cell is brought about by cleavage of specific substrates by members of the caspase family of enzymes. Immunoblotting of the AV and OT regions revealed the presence of the 10 kDa cleavage fragment of active caspase-9 throughout the time course examined (Figure 5A), providing evidence for involvement of this initiator caspase in these tissues. Evidence for the downstream activation of caspase-3 was also obtained in both the AV and OT cushions between ED 4 and 7, by detection of the 12kDa cleavage fragment of this molecule thoughout this period (Figure 5B).

One known substrate of the caspase family, which is characteristically cleaved during apoptosis, is the DNA repair enzyme poly (ADP)-ribose polymerase (PARP). Immunoblotting for this protein revealed the 24 kDa cleavage fragment to be dramatically up-regulated at days 7-8 in the AV cushions and to be present throughout days 3-8 in the OT (Figure 6), suggesting that the apoptotic characteristics of these cells are caspase-dependent.
Inhibition of Apoptosis in AV Cushion Cell Cultures by Caspase Inhibitors

Primary cultures of dissociated AV cushions were made and were induced to undergo apoptosis by serum withdrawal. Peptide inhibitors of caspase-3 and -9 and a universal caspase inhibitor were added to the cultures. Following this treatment, nuclei were stained with DAPI (Figure 7A), and apoptotic nuclei were labeled with the TUNEL technique (Figure 7B). The total number of nuclei and the number of TUNEL-positive nuclei were then counted. Figure 7C shows the effects of the inhibitors on the number of TUNEL labeled nuclei in each treatment. Each treatment significantly inhibited cell death in these cultures, with the universal and caspase-9 inhibitors being more effective than the inhibitor of the further downstream caspase-3.

Inhibition of Apoptosis in AV Cushion Cell Cultures by Bcl-2 Over-Expression

As described above, primary cultures of dissociated AV cushions were made and induced to undergo apoptosis by serum withdrawal. The cultures were infected with the replication-competent retrovirus RCASBP(B), carrying either the human bcl-2 transcript insert, or no insert as a negative control. Cultures were then incubated for a further 5 days. The cultures were stained with DAPI to label all the nuclei, and TUNEL to show the apoptotic nuclei. Cultures were also stained (green) either with the monoclonal antibody AMV 3C2, to immunolabel the viral coat protein, in order to confirm the infection efficiency, or for the over-expressed bcl-2 protein. Uninfected control cells were negative for viral coat protein (not shown) and contained endogenous bcl-2, as reflected by faint green fluorescence (Figure 8A). All cells in the infected cultures were positive for bcl-2 over-expression as seen by increased green fluorescence (Figure 8B), and for viral coat protein (Figure 8C). Because bcl-2 protein is associated with mitochondria, cultures were labeled with MitoTracker Red7. Unlike the endogenous bcl-2, the over-expressed bcl-2 was seen to partially co-localize with mitochondria (Figure 8B, yellow).
Figure 8D shows the number of TUNEL-positive cells in each treatment. Infection with the bcl-2-carrying virus significantly inhibited apoptosis in comparison with the untreated control cultures and cultures infected with the negative insert control. Bcl-2 over-expression by RCAS/bcl-2 was also shown to abolish caspase-9 and caspase-3 cleavage in cultured cells (Fig. 8E), in comparison with cultures treated with the negative insert, RCAS/(-). Untreated cultures showed a background level of caspase-9 cleavage, but no detectable caspase-3 cleavage, in contrast to uncultured OT cushion tissue, in which cleavage of both caspases was detectable (Fig. 8E).

