Bcl-xL Gene Transfer Inhibits Bax Translocation and Prolongs Cardiac Cold Preservation Time in Rats

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Methods and Results—Lewis rat hearts were transduced with an adenovirus vector harboring Bcl-xL cDNA (AxCAhB-clxL) 4 days before collection of tissue. After preservation in University of Wisconsin solution at 4°C for 24 hours, the heart was either perfused with a Langendorff device ex vivo or used for heterotopic heart transplantation in vivo. Bcl-xL gene transfer significantly reduced the infarct size (23.0 ± 2.6% versus 47.7 ± 7.0% in saline control and 48.6 ± 6.1% in vector control; P < 0.01) after 2-hour reperfusion at 37°C with the Langendorff device and significantly decreased creatine kinase release (0.82 ± 0.27 IU, versus 1.57 ± 0.33 and 1.50 ± 0.37 IU in saline and vector controls, respectively; P < 0.05). In heart transplantation, overexpression of Bcl-xL inhibited Bax translocation from the cytosol to the mitochondria, resulting in decreased cytochrome c release from the mitochondria; it also significantly decreased cardiac cell apoptosis and improved graft survival rate after long cold preservation, followed by warm reperfusion.

Conclusions—Bcl-xL gene transfer inhibited the translocation of Bax and prolonged the cold preservation time of cardiac transplants. This may be a potential therapeutic method in clinical practice. (Circulation. 2005;112:76-83.)

Key Words: gene therapy • ischemia • reperfusion • transplantation • apoptosis

With the development of new technologies and improvements in immunotherapy methods, there has been a worldwide increase in the number of cases of cardiac transplantation in recent decades. One of the limitations of heart transplantation is the lack of donor hearts, attributable at least in part to the current duration of heart preservation time, which is limited to 4 to 6 hours in clinical practice. Although using cardiac preservation liquids such as the University of Wisconsin solution (UWS) prolongs the preservation time of heart grafts, 18 hours is the maximum preservation time for functional recovery of the heart in different experimental protocols. With the development of new technologies and improvements in immunotherapy methods, there has been a worldwide increase in the number of cases of cardiac transplantation in recent decades. One of the limitations of heart transplantation is the lack of donor hearts, attributable at least in part to the current duration of heart preservation time, which is limited to 4 to 6 hours in clinical practice. Although using cardiac preservation liquids such as the University of Wisconsin solution (UWS) prolongs the preservation time of heart grafts, 18 hours is the maximum preservation time for functional recovery of the heart in different experimental protocols. With the development of new technologies and improvements in immunotherapy methods, there has been a worldwide increase in the number of cases of cardiac transplantation in recent decades. One of the limitations of heart transplantation is the lack of donor hearts, attributable at least in part to the current duration of heart preservation time, which is limited to 4 to 6 hours in clinical practice. Although using cardiac preservation liquids such as the University of Wisconsin solution (UWS) prolongs the preservation time of heart grafts, 18 hours is the maximum preservation time for functional recovery of the heart in different experimental protocols. With the development of new technologies and improvements in immunotherapy methods, there has been a worldwide increase in the number of cases of cardiac transplantation in recent decades. One of the limitations of heart transplantation is the lack of donor hearts, attributable at least in part to the current duration of heart preservation time, which is limited to 4 to 6 hours in clinical practice. Although using cardiac preservation liquids such as the University of Wisconsin solution (UWS) prolongs the preservation time of heart grafts, 18 hours is the maximum preservation time for functional recovery of the heart in different experimental protocols. With the development of new technologies and improvements in immunotherapy methods, there has been a worldwide increase in the number of cases of cardiac transplantation in recent decades. One of the limitations of heart transplantation is the lack of donor hearts, attributable at least in part to the current duration of heart preservation time, which is limited to 4 to 6 hours in clinical practice. Although using cardiac preservation liquids such as the University of Wisconsin solution (UWS) prolongs the preservation time of heart grafts, 18 hours is the maximum preservation time for functional recovery of the heart in different experimental protocols.

