

Viral Gene Transfer of the Antiapoptotic Factor Bcl-2 Protects Against Chronic Postischemic Heart Failure

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Background—Apoptosis secondary to acute ischemia and chronic remodeling is implicated as a mediator of heart failure. This study was designed to assess the effect of in vivo viral gene transfer of the anti-apoptotic factor Bcl-2 to block apoptosis and preserve ventricular geometry and function.

Methods and Results—In a rabbit model of regional ischemia followed by reperfusion, an experimental group treated with adeno-Bcl-2 was compared with a control group receiving empty vector adeno-null. Function was assessed by echocardiography, and sonomicrometry of the border zone was compared with the normal left ventricle (LV). Animals were killed at 6 weeks, and an additional group was killed after 3 days to see whether virus administration conferred an immediate effect. Animals that were administered Bcl-2 maintained higher ejection fractions at 2, 4, and 6 weeks compared with controls. Sonomicrometry demonstrated greater protection of border zone fractional shortening at 6 weeks. The Bcl-2 group had superior preservation of LV geometry with less ventricular dilatation and wall thinning. There was also reduced apoptosis compared with the controls. Finally, in the animals killed at 3 days, no functional difference was observed between the Bcl-2 and control groups.

Conclusions—Gene transfer of Bcl-2 preserves LV function after ischemia despite the absence of an observed acute protective effect. The benefit at 6 weeks is postulated to result from a Bcl-2-mediated reduction in apoptosis and ventricular remodeling. Adeno-Bcl-2 administration offers a potential strategy to protect the heart from late postischemic heart failure. (*Circulation*. 2002;106[suppl I]:I-212-I-217.)

Key Words: apoptosis ■ gene therapy ■ heart failure ■ myocardial infarction ■ remodeling

Ventricular injury after myocardial infarction can lead to progressive chamber enlargement. This change occurs in concert with a corresponding decline in ventricular function.¹ As the injured ventricle dilates, there is a failure to maintain a compensatory proportional increase in wall thickness. Consequently, the ratio of wall thickness to chamber radius is not preserved, and the transition to decompensated ventricular hypertrophy occurs.² Not only does this thinning of the ventricular wall occur in the infarcted necrotic zone, but it has also been demonstrated in the border zone region between the ischemic segment and the nonischemic viable myocardium.³ The interaction of the area of infarction with the remaining viable myocardium results in supraphysiological stretch across the border zone, which augments the tension load.⁴ As a result of the heightened wall tension, the ventricle begins to remodel and to undergo dilatation.

Initially, attempts to explain this expansion focused on analyzing length changes in individual myocytes or determining variability in the collagen content of the extracellular

matrix.³ However, it became apparent that the increase in wall tension, changes in wall dimensions, and further ventricular dilatation could result from the side-to-side slippage of myocytes from one another in an effort by these myocytes to distribute themselves to cover a larger ventricle, a phenomenon known as cell-cell slippage.³ Recent work has suggested that lateral slippage of myocytes is an early event that may condition the onset, development, and progression of a dilated cardiomyopathy that evolves after an acute myocardial infarction.⁵ Moreover, it has recently been demonstrated that cell loss within the myocardium may further destabilize the ventricular scaffolding, resulting in this cell-cell slippage of the remaining myocytes.⁶

This cell loss may be secondary to apoptosis or programmed cell death. Myocardial apoptosis has been demonstrated in animal models and humans after ischemia/reperfusion injury, myocardial infarction, congestive heart failure, and ischemic cardiomyopathy.^{6–11} Human autopsy data have revealed evidence of apoptosis within the infarct border zone

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but not in other remote areas of the heart outside the infarct.¹² It appears that apoptosis is an ongoing process associated with cardiac remodeling in the diseased heart. However, data are inconclusive in correlating the presence of apoptosis to cardiac pathophysiology and ventricular dysfunction.