**DiI Labeling of Neural Crest Cells**

Premigratory neural crest cells were labeled by injecting the lipophilic dye, DiI, into the lumen of the neural tube. This procedure permanently labels all cells that were in contact with the lumen at the time of injection, including the pre-migratory neural crest cells (Figure 9A). The cardiac neural crest cells enter the distal OT by way of the pharyngeal arches, and form the AP septum, which facilitates division of the OT. Our results confirm the presence of neural crest cells in the septum of the distal OT (Figure 9B, arrows). Sections through the cushions were stained with DAPI to identify whether the nuclei of the DiI-labeled cells were apoptotic. In the OT cushions, many apoptotic cells were seen, as evidenced by their characteristically condensed and fragmenting nuclei. Some of these dying cells were seen to be carrying the DiI label (Figure 9C), indicating that neural crest cells were indeed dying in the environment of the cushions. Dying neural crest cells were also seen in the prongs of the AP septum (Figure 9D), but we saw no overlap of DiI labeling and apoptotic cells in the condensed mesenchyme of the AP septum (not shown). These findings further support the contention that some neural crest cells undergo apoptosis in the cushions and distal tips of the AP septum.
DISCUSSION

Interest in the mechanisms and significance of episodes of apoptosis in heart development has dramatically increased in recent years (11, 19, 32, 43). Much of this recent work has concentrated on the distribution and occurrence of apoptosis in the developing heart, with a reassessment of the detailed work of Pexieder (29), using more modern techniques. These techniques indicate a more localized distribution of dying cells than previously thought, with a smaller number of main foci of cell death than Pexieder described. Atrial and ventricular tissue seem to have little cell death in very early development, while the AV and the OT cushions seem to be the principal foci, with the largest numbers of dying cells occurring during ED 4-8 (19, 31). Outflow tract myocytes have also been shown to be eliminated in large numbers during this time (47), and some neural crest cells undergo apoptosis in the AV region after entering the heart through its venous pole (30). A large number of dying cells are also reportedly seen in the superior aspect of the interventricular septum, at the site of its fusion with the atrial septum and the OT septum (11, 43). This area includes the sites of formation of the AV node, the bundle of His, and the left and right bundle branches. It is also worth noting here, that this site is the final destination of some of the cardiac neural crest cells. However, understanding of the regulation of the apoptotic process or the exact developmental role of the cell death in these tissues is rudimentary.

In this paper, we demonstrate the involvement in the endocardial cushions of the main, and best characterized, regulators and effectors of cell death pathways. We also lend support to the idea that some of the apoptotic cells in the endocardial cushions may be of neural crest origin. Abnormalities in the development of the endocardial cushions and OT are implicated in many life-threatening congenital heart defects, which arise from structural defects or impaired
alignment (e.g. Tetralogy of Fallot, persistent truncus arteriosus, double outlet right ventricle, and ventricular septal defects). An understanding of the molecular aspects of programmed cell death in these areas will be essential to understanding the etiology of some of these defects.

*Members of the Bcl-2 and Caspase Families are Involved in the Regulation of Apoptosis in the Endocardial Cushions of the Developing Heart*

The suggested involvement of the bcl-2 family of cell death regulators in heart development arises from inconclusive results using knockout mice or mRNA localization in heart extracts (reviewed by 43). Some evidence for caspase involvement in heart development has also been demonstrated by deletion of the caspase-8 gene in mice which results in, amongst other features, impaired ventricular musculature (44). Disruption of the upstream receptor linked to caspase-8, FADD/MORT1 (53, 54) results in a similar phenotype, as does inactivation of casper (c-FLIP), the upstream inhibitor of this pathway (52). These gene knockouts do not however, appear to affect cushion development, and it has therefore been suggested that these molecules may act in a cell death-independent manner (52). Also, some evidence for caspase-3 activity has been found by Watanabe *et al*. (47), who demonstrated the activity of this enzyme in homogenates of whole chick OT.

Here, we show that anti-apoptotic bcl-2 and pro-apoptotic bax, bak and bid proteins are present in the endocardial cushions of the heart. Bcl-2 itself is present throughout the main phase of apoptosis (ED 4-8) with a transient down-regulation of the protein seen at day 4 in the OT cushions and at day 5 in the AV cushions, corresponding with times of elevated cell death in these tissues. Bcl-2 acts in a cytoprotective manner in cells, by interacting with, and hindering, the function of pro-apoptotic stimuli (41). The pro-apoptotic molecule bak appears to be up-regulated at the time of the highest incidence of cell death in the cushions, further implicating
members of this family in regulating cell death in these tissues. By contrast, levels of the pro-
apoptotic bax protein appear to remain constant throughout the time course examined. Such
constant levels of bax during episodes of apoptosis have been reported previously in other
tissues (50). Bid, a pro-apoptotic molecule linking the death-receptor pathway with the
mitochondrial pathway, is also up-regulated in the cushions at times of high levels of cell death,
implying that the receptor-mediated cell death pathway may also be involved in apoptosis in
these tissues.

