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Bcl-xL gene transfer protects the heart against ischemia/reperfusion injury

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Abstract

Ischemia and reperfusion (I/R) injury causes the progression of cardiac dysfunction. The prevention of cardiomyocyte-loss due to I/R injury is important for the treatment of heart failure. Therefore, we employed antiapoptotic Bcl-xL protein to prevent I/R injury in the heart and evaluated the cardioprotective effect of Bcl-xL transduction by adenoviral vector (Adv) after I/R injury. Adv with Bcl-xL gene was injected in the rat heart 4 days prior to I/R. The prevention of cardiac performance-loss and the reduction of cardiac apoptosis, after 30 min ischemia and 30 min reperfusion of global I/R, were demonstrated in the heart with adenoviral Bcl-xL transduction. Also, significant reductions of the infarct size and serum creatine kinase levels were observed in the heart transduced with Bcl-xL gene compared with control after 30 min ischemia and 24 h reperfusion of the left anterior coronary artery. Thus, Bcl-xL may serve as a potential therapeutic tool for cardioprotection.
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Keywords: Bcl-xL; Adenoviral vector; Ischemia and reperfusion injury; Myocardial infarction; Apoptosis

Apoptosis is an important mode of both physiological and pathological death of cardiomyocytes. Ischemia and reperfusion (I/R) injury elicits an apoptosis of cardiac myocytes via the production of reactive oxygen species and mitochondrial damage. This massive cardiac myocyte death facilitates the progression of cardiac dysfunction, resulting in heart failure. Therefore, the prevention of cardiomyocyte-loss due to I/R injury has particularly important implications in the interventional treatment of coronary ischemia and also in cardiac surgery for cardiopulmonary bypass and heart transplantation.

The mitochondrion is a key organelle for cardiac I/R injury and mitochondrial damage generated by I/R injury results in the release of cytochrome *c* from mitochondria as a trigger of apoptosis. The antiapoptotic Bcl-2 family proteins prevent apoptosis by inhibiting the cytochrome *c* release through interaction with proapoptotic Bax [1,2]. Therefore, several therapeutic approaches to ameliorate I/R injury have been attempted through the modulation of pro- and anti-apoptotic Bcl family protein expression. One of the therapeutic approaches for the prevention of I/R injury is ischemic preconditioning, and it has been reported that cardiomyocytes can acquire resistance to I/R injury after brief ischemia through Bcl-2 induction [3]. Protection against I/R injury has also been accomplished by the administration of transforming growth factor-β [4], superoxide dismutase [5], hemeoxygenase-1 [6], and caspase-3 inhibitor [7], and the upregulation of Bcl-2 was proposed as a protective molecule in these studies. Some of the direct evidence for the cardioprotective effect of Bcl-2 against I/R injury has been demonstrated by studies utilizing transgenic animals [8,9]. However, we previously reported that adenoviral overexpression of Bcl-2 paradoxically exerted a proapoptotic effect in glioma

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cells. Moreover, Oshiro et al. [10] reported a similar phenomenon in the reperfused liver transduced with adenoviral Bcl-2. These findings suggest that adenoviral overexpression of Bcl-2 in the heart might accelerate I/R injury.

On the other hand, another Bcl-2 family member, Bcl-xL, has strong antiapoptotic effects, and it has been reported that Bcl-xL expression is modulated by a distinct mechanism with Bcl-2 in the context of I/R. Administration of insulin-like growth factor [11], hepatocyte growth factor [12], endothelin-1 [13], or angiotensin-converting enzyme inhibitor [14] can clearly attenuate cardiac I/R injury, and Bcl-xL induction by these signals has been suggested as a mechanism of cardioprotection. However, direct evidence of cardioprotection by Bcl-xL in cardiac I/R injury has not been reported.

In the present study, we investigated whether adenovirus-mediated *Bcl-xL* transfer could provide a direct cardioprotective effect in the context of cardiac I/R injury, and evaluated the effects of *Bcl-xL* transduction on cardiac apoptosis and function after global I/R. We also evaluated the effect of Bcl-xL gene transfer on myocardial infarction after regional I/R injury in the heart.

Materials and methods

Adenoviral vectors. The adenoviral vector (Adv) encoding human *Bcl-xL* (AxCAhBclxL) [15] or *Escherichia coli β-galactosidase* (*LacZ*) (AxCAZ3) [16,17] was used in these experiments. Adv propagation and purification were described previously [16]. Before use, the viral titer (pu/ml, particle units/ml) and the contamination of replicate competent Adv in the viral stock were evaluated according to previous reports [16,18].