Both proapoptotic and antiapoptotic factors exist simultaneously in the cell environment, and the balance between cell death and survival is mediated by a convergence of these factors. Apoptosis appears to be a universal mechanism by which organisms eliminate damaged or unnecessary cells. The apparent benefit of the energy-requiring process of apoptosis as opposed to cellular necrosis is that injured cells do not release their intracellular contents, which would result in adjacent cell injury and trigger an inflammatory response. It is likely that ischemic myocytes undergo apoptosis at a relatively early stage long before the onset of necrosis.¹³ It is unknown, however, whether the stimulus for the cell to initiate apoptosis is a sign of irreversible damage to cellular integrity. Blocking apoptosis in such a cell may theoretically prove to be detrimental, since the cell may instead go on to necrosis instead of apoptosis. On the other hand, it may be that blocking apoptosis prevents cell death.

The mitochondria play a critical role in apoptosis.¹⁴ The cytochrome *c* step in the electron transport chain is the most vulnerable to disruption^{15,16}. When a breakdown occurs, cytochrome *c* is released from the mitochondria into the cytosol to form a complex with the apoptosis activating factor (Apaf-1) and procaspase-9.¹⁷ Caspases are a family of cysteine proteases, and some are implicated as mediators of apoptosis.¹⁸ Synthesized as inactive precursors, they are proteolytically cleaved at specific aspartate residues into their active form. Thus, the cytochrome *c*/Apaf-1/procaspase-9 complex activates caspase-9, which in turn activates other effector caspases, resulting in programmed cell death.

One antiapoptotic factor, Bcl-2, has the potential to block apoptosis. The Bcl-2 protein family derives its name from B-cell lymphoma, where it was originally discovered. It is a potent and well-characterized regulator of cell survival.¹⁹ This 28-kDa protein is localized on the mitochondrial outer membrane, and it is ideally positioned to control cell survival at an early point in the apoptotic pathway. It binds to and inactivates the Bax protein by preventing the formation of Bax heterodimers that disrupt mitochondrial membrane function.^{20,21} Bcl-2 can prevent the opening of mitochondrial transition pores and the resultant release of cytochrome *c* that directly triggers apoptosis.²² Finally, Bcl-2 can block cytokine-mediated apoptosis via its ability to reduce inhibitor $I\kappa B\alpha$ levels, maintaining the antiapoptotic effects of the nuclear factor- κB signaling pathway.²³

In this study, we proposed to administer adeno-Bcl-2 after a period of myocardial ischemia to determine its role in preventing the development of postischemic heart failure. The hypothesis of this study is that chronic, progressive ventricular dilatation and dysfunction seen after myocardial ischemia may be exacerbated by apoptosis in the surviving myocyte pool. After the initial ischemia and reperfusion injury, immediate cell death occurs in the infarcted zone, mostly by necrosis with some apoptosis. As the ventricle remodels and the infarction proceeds to stiffen, we postulate

that apoptosis persists at a low level in the border zone. Continued cell death and the loss of myocytes in this region may lead to ventricular dilatation and dysfunction. As a result, blocking apoptosis may reduce the drive toward continued ventricular dilatation and preserve myocardial function. This would allow targeted gene therapy to be an adjunct to standard therapies after infarction in an effort to prevent subsequent heart failure.

Methods

Animal Care

This study was performed in accordance with the animal care guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Adenoviral Vector Construction

A replication-deficient (E1, E3 deleted) adenoviral vector containing the transgene encoding human Bcl-2 and the constitutively active cytomegalovirus (CMV) promoter was obtained from the University of Iowa Vector Core.

Animal Surgery

Thirty New Zealand White rabbits (3 to 4 kg) were used in the study. All rabbits were fully anesthetized with intramuscular doses of ketamine (40 mg/kg), xylazine (2.5 mg/kg), glycopyrrolate (0.02 mg/kg), and buprenorphine (0.05 mg/kg) and then mechanically ventilated (Hallowell EMC model 2000). The left hemithorax was opened through the third intercostal space, exposing the base of the heart. The circumflex artery was identified, and a 6-0 polypropylene suture was placed around its circumference at a point halfway between the atrioventricular groove and the left ventricular (LV) apex. The circumflex tourniquet was tightened to achieve complete cessation of flow as demonstrated by both electrocardiographic changes and visual blanching of the myocardium. After 30 minutes, the tourniquet was released, allowing for reperfusion.