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Long cold preservation and warm reperfusion are considered important causes of early cardiac graft loss. Although calcium flux, 3 acidosis, 4 ATP depletion, 5 and reactive oxygen species 6 have been defined as putative causative factors, there may be other mediator factors that remain to be identified. Indeed, besides necrosis, apoptosis is one of the early events of either warm ischemia and reperfusion injury 7,8 or cold ischemia and reperfusion injury 2 in the heart. Several studies proved that cardiac dysfunction could be prevented by the antiapoptotic Bcl-2 gene in rats 9 or transgenic mice. 10 On the other hand, cobalt protoporphyrin (heme oxygenase-1 inducer) was reported to prolong graft survival after 24-hour preservation at 4°C in a cardiac transplantation model in which Bcl-2 expression was elevated. 11 Thus, the strategy of a combination of antiapoptotic gene transfer and cold preservation seems to be reasonable for success of heart transplants.

Bcl-xL belongs to the same family as Bcl-2, which was shown to play an important role in cardiac protection by hepatocyte growth factor 12 and insulin-like growth factor-1. 13 Bcl-xL was also reported to inhibit Bax translocation to the mitochondria, decrease the release of cytochrome c (cyto-c) from the mitochondria to the cytosol, and protect retinal cell apoptosis in transgenic mice. 14 Because cold ischemia and reperfusion cause mitochondrial dysfunction and release of cyto-c from the mitochondria to the cytosol, 15 we hypothesized that Bcl-xL would be useful in...
preventing cold ischemia and warm reperfusion injury of the heart.

We previously demonstrated that adenovirus-mediated Bcl-xL gene transduction to the heart improved cardiac function and decreased the infarct size of the heart after warm ischemia and reperfusion. In the present study, we used adenovirus-mediated Bcl-xL gene transfer to the heart to ascertain whether it could inhibit cold ischemia and warm reperfusion injury and prolong the cold preservation time of heart grafts in a rat heterotopic transplantation model.

Methods

Adenoviral Vectors

The adenoviral vector (Adv) encoding human Bcl-xL or Escherichia coli β-galactosidase (LacZ) was used in the experiment. Adv propagation and purification were described previously. Before use, the viral titer (particle units/mL [PU/mL]) and the contamination of replication-competent Adv in the viral stock were evaluated as previously reported.

Animal Model

Syngeneic Lewis rats (male, 250 to 350 g) were purchased from Sankyo Labo Service (Tokyo, Japan). The rats were anesthetized by intramuscular injection with ketamine (40 mg/kg) and xylazine (4 mg/kg). The donor rat was intubated with a 17-gauge needle tube and supported by a rodent respirator. A median thoracotomy was made and the aorta was exposed. AxCAhBclxL (1010 PU in 100 μL of 0.9% saline) was injected into the heart at the root of the aorta. The aorta and pulmonary artery were clamped for 30 seconds after Adv injection and then released (n = 10). For control, 1010 PU AxCAZ3 in 0.9% saline (PBS), and blocked with 3% bovine serum albumin in PBS. The sections were incubated with anti-human Bcl-xL antibody (65186E, BD PharMingen) and anti–β-actin antibody (65186E, BD PharMingen) was applied, and the Vector ABC kit (Vector Laboratories) was used as described by the manufacturer.

Langendorff Perfusion and CK Measurement

To determine the effect of long cold preservation and warm reperfusion on the heart, we first performed the Langendorff perfusion of the heart ex vivo. After 24 hours of cold preservation, the heart was transferred to the Langendorff perfusion system and perfused with a modified Krebs-Henseleit solution gassed with 95% O2 and 5% CO2 through the aorta at 85 mm Hg of perfusion pressure. The heart was perfused at 37°C for 2 hours, the coronary effluent flow for the first 15 minutes after the start of reperfusion was collected for creatine kinase (CK) measurement, and CK activity was measured by an enzymatic assay.