Myocardial adenoviral injection. Lewis rats (male, 8 weeks old, 250–300 g weight) were purchased from Japan SLC (Hamamatsu, Japan). Adenovirus-mediated gene transduction to the heart was carried out by direct myocardial injection of adenoviral vector 4 days prior to I/R injury. Briefly, a left thoracotomy was performed and adenovirus particles (10^{10} pu) in 0.1 ml of 0.9% saline solution were injected into the anterior wall of the left ventricle using a 1-ml syringe with a 27-gauge needle. Three experimental groups were evaluated. Saline control group rats were injected with 0.9% saline solution alone. In the LacZ group, rats were injected with adenoviral vector encoding *LacZ* (AxCAZ3) as a vector control. In the Bcl-xL group, rats were injected with *Bcl-xL* (AxCAhBclxL). The study was performed in accordance with the Institutional Guidelines for Animal Experiments.

Determination of β-galactosidase expression in heart. The detection of LacZ expression by X-gal staining in the heart was described previously [17,19]. Briefly, the heart transduced with AxCAZ3 was fixed by systemic perfusion with 2% paraformaldehyde. The isolated heart was then rinsed with cold phosphate buffered saline (PBS) and immersed in X-gal (Sigma–Aldrich, St. Louis, MO) solution (1 mg/ml X-gal in PBS, pH 7.2, 2 mM $MgCl_2$, and 4 mM potassium ferricyanide) for 16 h at 30 °C.

Determination of Bcl-xL, Bcl-2, Bax, and cleaved caspase-3 expression in heart. Hearts were homogenized in lysis buffer (100 mM Tris–HCl, pH 7.4, 15% glycerol, 2 mM EDTA, 2% SDS, and 0.1 mM phenylmethylsulfonyl fluoride). Homogenates were then heated at 95 °C for 10 min and centrifuged at 12,000*g* for 10 min. After determination of protein concentration in supernatants by BCA Protein Assay (Pierce, Rockford, IL), aliquots (40 μg of cellular protein) were electrophoresed in 15% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane. Immunoblotting analysis was carried out by incubating the membranes with either rabbit anti-human Bcl-xL polyclonal antibody (Ab) (Transduction Laboratories, Lexington, KY), mouse anti-Bcl-2 monoclonal Ab (C-2, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Bax monoclonal Ab (YTH-6A7, Trevigen, Gaithersburg, MD), or rabbit anti-cleaved caspase-3 polyclonal Ab (#9662, Cell Signaling Technology, Beverly, MA). These antibodies cross-reacted with the respective rat antigens as well as human antigens. As an internal control, mouse anti-α-tubulin monoclonal Ab (B-5-1-2, Sigma–Aldrich) was used. To detect rabbit Ab, horseradish peroxidase (HRP)-conjugated $F(ab)'_2$ fragment of donkey anti-rabbit immunoglobin Ab (Amersham Biosciences UK, Buckinghamshire, England) was used as a second Ab and visualized by the ECL-plus kit (Amersham Biosciences UK, Little Chalfont, UK). To detect mouse monoclonal Ab, HRP-conjugated rabbit anti-mouse IgG + A + M (H + L) Ab (Zymed, South San Francisco, CA) was used.

The heart isolation and global ischemia. Rats were sacrificed 4 days after gene-transduction. The excised heart was perfused with a modified Krebs–Henseleit solution gassed with 95% O_2 and 5% CO_2 through the aorta with 85 mmHg fixed perfusion pressure by the Langendorff technique with a fixed pacing rate of 300 bpm [8,20]. After equilibration for 20 min of perfusion at 37 °C, I/R (30 min of ischemia followed by 30 min reperfusion at 37 °C) was applied to the heart. Coronary effluent was collected for the measurement of coronary flow.

Measurement of cardiac function. The ventricular function of the rat heart was measured by insertion of a latex balloon connected to a pressure transducer into the left ventricle via the left atrium [8]. Maximum and minimum rates of pressure development ($\pm dP/dt$) and coronary effluent flow rate at 10 mmHg of left ventricular end-diastolic pressure were measured before and after I/R injury. Percentages of functional recovery were calculated by dividing each of the functional recovery values at the end of reperfusion by their corresponding pre-ischemic values. Coronary flow rate was determined by the amount of perfusate measured in a specific time period.