Next, the main pulmonary artery and ascending aorta were each dissected free and encircled with a 0-0 silk suture. Each silk tie was passed through a 14F red rubber catheter creating a tourniquet. The pulmonary tourniquet was then tightened for complete pulmonary artery occlusion. The LV was allowed to empty for 5 seconds, and then the aortic tourniquet was tightened to achieve complete aortic occlusion. A solution (1000 μ L) containing 5.0×10^{10} particle-forming units of recombinant human adeno-CMV-Bcl-2, suspended in 10% glycerol, was injected into the LV cavity in the experimental group ($n=11$). The control group ($n=7$) received 1000 μ L of a solution containing 5.0×10^{10} particle-forming units of empty vector adeno-null. After 30 seconds of complete outflow occlusion, the tourniquets were removed. The chest was closed, and the animal recovered without operative intervention for 6 weeks. During this interval, the animal was studied with transthoracic echocardiography at 2-week intervals (weeks 2, 4, and 6).

The animals returned for nonsurvival surgery after 6 weeks. A repeated left thoracotomy was performed with exposure of the LV free wall. A polypropylene suture that marked the site of circumflex artery occlusion identified the border zone. Four 1-mm piezoelectric sonomicrometers were implanted into the myocardium to measure fractional shortening. This included the placement of 2 crystals in the infarcted region and 2 crystals in the normal LV away from the infarcted region. (Figure 1) After sonomicrometry, the heart was arrested in diastole by an intravenous injection of 1 mL KCl (1 mEq/mL), and the right atrium was incised to allow drainage of blood. The heart was procured, and the LV cavity was filled with OCT embedding compound retrograde through the transected aortic root at a constant intracavitary pressure. The catheter was removed and the aortic root was ligated. The heart was placed in a container of OCT embedding compound, bathed in isopentane, frozen in liquid nitrogen, and stored at -80°C .

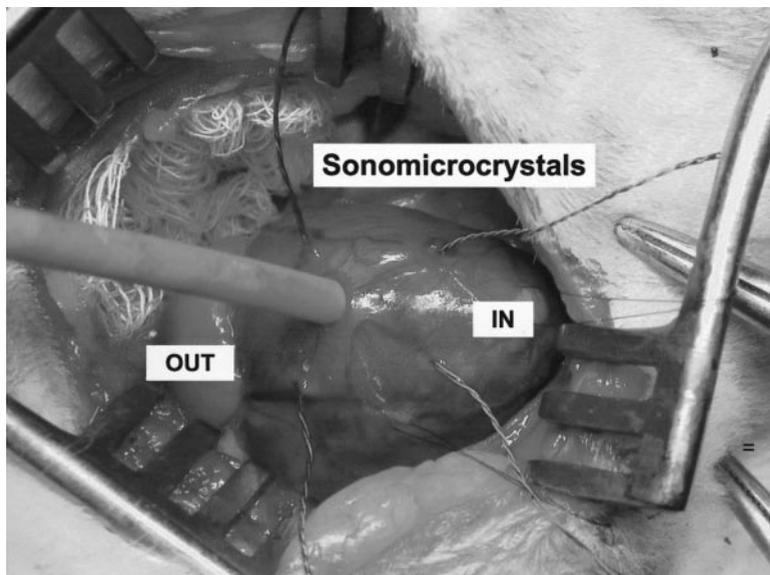


Figure 1. Representative composite intraoperative photograph, which demonstrates the circumflex artery tourniquet positioned on the left ventricle for regional ischemia. In addition, it illustrates the position of the 2 sonomicrocrystals within the ischemic segment ("IN" position) 10 mm apart from each other, and the 2 sonomicrocrystals in the viable myocardium outside the infarct zone ("OUT" position) 10 mm apart. Fractional shortening is defined as the difference between the end-diastolic length and the end-systolic length divided by the end-diastolic length (the distance between the crystals). Contractility across the border zone was measured by assessing segmental length changes between a crystal in the "IN" position with the ipsilateral (in relation to the tourniquet site of occlusion) "OUT" crystal 10 mm apart. Normal contractility was measured between the 2 "OUT" crystals.

An additional group of 6 rabbits ($n=2$, Bcl-2; $n=3$, adeno-null) underwent the identical surgical procedure but were killed on postoperative day 3 after sonomicrometry and echocardiography. The heart was arrested in diastole with 1 mL of KCl (1 mEq/1 mL) and procured as previously described. This group was used to determine whether Bcl-2 administration would demonstrate an effect immediately within 3 days or whether it would be apparent over time. In addition, a group of normal rabbits ($n=5$) was killed without intervention to determine baseline ventricular wall thickness and diameter. There was 1 death during the procedure from ventricular dysrhythmia and 1 death in the follow-up period (2/30, or 6.7% total mortality).