TTC Staining

Triphenyltetrazolium chloride (TTC) staining has been a standard for the measurement of infarct size and has been used previously for assessment of infarct size resulting from apoptosis and necrosis. Thus, we performed TTC staining to detect the infarct size of the heart after 2 hours of Langendorff perfusion. The heart was cut evenly into 4 slices transversely from the apex to the base and immersed into 1% TTC (Sigma-Aldrich) in PBS at 37°C for 30 minutes, and then the reaction was stopped by addition of 10% neutralized formalin. The heart sections were photographed and captured as a digital image. Infarct size was analyzed with NIH software, and infarct size percentage was calculated as infarct area (white area)/total area of the left ventricle (brick red area + white area).

Histological Examination

After 2-hour perfusion in the Langendorff device or 24 hours of heterotopic heart transplantation, tissue was excised from the middle of the heart between the apex and base, fixed with 10% neutralized formalin, dehydrated, and embedded in paraffin. Five-micron sections were cut, and randomly selected sections were stained with hematoxylin and eosin. The sections were observed under the light microscope.

TUNEL Staining

After 24 hours of cold preservation and 15 minutes after heart transplantation, tissue was excised from the middle of the heart between the apex and base, and cryosections were cut and randomly selected for terminal dUTP nick end-labeling (TUNEL) staining as previously described. Nuclei were counterstained with methyl green. The total numbers of nuclei and of TUNEL-positive nuclei were counted in 5 randomly chosen fields of view per tissue section in a blinded manner, and results were expressed as the number of TUNEL-positive nuclei divided by the number of counterstained nuclei.

Imunochemical Analysis of Bcl-xL, Bax, and Cyto-c in the Heart

After 24 hours of cold preservation and 15 minutes after transplantation, the heart was collected. Bcl-xL expression was determined by Western blotting as previously described. To determine Bax translocation and cyto-c release after 15 minutes of heart transplantation, the heart was immersed in 4 mL lysis buffer containing 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5, and incubated for 5 minutes on ice. The heart was homogenized with a mixing homogenizer (Kinematica AG), and the suspension was centrifuged at 750g for 10 minutes at 4°C to sediment the nuclear fraction. The supernatant was collected and centrifuged at 12 000g for 10 minutes at 4°C to sediment the mitochondrion fraction. The resultant supernatant was further centrifuged at 14 000g for 10 minutes at 4°C and then filtered through a 0.22-μm ultrafilter (Millipore) to generate a purified cytosolic fraction.
Immunoblotting was performed according to standard protocols. The protein concentration was measured by the bicinchoninic acid method (Pierce). Aliquots of 40 μg of each sample were loaded on 15% sodium dodecyl sulfate polyacrylamide gels, subjected to electrophoresis, and then transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 at 4°C overnight and then probed with rabbit antibody to Bax (sc-526, Santa, Cruz Biotechnology) and mouse antibody to cyto-c (7H8.2C12, PharMingen), followed accordingly by horseradish peroxidase–conjugated anti-rabbit IgG (Zymed) or horseradish peroxidase–conjugated anti-mouse IgG (Amersham Biosciences) at room temperature for 1 hour. The horseradish peroxidase was detected with a chemoluminescence ECL-Plus kit (Amersham Biosciences UK). Cyto-c oxidase IV was detected with mouse antibody to cyto-c oxidase IV (COX, A-6431, Molecular Probes) as the mitochondrial marker, and α-tubulin was detected with mouse antibody to α-tubulin (B-5-1-2, Sigma-Aldrich) as an internal protein control.

Statistical Analysis
Data were expressed as mean±SD. Statistical comparisons were performed with ANOVA followed by Bonferroni/Dunn testing. A probability value <0.05 was considered statistically significant.