Determination of TUNEL positive cardiomyocytes. After global I/R, the heart was snap-frozen in liquid nitrogen and a 10-μm thickness cryo-section was prepared. The section was fixed with 4% paraformaldehyde at room temperature for 20 min, incubated with blocking solution (3% H_2O_2 in methanol) for 10 min at room temperature, and then incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. After washing with PBS, the section was incubated with the TdT-mediated dUTP-biotin nick end labeling (TUNEL) reaction mixture (Rosche Diagnostics, Mannheim, Germany) for 60 min at 37 °C. After washing, the section was further incubated with converter-POD (Rosche) for 30 min at 37 °C and then incubated with DAB-substrate (Rosche) solution at room temperature for 10 min in order to visualize TUNEL positive nuclei.

In vivo ischemia and reperfusion (regional I/R) model. Four days after the injection of either AxCAZ3 or AxCAhBclxL (10^{10} pu) into the heart, the left anterior descending coronary artery (LAD) was ligated 3 mm below the left atrial appendage with a 6–0 suture. After 30 min of ligation, the suture was released and reperfusion was carried out. Rats were sacrificed 24 h after reperfusion and infarct size was evaluated.

TTC staining and infarct size. To evaluate the effect of 30 min ischemia followed by 24 h reperfusion of the LAD, the area at risk (AAR) and infarct region were determined by Evan's blue dye perfusion and triphenyltetrazolium chloride (TTC) staining [5]. At the end of reperfusion, the LAD was religated at the same position and perfused with 2% Evan's blue (Sigma–Aldrich) via the left ventricle. After washing with PBS (pH 7.4) at room temperature, the heart was sectioned at the middle point between the ligation site and the apex at 2–3 mm thickness, and then immersed in PBS containing 1% TTC (Sigma–Aldrich) for 20 min at 37 °C to stain the viable myocardium

with a brick-red color. To block over-staining, the section was rinsed with 10% neutralized formalin for 12 h. The sectioned heart was then photographed and captured as a digital image. Total area, AAR, and infarct area were analyzed by NIH image software. Percentage AAR was defined as AAR (brick-red area + white area)/total area × 100 and percentage infarct size was calculated as the infarct area (white area)/AAR (brick-red area + white area) × 100.

Creatine kinase measurement. Twenty-four hours after temporary ligation of the LAD, blood samples were collected to determine serum creatine kinase (CK) release. CK activity was measured by an enzymatic assay.

Statistical analysis. Data were expressed as means ± standard deviations (SD). Statistical comparisons were performed using ANOVA followed by Bonferroni/Dunn testing. A p value less than 0.05 was considered to be statistically significant.

Results

Adenoviral gene expression in rat heart

Initially, we confirmed adenoviral gene transfer into normal hearts by the adenoviral vector, AxCAZ3. LacZ-positive cardiac muscle cells were widely distributed around the viral injection sites on the pericardium 4 days after gene-transduction (Figs. 1A and B). Bcl-xL expression 4 days after adenoviral transduction to normal heart was also evaluated by Western blot analysis. As shown in Fig. 1C, rat endogenous Bcl-xL was constant among normal heart, saline control, and adenoviral control. However, robust Bcl-xL expression was detected in the heart transduced with Bcl-xL gene and this Bcl-xL overexpression level reached at a peak 4 days after adenoviral injection (data not shown). In order to obtain sufficient cardiac protection while allowing the heart to recover from the myocardial damage due to the adenoviral injection, we delivered the gene 4 days before I/R injury.

Expression of Bcl-2 and Bax in the rat heart transduced with Bcl-xL

We also evaluated Bcl-2 and Bax expression in the hearts transduced with the Bcl-xL gene, because Bcl-xL transduction might alter the Bcl-2/Bax ratio and modify the results of I/R injury. In this determination, we could not observe any difference in either the Bax or Bcl-2 expression levels between the hearts with or without adenoviral transduction (Fig. 1C).