Functional Analysis and Assessment of Infarct Percentage

Functional analysis was studied by transthoracic echocardiography to evaluate global cardiac function. Each animal underwent echocardiography at 2, 4, and 6 weeks after surgery under light sedation (ketamine/xylazine). The group that was killed after 3 days underwent echocardiography before euthanasia. Five normal rabbits were studied to determine baseline ejection fraction (EF). Each of the investigators and an echocardiography specialist were blinded to the treatment group.

Sonomicrometry, a well-established technique that measures LV segmental length changes during each cardiac cycle, was used to measure regional contractility. Contractility across the border zone was measured between a crystal in the infarcted region and the ipsilateral (in relation to the tourniquet site of occlusion) crystal 10 mm apart in the viable myocardium. Normal contractility was measured between the 2 crystals in the viable myocardium. Fractional shortening is defined as the difference between the end-diastolic length and the end-systolic length divided by the end-diastolic length (the distance between the crystals). The data were analyzed with Sonoview software (Sonometrics Corp.) and reported as a percentage of baseline \pm SEM.

Four sections from each heart were analyzed for infarct percentage of the LV by triphenyl tetrazolium chloride (TTC) incubation, as previously described.²⁴ Images of both pre-TTC- and post-TTC-incubation hearts were scanned into Adobe Photoshop. The area that was not stained by TTC was measured and calculated as the infarct area. Infarct percentage size for each heart was defined as (infarct area of each slice/total area of LV) \times 100.

Chamber Size and Wall Thickness

In both groups, 4 adjacent sections, midway between the base and the apex spanning the border zone and perpendicular to the longitudinal

axis of the ventricle, were obtained to measure chamber diameter and thickness of the free wall of the ventricle. Five equally spaced measurements of the free wall of the LV were collected from each slice, and the values were averaged by each of 2 investigators blinded to the treatment groups. Measurements were obtained with a dissecting microscope with a micrometer accurate to 0.03 mm. Five native rabbits were also killed to evaluate the chamber diameters and wall thickness in uninfarcted and untreated animals.

TUNEL Assay

Specimens were obtained from storage in a -80°C freezer and allowed to warm to approximately 22°C . Five transverse $10\text{-}\mu\text{m}$ sections of the border zone were prepared with a cryostat at 0.25-cm intervals from the point of circumflex occlusion to the apex of the heart. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed with a TdT-FragEL DNA fragmentation detection kit (Oncogene Research Products). The apoptotic index was taken as the number of apoptotic nuclei per high-powered field (HPF) examined in 5 measurements obtained from the free wall of each section, by each of 2 investigators blinded to the treatment group. The apoptotic index was expressed as a percentage of the number of apoptotic cells per total nuclei in each HPF examined.

Western Blotting

Specimens obtained for Western blotting were snap-frozen in liquid nitrogen after removal from the animal, without OCT fixative. Specimens were pulverized, homogenized in 10 volumes of sodium dodecyl sulfate (SDS) lysis buffer (100 mmol/L Tris, pH 8.0; 10% SDS, 10 mmol/L EDTA, 50 mmol/L dithiothreitol), and sheared with a 25-gauge needle. Samples were normalized for total protein content, and 50 μg of each sample was electrophoresed on a 12.5% SDS-polyacrylamide gel after addition of 6 \times sample loading buffer and 3 minutes of denaturation at 100°C . Proteins were then transferred to Immobilon-P (Millipore) by using a wet transfer apparatus. The membrane was subsequently blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20. Immunoblotting was performed using the mouse monoclonal antibody against human Bcl-2 at a 1:100 dilution (DAKO). Detection was performed using the ECL kit (Amersham). Subsequently, the Immobilon-P membrane was stained with Coomassie blue, and the actin band was identified to confirm equal loading conditions.