Results
Efficiency of Bcl-xL Gene Transfer and Duration of Expression of Bcl-xL in the Heart
X-gal staining showed that the Adv expression was distributed in both the left and right ventricles after aortic root injection (Figure 1A) and that the transductive efficiency for cardiomyocytes was ≈45% to 50% (data not shown). Bcl-xL expression was also clearly proved by immunostaining methods (Figure 1B). Both immunostaining and Western blotting showed that after Adv gene transduction, the expression of Bcl-xL was strong for 2 to 7 days after gene transfer, then decreased, and almost reached normal levels after 21 days (Figure 1C and 1D).

Bcl-xL Gene Transfer Decreased Infarct Size and Inhibited CK Release in the Heart
The effects of Adv-mediated Bcl-xL gene transfer on infarct size and CK release were assessed in isolated rat hearts perfused with the Langendorff device. As shown in Figure 2A and 2B, after 24-hour preservation in UWS at 4°C and 2-hour reperfusion at 37°C, infarct size was 23.0 and 2.6% in the AxCAhBclxL-treated group, 47.7±7.0% in the saline group, and 48.6±6.1% in the AxCAZ3 group (n=4 in each group). Bcl-xL gene transfer significantly reduced the infarct size compared with saline or AxCAZ3 (P<0.01). Histological examination showed that in control hearts, a large proportion of cardiomyocytes underwent cell membrane rupture, whereas AxCAhBclxL-treated heart did not show such changes (data not shown). The coronary effluent was collected during the first 15 minutes of reperfusion, and total CK level was measured as an index of myocardial damage. As shown in Figure 2C, the CK level in hearts treated with AxCAhBclxL was 0.82±0.27 IU; in contrast, the CK level was 1.57±0.33 IU in saline-treated hearts and 1.50±0.37 IU in AxCAZ3-treated hearts (n=4 each). Bcl-xL gene transfer significantly decreased the CK level in the perfusate (P<0.05).

Bcl-xL Gene Transfer Prolonged Survival of Cardiac Isografts After Cold Ischemia and Warm Reperfusion Insult
To determine whether Bcl-xL prolonged the preservation time, rat hearts that had been transduced with Bcl-xL gene were harvested and preserved in UWS at 4°C for 24 hours; subsequently, heterotopic heart transplantation was performed. As shown in Figure 3A and 3B, 70% (7 of 10) of the control hearts that underwent 24 hours of cold ischemia ceased to beat as early as <15 minutes after transplantation.
syngeneic Lewis rats. Only 1 graft survived >14 days in the control group. In contrast, 88% (8 of 9 rats [1 rat died because of complications of surgery]) of cardiac isografts with Bcl-xL gene transfer survived >14 days.

**Bcl-xL Gene Transfer Decreased Cardiac Cell Death and Inflammatory Cell Infiltration After Heart Transplantation**

Cardiac cell apoptosis was detected after 24 hours of cold preservation and 15 minutes after heart transplantation. The average number of TUNEL-positive nuclei was 6.5±1.9% in AxCAhBclxL-treated hearts compared with 18.9±6.1% in saline-treated hearts (Figure 4). Thus, Bcl-xL significantly decreased cardiac cell apoptosis (P<0.01).

Hematoxylin-eosin staining was performed after 24 hours of heart transplantation. As shown in Figure 5, there were large numbers of cardiomyocytes that underwent necrosis in the control saline and AxCAZ3-treated rat hearts, as evidenced by infiltration of polymorphonuclear and mononuclear leukocytes in the interstitium of the myocardium. In contrast, in the AxCAhBclxL-treated hearts, myocardial structure was well preserved, and only a few infiltrating leukocytes were observed in the myocardium.

**Bcl-xL Expression After 24 Hours of Cold Preservation and 15 Minutes After Heterotopic Heart Transplantation**

We previously reported that preadministration of AxCAhBclxL resulted in a robust Bcl-xL expression in the rat heart.16 To determine whether cold preservation and warm reperfusion had some effect on the expression of Bcl-xL, immunoblot analysis of the heart after 24 hours of cold preservation with or without heart transplantation was performed.