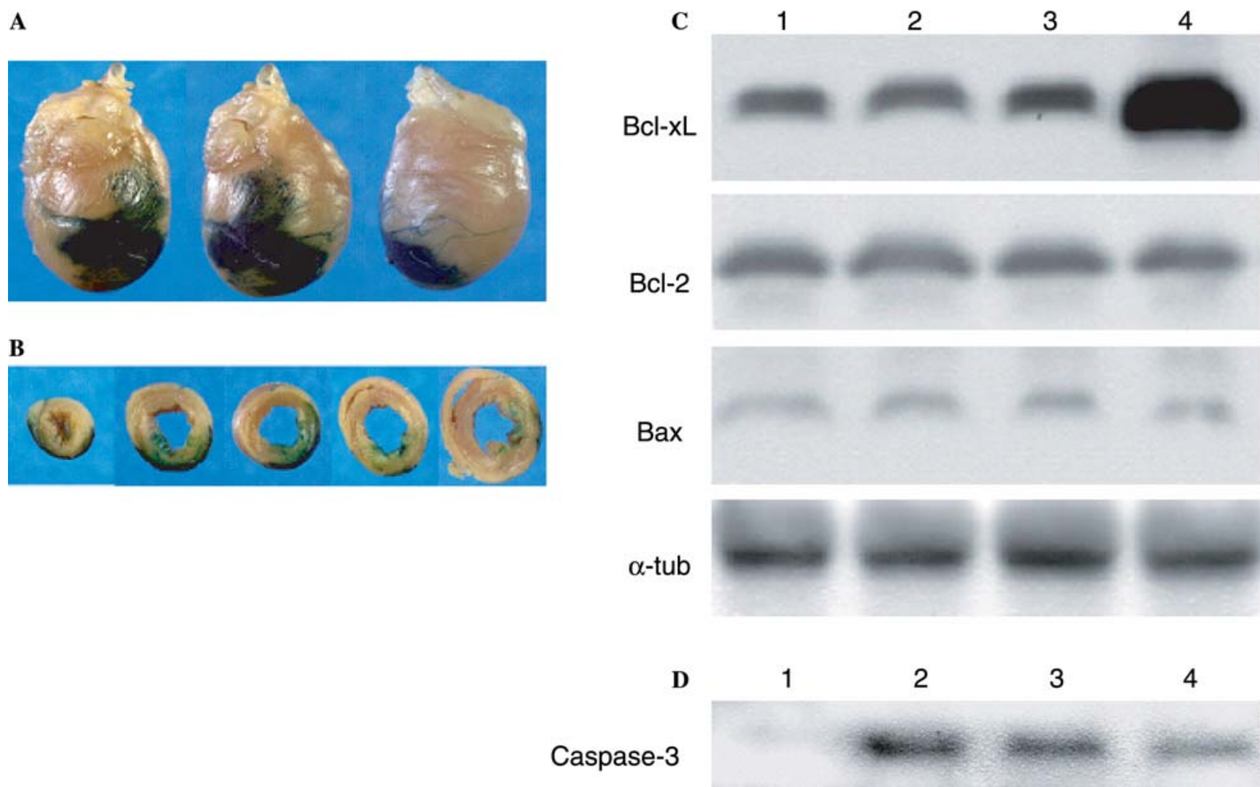

Fig. 1. Transgene expression in the rat heart was determined by X-gal staining and immunoblotting. Four days after myocardial LacZ transduction, the heart was fixed with in vivo perfusion with 2% paraformaldehyde and then immersed in X-gal solution. LacZ-positive myocardium was stained as a dark blue area and shown as whole heart views (A) and horizontal sections (B). Bcl family protein (Bcl-xL, Bcl-2, and Bax) expression, 4 days after Bcl-xL-gene transduction in the rat heart was detected by Western blotting (C). Lane 1, normal heart; lane 2, saline injected heart; lane 3, LacZ-gene-transduced heart; and lane 4, Bcl-xL-gene-transduced heart; α-tub, α-tubulin. (D) Cleavage products of caspase-3 after global I/R injury were detected by Western blotting. Cleaved caspase-3 was detected before and after I/R in the heart transduced with Bcl-xL. Lane 1, saline injected heart before I/R; lane 2, saline injected heart after I/R; lane 3, LacZ-gene-transduced heart after I/R; and lane 4, Bcl-xL-gene-transduced heart after I/R.