Statistical Analysis

All data were expressed as mean value \pm SEM. Statistical analyses were performed using INSTAT^T (GraphPad Software). The un-

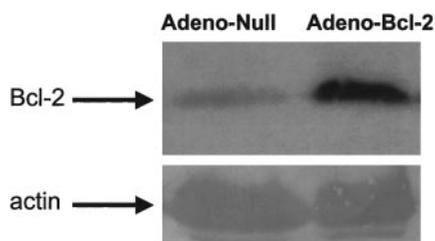


Figure 2. Western blot, showing mouse anti-human monoclonal Bcl-2 that was used to probe samples at 6 weeks postinfarction. Lane 1 is an adeno-null sample, and lane 2 is a Bcl-2-treated specimen.

paired, 2-tailed Student's *t* test was used to calculate the probability value. Multiple comparisons against the control were first analyzed by 1-way analysis of variance followed by Tukey-Kramer's modified unpaired student's *t* test to determine significance between groups. A probability value <0.05 was considered significant.

Results

Western Blot

At 6 weeks, increased expression of Bcl-2 was demonstrated in the adeno-Bcl-2-treated group compared with the controls (Figure 2).

Echocardiography

At 3 days, no difference in EF was observed in the Bcl-2-treated group compared with the controls ($33 \pm 2.5\%$ versus $31 \pm 6.0\%$; $P=NS$). However, Bcl-2 animals maintained a higher EF than controls at week 2 ($41 \pm 1.8\%$ versus $32 \pm 1.5\%$, $P<0.05$), week 4 ($41 \pm 2.0\%$ versus $29 \pm 2.0\%$, $P<0.01$), and week 6 ($40 \pm 1.8\%$ versus $27 \pm 1.8\%$, $P<0.001$) as shown in Figure 3. For comparison, baseline EF in a group of native rabbits was $42 \pm 1.2\%$ ($P=NS$ compared with Bcl-2).

Sonomicrometry

At 3 days, there was no statistical difference in fractional shortening seen in the Bcl-2-treated groups compared with the controls (Bcl-2, $50.4 \pm 7.6\%$ versus control, $62.2 \pm 13.8\%$; $P=0.57$). However, at 6 weeks, there was significantly greater preservation of fractional shortening in the Bcl-2 group (Bcl-2, $72.1 \pm 7.0\%$ versus control, $49.8 \pm 2.0\%$; $P<0.03$). Results are depicted in Figure 4. Echocardiography

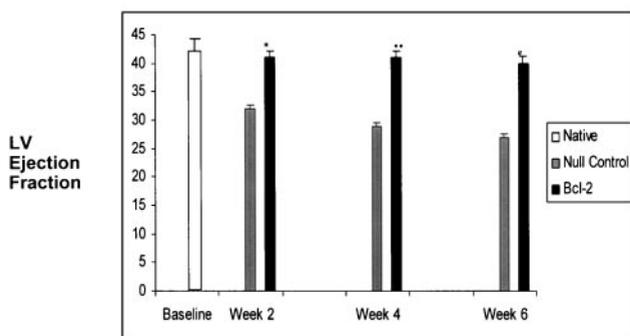


Figure 3. Echocardiography. Global myocardial function measured by left ventricular ejection fraction. Although no significant difference is seen at 3 days between the 2 groups, at weeks 2, 4, and 6, the benefit is apparent ($*P<0.05$, $**P<0.01$, $\#P<0.001$).

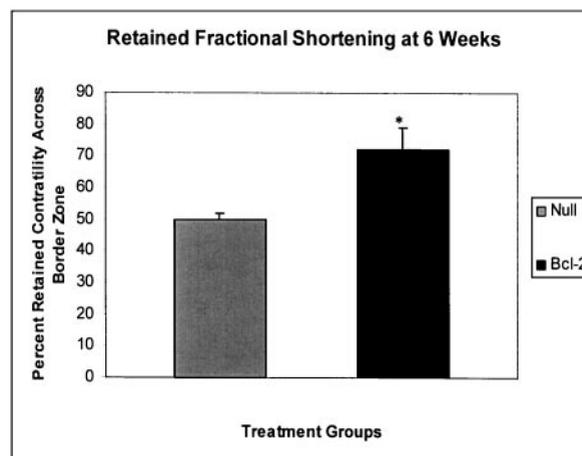


Figure 4. Sonomicrometry, showing superior preservation of fractional shortening (FS) in the Bcl-2 group at 6 weeks. Results are reported as a percentage of baseline FS of the border zone compared with the remote viable myocardium ($*P<0.03$).

and sonomicrometry together consistently demonstrated greater preservation of myocardial contractility in the Bcl-2-treated animals.