The pro-apoptotic bax and bak appear to associate predominantly with the prongs of the
differentiating AP septum. However, the previously shown distribution patterns of apoptotic
cells appear not to overlap exactly with the pattern of these pro-apoptotic molecules (19). One
possible explanation for this is that the pro-apoptotic members may not be immediately active at
this site. Inactive bax and bak are often normally found to reside in the cytosol of healthy cells.
On receiving an appropriate signal, these molecules are activated and they translocate to the
mitochondria where they contribute to the downstream apoptotic signaling pathways (15). The
antibodies used in the current study do not distinguish between the inactive and active forms of
these molecules. Our results show that bax is present throughout the cushions and that it is
expressed in the cytoplasm of all apoptotic cells. It is possible that migrating and
morphogenetically active cells express inactive pro-apoptotic molecules during the early
development of the heart, notably in the endocardial cushions, and at the time of differentiation
they receive signals that bring about translocation and activation of the pro-apoptotic molecules.

We also show that bcl-2 over-expression can protect primary cultures of cushion cells from
serum starvation-induced apoptosis, and concomitantly abolishes caspase-9 and caspase-3
activation. Bcl-2 has been shown to protect numerous cell types from a variety of insults. One
interesting observation in our culture model was the fact that when the serum-starved cells were stained using the TUNEL technique, only 3-5% of the cells in the cultures were ever apoptotic at any one time (data not shown), even though bcl-2 seems to be present in the majority of the cells. This suggests that it is a specific population of cells in these cultures that is dying. Whether these are the neural crest-derived cells or not remains to be determined.

Western blotting of the dissected AV cushions and the OT provides evidence of caspase activation in these tissues. The caspase family of enzymes are the downstream effectors of cell death, that bring about the demise of the cell by cleaving and inactivating cellular proteins necessary to maintain cellular homeostasis, or by activating other cell death proteins (39). Different pathways of caspase activation may be present, with processes being either receptor-mediated or mitochondrial-initiated. Here we show evidence of activation of one of the upstream mitochondrial-associated caspases, and also a downstream effector caspase. Caspase-9 normally resides in an inactive state and is activated via the release of cytochrome c from the mitochondria. Activation results in cleavage of caspase-9 by a cytochrome c/Apaf complex (24) which then activates the downstream caspases, such as caspase-3 (37). The antibody used in this study recognized the 10 kDa cleavage fragment characteristic of activated caspase-9. We also provide evidence for the downstream activation of caspase-3 uniformly throughout the period examined. The activation of these two caspases does not always directly reflect the levels of expression of molecules of the bcl-2 family. However, we note that both pro-apoptotic bax and the caspase cleavage fragments are found to be present in the cushions throughout ED 4 – 7. Further evidence of caspase activity in the endocardial cushion cells is demonstrated by the in vitro studies on primary cultures of dissociated cushions. An inhibitor of the upstream mitochondrial pathway-associated caspase-9 was as effective as a universal inhibitor, while an
inhibitor of the downstream caspase-3 was significant but less effective, further suggesting the probable involvement of other caspases downstream from caspase-9.

A further hallmark of caspase activity is the cleavage of PARP, a DNA repair enzyme. In dying cells this protein is cleaved and inactivated from a 113 kDa molecule to 89 and 24 kDa cleavage products (8). PARP cleavage is seen throughout ED 3-8 in the OT, which may reflect the prolonged time course of apoptosis in the OT myocardium as well as the slightly earlier phase in the cushions. In the dissected AV cushions, PARP was consistently cleaved during ED 7-8, which closely follows the phase of peak cell death. The cleavage of PARP did not directly correlate with the apparent activation of caspase-9 and caspase-3. This points to the likelihood that PARP cleavage is associated with the activity of factors other than caspase-3, such as inhibitors of apoptosis (IAPs; 13), caspase-7 (12), or with caspase-independent mechanisms such as apoptosis-inducing factor (AIF; 7).