Adenoviral Bcl-xL transduction protects the heart from global I/R injury

Since Bcl-xL overexpression was confirmed 4 days after gene transduction in rat heart, we evaluated the effect of this Bcl-xL overexpression on global I/R injury. Adenoviral gene transduction of AxCAZ3 did not affect indicators of cardiac performance, such as % $\pm dP/dt$ recovery and % coronary flow recovery (%CFR) after global I/R. However, marked improvement of % $\pm dP/dt$ recovery and %CFR was observed in the heart injected with AxCAhBclxL in comparison with either the saline or adenoviral control groups ($p < 0.01$, Figs. 2A–C).

Inhibition of apoptosis by Bcl-xL overexpression after global I/R in the heart

Adenoviral transduction of the Bcl-xL gene clearly preserved the function of the hearts after global I/R injury. We therefore evaluated the effect of Bcl-xL gene transduction in terms of apoptosis after global I/R injury. By Western blot analysis, an increase of 17-kDa cleaved caspase-3 was observed in the lysate of saline or AxCAZ3 injected heart after I/R injury. However, a decrease of caspase-3 cleaved product after I/R injury was clearly demonstrated in the heart injected with AxCAhBclxL (Fig. 1D), suggesting an inhibition of apoptosis. This inhibition after global I/R injury in the heart with Bcl-xL transduction was also confirmed by TUNEL staining. As shown in Figs. 2D and E, numerous TUNEL-positive nuclei in the myocardium were observed in the control heart (197 ± 16.5/field) after global I/R injury. LacZ-gene transduction did not reduce the number of TUNEL-positive nuclei (204 ± 29.2/field). However, only a few apoptotic cardiomyocytes were displayed in the specimens from the Bcl-xL-overexpressed heart (77.8 ± 11.1/field, $p < 0.01$ vs. saline or LacZ). This dramatic reduction of apoptosis was observed only at the adenoviral AxCAhBclxL injection site and no inhibition of apoptosis was observed at the surrounding area outside the AxCAhBclxL-transduced myocardium (181 ± 15.3/field).