Infarct Size

There was no difference in the infarct size in the Bcl-2 group ($28.3 \pm 2.7\%$) compared with the control group ($28.6 \pm 2.0\%$, $P=NS$).

Ventricular Geometry

Control rabbits had significant ventricular dilatation with a greater mean LV midchamber diameter (16.87 ± 0.67 mm) compared with the uninfarcted and untreated native rabbits (13.44 ± 0.56 mm, $P<0.01$). There was significant wall thinning in the control group (2.57 ± 0.15 mm) compared with native rabbits (3.61 ± 0.60 mm, $P<0.001$). On the other hand, rabbits treated with adeno-Bcl-2 showed no statistically significant difference from native rabbits with respect to ventricular diameter (13.70 ± 0.46 mm, $P=NS$) or mean free-wall thickness (3.34 ± 0.10 mm, $P=NS$). In addition, there was significant preservation of chamber dilatation ($P<0.01$) and free-wall thickness ($P<0.01$) in the Bcl-2 group compared with controls. The results are summarized in Figures 5 and 6. A representative cross section from the border zone from each group is shown in Figure 7.

Demonstration of Apoptosis

There was a significant reduction in the apoptotic index in the Bcl-2 group compared with the controls after 6 weeks (control, $2.77 \pm 0.35\%$ versus Bcl-2, $1.01 \pm 0.14\%$; $P<0.0002$). The results are shown in Figure 8.

Discussion

Myocardial cell death after infarction results in progressive ventricular dilatation. Olivetti et al³ noted a 20% increase in midchamber luminal diameter and a 33% thinning of the ventricular wall in a rat ischemia model consisting of a 63% infarction in the area at risk. This is consistent with the results observed in our 30% total LV infarction model. This study

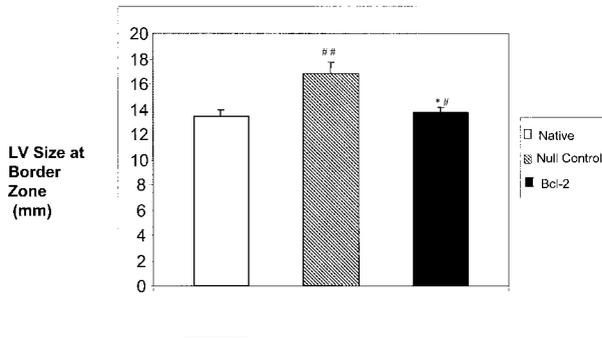


Figure 5. Assessment of left ventricular dilatation, s measured by ventricular diameter at the border zone (* $P < 0.01$ when compared with adeno-null control group; # $P = NS$ and ## $P < 0.01$ when compared with native group).

demonstrates a protective effect of Bcl-2 with respect to preservation of ventricular geometry and prevention of ventricular dilatation. This was paralleled by superior preservation of ventricular function over time.

In addition to a significant number of myocytes undergoing apoptosis in the ischemic segment during the initial period of ischemia/reperfusion, it appears that apoptosis is present at the border zone beyond the initial injury. Although the TUNEL assay is not specific for cardiomyocytes, recent reports have determined that nearly all apoptotic bodies are cardiomyocytes.^{25,26} Olivetti et al³ also noted that the mechanism of ventricular wall thinning of the remaining myocardium was the result of a 36% reduction in the mural number of myocytes over time. Initial myocyte loss may be coupled with additional cell loss over time due to apoptosis.

The pathophysiological contribution of chronic apoptosis is suggested by the comparison of ventricular function at 3

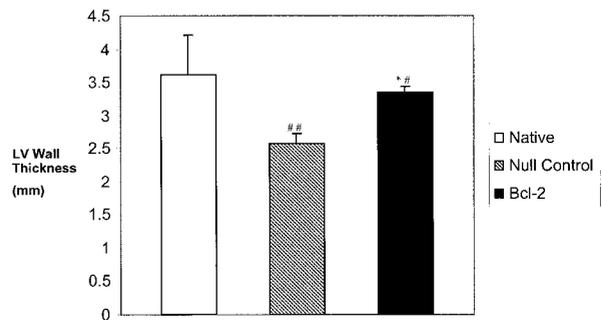


Figure 6. Assessment of left ventricular (LV) free-wall thinning at the border zone was measured by LV free-wall thickness (* $P < 0.01$ when compared with adeno-null control group, # $P = NS$ and ## $P < 0.01$ when compared with native group).