Another question that needs to be addressed is whether similar mechanisms of cell death are occurring in both sets of cushions. The derivation of the endocardial cushions in both the AV and OT regions appears to be similar (27), and a number of our findings would suggest that similar apoptotic pathways are in effect in both sets of cushions. Bcl-2 appears to be down-regulated at the onset times of peak cell death in both areas, whereas the pro-apoptotic bak appears to be up-regulated in each area. Immunoblotting also shows the cleavage fragment of active caspase-9 in both sets of cushions. Evidence of PARP cleavage in the AV cushions and the OT further support the idea that it is indeed apoptosis that is occurring in the cushions of the heart, and that similar processes are in effect in both regions.

Are the Dying Cells of Neural Crest Origin?
The pathways of neural crest cell migration have been well-studied, and the cardiac contribution of neural crest cells is the focus of much ongoing attention (5,18,45). There is debate as to whether or not the apoptotic cells observed during cushion morphogenesis are of neural crest origin. Retroviral and TUNEL labeling of neural crest cells (31) suggests that some of the apoptotic cells in the conal cushions of the outflow tract may be crest-derived. However, quail-chick chimeras imply a fate of differentiation as opposed to apoptosis for most neural crest cells (42, 46). Furthermore, the mismatch of timing of invasion and arrival at their final destination of migrating neural crest cells, with the distribution and numbers of apoptotic cells, further adds to the complexity. Probably, some neural crest cells are eliminated by apoptosis (31) but whether or not it is the final fate of the majority of them remains to be shown conclusively.

Here we provide further evidence that neural crest cells in the OT cushions and in the prongs of the AP septum undergo apoptosis. The co-localization of DiI labeling with the fragmenting nuclei, characteristic of apoptotic cells, suggests that, for at least some neural crest cells, cell death is the final fate. This is further supported by the conspicuous association of the pro-apoptotic bax and bak proteins with the prongs of the AP septum, which is the neural crest-derived mesenchyme that invades the endocardial cushions. Immunoreactivity for these proteins was much lower in the condensed mesenchyme of the AP septum. The significance of this is uncertain, but it is known that the prongs of the AP septum are located in an area that will become muscularized, as opposed to the mesenchymal fate of the condensed septum itself (32). Poelmann et al. (31) contend that only a subpopulation of neural crest cells in the distal tips of the prongs undergoes apoptosis, and it is this view that best fits our results. However, the patterns of apoptosis shown by us previously (19) do not exactly overlap with neural crest distribution. This would suggest a more complex process, in which the dying cells are not exclusively neural crest cells. In neural crest-ablated embryos, the cushions of the outflow tract
fail to fuse, resulting in a heart defect known as persistent truncus arteriosus (PTA; 22, 46), amongst other phenotypic changes, depending on the completeness of ablation. However, it remains to be seen if levels of apoptosis in the developing heart are affected in neural crest-ablated embryos.

What is the Role of Apoptosis in the Endocardial Cushions?

One prevailing view is that the apoptotic cells in the cushions are important for the process of muscularization of this tissue which occurs during the differentiation of the cushions into the valve leaflets (30). It has also been suggested that these myocardializing apoptotic cells are neural crest-derived (31). During cushion differentiation, myocardial cells invade the mesenchymal tissue by migration from the adjacent muscle layer (42, 51). The timing of this muscle cell migration follows the period of peak cell death in the cushions, and also corresponds with the timing of invasion of neural crest cells. Poelmann and Gittenberger-de Groot (30) postulate that apoptotic neural crest cells may release "molecules" that stimulate myocardialization of the valves and septa. However, there is as yet little evidence of apoptotic cells acting as in such a signaling manner. Similar roles have also been postulated for some of the other apoptotic populations of cells in the heart. For example, apoptosis of the neural crest cells that enter the heart via the venous pole is thought to induce the final differentiation of cardiomyocytes into the specialized conduction system. (30). Our results suggest that the apoptotic cells in the developing heart are not exclusively of neural crest origin and that it is premature to ascribe a central role for crest-derived cell death in cushion morphogenesis.