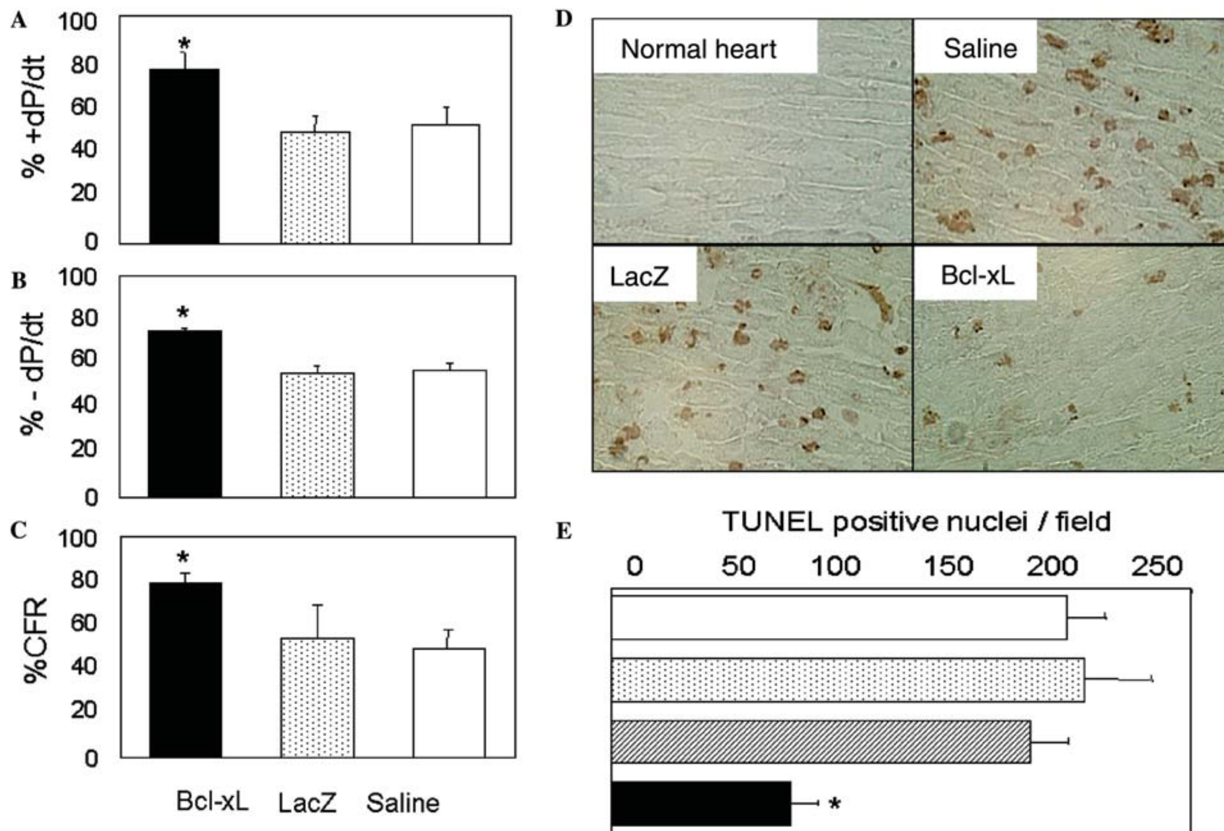

Fig. 2. Cardiac function after global I/R injury. Percentage recovery of maximum dP/dt (%+dP/dt) (A), percentage recovery of minimum dP/dt (%−dP/dt) (B), and percentage recovery of coronary flow (%CFR) (C) are shown. *$p < 0.01$ vs. either saline or LacZ control. (D) Apoptotic nuclei in the myocardium after global I/R were detected by TUNEL staining. Normal heart, the heart injected with saline before I/R; saline, the heart injected with saline after I/R; LacZ, the heart transduced with *LacZ* after I/R; and Bcl-xL, the heart transduced with *Bcl-xL* after I/R. (E) The number of apoptotic nuclei in the myocardium after I/R. The number of apoptotic nuclei was counted in the specimen of heart after global I/R and expressed as the means ± SD. Open column, the myocardium injected with saline as a control; dotted column, the myocardium injected with AxCAZ3 (LacZ); hatched column, the surrounding myocardium outside the *Bcl-xL* transduced area; and closed column, the *Bcl-xL* transduced myocardium.

Reduction of serum CK level following I/R by Bcl-xL transduction

As shown above, the strong cardioprotective effect against global I/R injury by the adenoviral *Bcl-xL* transduction was confirmed. As a next step, utilizing a regional I/R model, we evaluated the feasibility of *Bcl-xL* transduction as a therapeutic tool for coronary ischemia. Bcl-xL gene transduction was achieved 4 days prior to temporary LAD ligation, for 30 min. Twenty-four hours after reperfusion, serum CK level was measured as an index of myocardial damage. In the animals treated either with saline ($n = 6$) or AxCAZ3 ($n = 7$), high levels of CK release in serum were observed (406 ± 130 and 504 ± 98 IU/l, respectively, Fig. 3C). These results indicated that massive cardiomyocyte death occurred within 24 h after reperfusion and that the control adenovirus injection did not affect the CK level. A significant reduction of serum CK levels was observed in the animals treated with the myocardial Bcl-xL gene transduction ($n = 12$, 285 ± 27 IU/l).

Bcl-xL reduced infarct size in the LAD-ligated heart

The strong cardioprotective effect of Bcl-xL transduction was also confirmed by evaluation of the infarct size. As shown in Fig. 3A, the %AAR, determined by the amount of negative staining region with Evan's blue after religation of the LAD, showed no difference among all groups (Saline, 44.7 ± 1.5; LacZ, 47.4 ± 7.5; and Bcl-xL, 46.3 ± 3.6). However, in agreement with the results of serum CK levels, a significant reduction of the % infarct size was observed in the AxCAhBclxL group ($23.3 \pm 10\%$) compared with either saline control ($47.5 \pm 7.4\%$, $p < 0.01$) or adenoviral control ($49.0 \pm 4.0\%$, $p < 0.01$) by TTC staining in the regional I/R model (Fig. 3B).