Figure 7. Representative sections at border zone. Representative midchamber sections through the border zone 6 weeks after ischemia are depicted above. The section on the left is a native (uninfarcted and untreated) rabbit heart. The section in the middle is a null control heart (no Bcl-2), and the section on the right is a heart transfected with adeno-Bcl-2. There is a significant increase in midchamber transverse diameter and decrease in wall thickness in the untreated infarcted group compared with the infarcted Bcl-2-treated group and native group.

days to 6 weeks. Since Bcl-2 overexpression would first be detected 48 to 72 hours after viral gene delivery, we would not expect to see an immediate difference in function, since the ischemia/reperfusion injury and infarct size are largely determined in the first 24 hours. This is supported by the data noting that at 3 days after ischemia, there is no functional difference between the groups treated with Bcl-2 or adeno-null. In addition, there was no difference in infarct size between the 2 groups, since Bcl-2 would not have had a chance to exert an immediate protective effect. However, the benefit over the course of the next 6 weeks seen in the Bcl-2 group would be from preventing a slow decline in function by blocking apoptosis that occurs after the initial injury.

The trigger for apoptosis over the chronic period may be due to stretch across the border zone. Pathological alterations of myocardial load as seen after infarction may be coupled with the generation of reactive oxygen species and the activation of gene products implicated in apoptosis.⁴ There is evidence to suggest that progressive stretch leads to an increase in cytosolic calcium levels, which destabilize the mitochondrial membrane and trigger the release of cytochrome *c* and apoptosis.²⁷ In addition, stretch sensors within the affected cells signal autocrine release of angiotensin II, which subsequently activates the transcription factor p53, favoring apoptosis by increasing Bax and decreasing Bcl-2 expression.²⁸ Thus, as the mechanical disadvantage of higher wall tension is augmented with infarct maturation, ventricular stiffening, and progressive chamber dilatation,² the continued stretch across the border zone may initiate and sustain further apoptosis.

Although this stretch may be a trigger of apoptosis, other initiators of apoptosis may be present. Tumor necrosis factor- α (TNF- α) has been demonstrated to be present in increased levels in congestive heart failure,²⁹ myocardial infarction,³⁰ and ischemia/reperfusion injury.³¹ TNF- α has been shown to trigger apoptosis as well by activation of caspase-8.³² In addition, pathological alterations in the border zone may occur because the oxygenation potential of the surviving myocardium is significantly reduced in this region, making the cells more susceptible to additional ischemic episodes.²³

Bcl-2 conferred protection from apoptosis during the entire 6 weeks examined while gene expression was maintained. It would be important to determine whether this protective effect of Bcl-2 would still be present over a longer period once the transgene was no longer expressed. It may be that further long-term protection requires continued Bcl-2 expression; on the other hand, it may be that Bcl-2 is only required

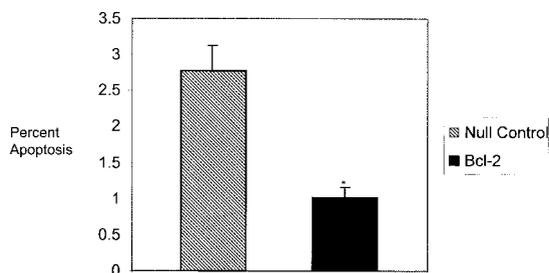


Figure 8. Apoptotic index. A significant reduction in the percentage of apoptotic cells in the Bcl-2 group compared with the controls ($*P < 0.0002$) after 6 weeks is demonstrated.

during a critical period soon after the infarction to “rescue” the heart by blocking apoptosis.

We postulate that apoptosis persists within the border zone region after infarction, which contributes to eventual ventricular dilatation and dysfunction. The data suggest that blocking apoptosis in this region can prevent pathological ventricular remodeling. Administration of adeno-Bcl-2 leads to a reduction in apoptosis, preservation of ventricular geometry, and prevention of postischemic heart failure.

Acknowledgments

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