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FIGURE CAPTIONS

**Fig. 1.** Immunoreactivity for bcl-2 at the time of peak cell death in the AV and OT cushions. Figs. 1A and 1B: the distal OT (ED 4), showing relatively low levels of immunoreactivity in the condensed mesenchyme of the AP septum (aps) and higher levels in the surrounding cushion cells (ec) and myocardium (m).

Figs. 1C and 1D: the proximal OT cushions (ED 4) showing that immunoreactivity for bcl-2 protein is widespread in the cushions (ec) and myocardium (m) at this time.

Figs. 1E and 1F: the AV cushions at early (ED 4) and later (ED 6) stages respectively show immunoreactivity in most cushion cells. Negative controls using antibodies preabsorbed with the control peptide (not shown) resulted in absence of staining in all cases.

Magnification bars: Figs. A,C,E X 100 μm; Fig. B X 50 μm; Figs. D,F X 20 μm.

**Fig. 2.** Immunoreactivity for bax at the time of peak cell death in the cushions.

Figs. 2A and 2B show positive immunoreactivity in the distal OT (ED 4), with lower levels of bax protein in the condensed core mesenchyme of the AP septum (aps), and higher expression in adjacent cushion cells (arrow) and in the myocardium (m). The boxed region of Fig. 2A is shown in Fig. 2B, where bax immunoreactivity can be seen in the cells at the point of cushion fusion. Figs. 2C and 2D: in the more proximal OT (ED 4), bax appears to be highly immunoreactive in the prongs of the AP septum, and is scattered throughout the cells of the cushions (ec) and myocardium (m).
Fig. 2E: expression of bax protein is widespread throughout the developing AV cushions (ED 6). Negative controls (not shown) using antibodies preabsorbed with the blocking peptide resulted in absence of staining in all cases.

Fig. 2F: confocal image of a section through the AV cushions double-labelled with TUNEL for apoptotic nuclei (green) and immunocytochemistry for bax (red cytoplasm). Bax is present in the cytoplasm of most cells in the AV cushions, and in the cytoplasm of all apoptotic cells.

Magnification bars: Figs. A,C,E X 100 μm; Figs. B,D X 20 μm.

**Fig. 3.** Immunoreactivity for bak at the time of peak cell death in the AV and OT cushions.

Figs. 3A and 3B: bak protein expression in some regions of the OT cushions (ec) and myocardium (m) at ED 4. Strong expression can be seen in the prongs of the AP septum (aps). Fig. 3C: bak expression in the AV cushions (ED 6), showing little immunoreactivity in the myocardium in comparison with the cushions (ec). Negative controls (not shown) using the antibody preabsorbed with the blocking peptide resulted in an absence of staining in all cases.

Magnification bars: Fig. A X 100 μm; Fig. B X 20 μm; Fig. C X 50 μm.

**Fig. 4.** Western blot on dissected AV cushions and whole OT, probed for members of the bcl-2 family through ED 4-8.

Fig. 4A: levels of bcl-2 protein at 26 kDa show a decrease at ED 5 in the AV cushions and at ED 4 in the OT. The significance of the 23 kDa isoform is not yet known.
Fig. 4B: bax protein is consistently expressed throughout the time course examined, in both the AV cushions and the OT, from ED 4-8.

Fig. 4C: bak protein expression appears to be up-regulated from ED 4-6 in the AV cushions and from ED 4-5 in the OT.

Fig. 4D: bid protein at 18kDa becomes up-regulated between ED 4 and 7 in the AV cushions and is expressed throughout this period in the OT cushions.

+ = Jurkat cell-positive control; WE = positive control using lysate from the whole embryo.

**Fig. 5.** Western blot on dissected AV cushions and whole OT, probed for activation cleavage fragments of caspase-9 and caspase-3.

Fig. 5A: Immunoblotting reveals the presence of the 10 kDa cleavage fragment from ED 5-8 in the AV cushions, with a slightly extended expression in the OT, from ED 4-8. The expression of the cleavage fragment is more intense in the cushions than in the lysate of the whole embryo (WE).