Discussion

In the present study, we demonstrated that adenoviral Bcl-xL gene transfer to the rat heart reduced the infarct size after regional I/R injury and preserved car-

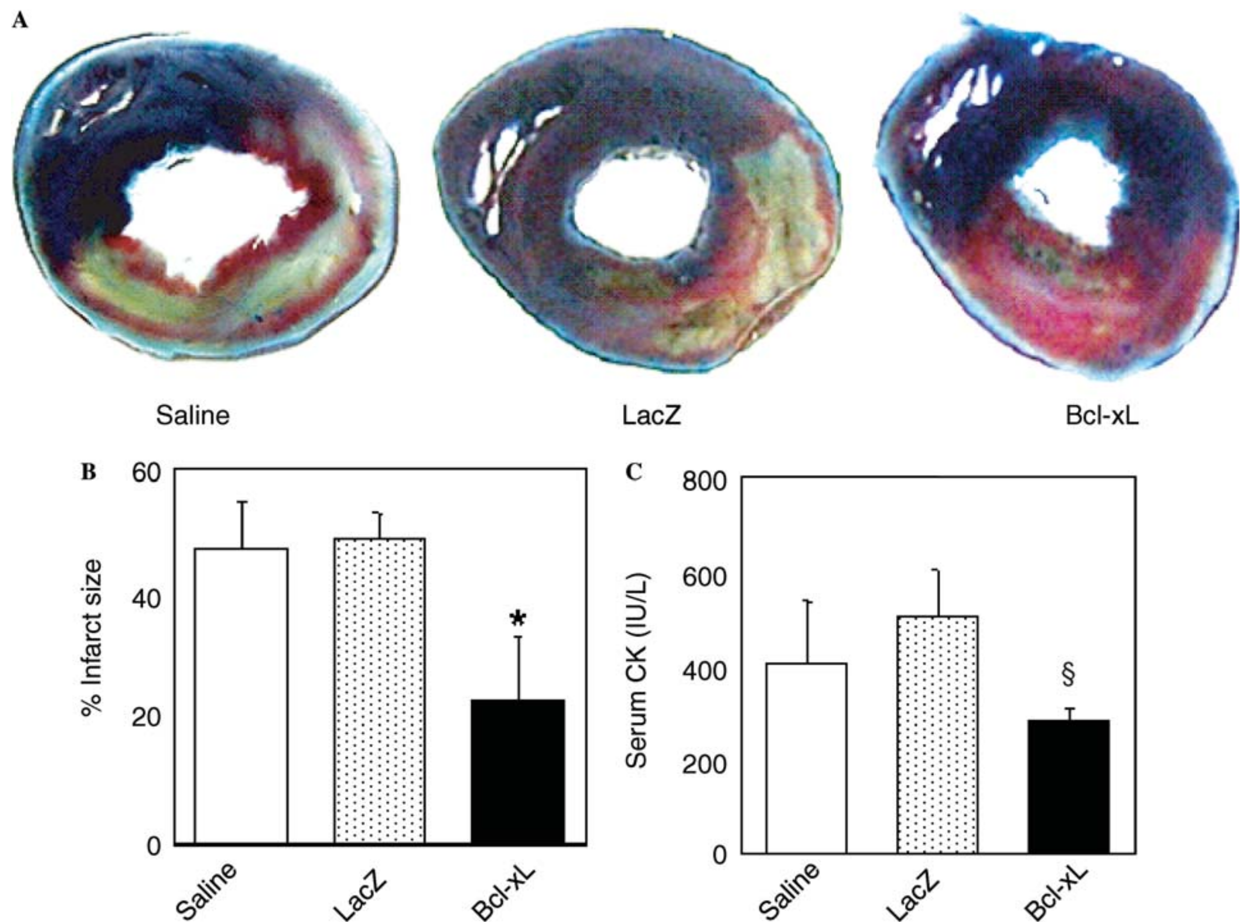

Fig. 3. Area at risk (AAR) and infarct size were determined after regional I/R (A). Negatively stained area with Evan's blue dye demonstrates AAR. The brick-red area indicates viable myocardium and the white area indicates infarction. Percentage infarct size was calculated as: %infarct size = infarct area/AAR × 100 (B). *$p < 0.01$, vs. either saline or LacZ. Serum creatine kinase (CK) level was measured 24 h after reperfusion (C). §$p < 0.05$ vs. either saline or LacZ.

diac function after global I/R injury. Our results suggest that Bcl-xL may serve as a potential therapeutic tool for ischemic heart diseases, heart failure, and in certain settings of cardiac surgery in clinical practice.