Fig. 5B: The 12kDa cleavage fragment of activated caspase-3 is detectable in both the AV and OT cushions throughout ED 4-7.

**Fig. 6.** Western blot on dissected AV cushions and whole OT, probed for poly(ADP)-ribose polymerase (PARP). Immunoblotting reveals that the expression of the 24 kDa PARP cleavage fragment is dramatically up-regulated in the AV cushions at ED 7-8 only, while it is present throughout the time course examined in the complete OT (ED 3-8). In
the negative control, (P.Ab), preabsorption of the antibody with the a control peptide resulted in complete abolition of the 24 kDa band.

**Fig. 7.** Primary cultures of dissociated AV cushion cells were serum-starved and were treated with caspase peptide inhibitors to assess the role of caspases in the apoptosis of these cells.

Fig. 7A and 7B: respectively, DAPI (blue) labeling of nuclei and TUNEL (green) labeling of apoptotic nuclei in the same field of view. The condensed nuclei labelled by DAPI also label with TUNEL, confirming that they are apoptotic.

Fig. 7C: there is a significant reduction in the number of TUNEL-positive nuclei in cultures treated with a universal caspase inhibitor (Univ. CI), caspase-3 inhibitor (C3I) and caspase-9 inhibitor (C9I) in comparison with untreated serum-starved cells (Ctrl.). (n=5 for each treatment).

**Fig. 8.** Primary cultures of dissociated AV cushion cells were serum-starved to induce apoptosis and bcl-2 protein was retrovirally over-expressed.

Fig. 8A: a combined confocal image of cells stained with DAPI (blue), MitoTracker (red) and for bcl-2 immunoreactivity (green), showing low levels of endogenous bcl-2 in uninfected control cells.

Fig. 8B: there is increased green fluorescence in cultures infected with the RCASBP(B)bcl-2 virus, immunolabelled for bcl-2 protein. Co-localization of over-expressed bcl-2 with mitochondria is indicated by yellow in this image.

Fig. 8C: as Fig. 8B, but stained for the viral coat protein (green).
Fig. 8D: the effect of infection of cushion cells with RCASBP(B)bcl-2 virus and negative control RCASBP(B)(-) virus on the number of TUNEL-positive cells in the culture. Bcl-2 infection significantly inhibits apoptosis in comparison with the uninfected control (Ctrl.) and the negative-insert control (n=5 for each treatment).

Fig. 8E: Western blots for the cleavage fragments of caspase-9 (at 10kDa) and caspase-3 (at 12kDa) in cultured cushions cells. Lane 1: treated with RCAS/bcl-2; lane 2: treated with RCAS/(-); lane 3: untreated cultures in medium plus FBS; lane 4: uncultured cushion tissue from the outflow tract.

**Fig. 9.** DiI and DAPI labeling of cardiac neural crest cells.

Fig. 9A: the neural tube and the neural crest-derived structures are DiI-positive after taking up the injected dye. The neural crest cells have migrated to the dorsal root ganglia (arrow) and the sub-ectodermal melanocytes (arrowhead) as expected.

Fig. 9B: DiI-positive cardiac neural crest cells (red) in the septum dividing the distal outflow tract (arrowhead) and the endothelial layers of the great vessels (arrows).

Fig. 9C: DAPI labeled nuclei in the OT cushions (blue). Shrunken or fragmenting apoptotic nuclei are seen in some of the neural crest cells carrying the red fluorescent label (arrows).

Fig. 9D: As Fig. 9C, showing apoptotic neural crest cells in the prongs of the aorticopulmonary septum (arrows).
Figure 1
Figure 2
Figure 3
Figure 4
**Figure 5**

**Caspase 9**

**AV**

**OT**

-10kDa

**Caspase 3**

**AV**

**OT**

-12kDa

**PARP**

**AV**

**OT**

-24kDa

**Figure 6**
Figure 7
Figure 8

Ctrl. RCAS/bcl-2 RCAS/(-)

TUNEL positive nuclei per culture

1          2           3         4

Caspase 9

-10kDa

Caspase 3

-12kDa

p<0.001

Figure 8