Gene therapy is a rapidly expanding field with potential applications to a number of human organs. In clear contrast to the case with soluble proteins, such as growth or angiogenic factors, since antiapoptotic Bcl family proteins are intracellular proteins, a highly efficient transduction of cardiomyocytes is required to obtain sufficient organ protection against I/R injury. In the case of liver, it has been reported that high transduction efficiency was easily achieved by simple intravenous adenovirus administration, and that strong hepatic protection against I/R injury was offered by the simple intravenous approach of adenoviral Bcl-2 transduction [21]. Although the adenoviral vector system is one of the most efficient and powerful methods for in vivo gene transfer into the myocardium, actual transduction efficiency achieved by direct myocardial adenoviral injection was usually limited to 10–20%. However, as shown in Fig. 1, direct adenoviral injection into the left ventricle clearly brought about a robust reporter gene expression that was widely distributed and capable of covering the area at risk perfused by the LAD. Indeed, Bcl-xL gene transduction utilizing the intramyocardial injection approach clearly provided significant cardioprotection from regional I/R injury (Fig. 3). Interestingly, even though adenoviral gene-transduced cardiomyocytes were limited to the anterior wall of the left ventricle, Bcl-xL gene transduction resulted in an obvious retention of cardiac function after global I/R damage in our study (Fig. 2).

In clinical practice, I/R injuries are often seen after thrombolysis, percutaneous coronary intervention, and coronary artery bypass grafting in acute myocardial infarction. Since the myocardial damage occurring in the early phase of reperfusion determines the later extent of infarct size and loss of cardiac function [22], it is necessary to inhibit the early I/R injury to improve the prognosis of acute myocardial infarction. As shown in the present study, our adenoviral *Bcl-xL* transduction dramatically reduced early cardiomyocyte death after regional I/R. In addition to the initial period of cardiac I/R injury, progressive cardiac apoptosis at the border zone between ischemic and nonischemic myocardium continues, due to the abnormal passive stretching signal during the compensation of impaired cardiac function and remodeling [23]. Recently, Chatterjee et al. [23] demonstrated that adenoviral antiapoptotic Bcl-2 transduction prevented progressive myocyte loss in the chronic phase of myocardial infarction and resulted in the preservation of heart function. Although Bcl-xL and Bcl-2 possess similar antiapoptotic effects, these molecules regulate the apoptotic pathway through independent molecular mechanisms [24]. Further studies are required to confirm whether adenoviral Bcl-xL transduction, like Bcl-2 transduction, can confer similar cardioprotective effects in the chronic phase of myocardial infarction [23]. Another typical clinical presentation of I/R injury is post-cardiac surgery reperfusion injury after cardioplegic arrest. It is well known that global I/R injury after deep hypothermic circulatory arrest can increase morbidity and mortality. Therefore, in order to limit myocardial damage, control of global I/R injury after cardioplegic arrest is a critical requirement for the improvement of prognosis in cardiac surgery with cardiopulmonary bypass. Cardioprotective agents such as insulin and adenosine for altering metabolism [25], antioxidants [26], and inhibitors of sodium-hydrogen antiports [27] have been employed to attenuate global I/R injury, but the success of these pharmacological interventions against I/R injury has unfortunately been limited. Our adenoviral Bcl-xL transduction strategy preserved cardiac function in the heart after global I/R injury. This approach might therefore offer a new therapeutic option in cardiac surgery with cardiopulmonary bypass.

We used Bcl-xL gene transduction to prevent I/R injury. This dramatic amelioration of I/R injury was achieved by the preventive administration of adenoviral vector prior to the I/R event. The fact that this approach required at least a few hours to obtain a sufficient expression level of the adenoviral transgene to exert an immediate cardioprotective effect after I/R injury suggests a limitation of therapeutic adenoviral gene delivery in the clinical I/R setting, such as post-thrombolysis and post-cardioplegic reperfusion. Therefore, an alternative strategy in Bcl-xL molecular therapy must be developed to overcome these obstacles to effective adenoviral gene transduction. For this purpose, direct delivery of Bcl protein into the heart may be one of the answers. Recently, membrane translocation proteins, such as human immunodeficiency viral TAT protein [28] and herpes simplex viral type 1 tegument protein VP22 [29], have been proposed as tools for protein delivery into cells. The combination of these membrane translocation proteins with Bcl protein might be promising for the clinical application of Bcl molecular therapy, since Bcl protein is a cytoplasmic protein which cannot pass through cellular membranes by itself. A similar attempt, utilizing a caspase recruit domain fused with TAT, was reported by Gustafsson et al. [30]. Further studies are required to elucidate whether the fusion of Bcl-xL proteins with membrane translocation proteins can effectively prevent the I/R injury of the heart.

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