As shown in Figure 6, expression of Bcl-xL decreased after 24 hours of cold preservation and 15 minutes after heart transplantation in control hearts (lanes 6, 9). In contrast, even after 24 hours of cold preservation with or without heart
transplantation, Bcl-xL still showed high expression in AxCAhBclxL-transfected hearts (lanes 2, 3).

**Overexpression of Bcl-xL Inhibited Bax Translocation**

Cold ischemia and reperfusion cause dysfunction of mitochondria and cyto-c release. To examine the effect of Bcl-xL overexpression on Bax and cyto-c after 24 hours of cold preservation and 15 minutes after heart transplantation, we determined the effect of Bcl-xL overexpression on the subcellular localization of Bax and cyto-c by immunoblot analysis.

As shown in Figure 7, 24-hour cold preservation and 15-minute reperfusion after heart transplantation resulted in Bax loss in the cytosol and release of cyto-c from the mitochondria to the cytosol in controls (lanes 2, 3). In contrast, overexpression of Bcl-xL by Adv transduction of the heart prevented Bax loss from the cytosol and decreased the cyto-c release (lane 4). The data show that cold preservation and reperfusion caused Bax translocation from the cytosol to the mitochondria, resulting in increased release of cyto-c from the mitochondria to the cytosol. Overexpression of Bcl-xL inhibited Bax translocation and cyto-c release.

**Discussion**

Miniati et al. found that Adv up-regulation of Bcl-2 inhibited oxidative stress and graft coronary artery disease in rat heart transplants. The authors performed the syngeneic-allogeneic retransplantation technique to allow for maximal translation of the Bcl-2 gene product at the time of allogeneic transplant reperfusion. In the present study, we preadministered Adv vector to the donor heart 4 days before transplantation and found that Bcl-xL was efficiently expressed in the heart after Adv vector transduction via the root of aorta. Overexpression of Bcl-xL in the heart inhibited the cold ischemia and warm reperfusion injury by decreasing cardiac cell apoptosis and prolonged the preservation time of heart grafts. Rat hearts

![Figure 4](image-url)

*P<0.01

![Figure 5](image-url)

*P<0.01
exposed to a prolonged period of cold ischemia (24 hours, 4°C) failed to function after transplantation into syngeneic recipients. Our study suggests that Bcl-xL can serve as a powerful cardiac preservation agent in heart transplantation.

In the field of heart transplantation, various attempts have been made to prolong cardiac preservation time. Most of them have been designed to limit energy loss, maintain intracellular ionic composition, and prevent hypothermic myocyte swelling; however, even with optimal preservation after a long period of cold preservation, reperfusion of the heart causes dysfunction and apoptosis and/or necrosis of cardiac myocytes. There have been reports that Adv-mediated Bcl-xL or TAT-BH4 inhibited apoptosis of cardiac myocytes caused by warm ischemia and reperfusion injury. In the present study, overexpression of Bcl-xL was shown to decrease cardiac injury in a stringent and clinically relevant model of 24-hour cold ischemia and warm reperfusion followed by syngeneic transplantation. Ex vivo experiments also showed that after 24-hour cold preservation and 2-hour reperfusion at 37°C, infarct size and CK levels were significantly reduced by Bcl-xL gene transfer.

Mitochondria play a critical role in cell apoptosis. In the heart, prolonged cold ischemia and warm reperfusion jeopardize the myocardial capacity to regenerate energy by mitochondrial oxidative phosphorylation and are correlated with a progressive loss of mitochondrial function. Cyto-c release from mitochondria occurs in both normothermic and cold ischemia and reperfusion, and cyto-c release activates the caspase pathway, with consequent induction of apoptosis. Furthermore, in pathophysiolog-

Figure 6. A, Representative Western blot analysis of Bcl-xL after 24 hours of cold preservation and 15 minutes after heart transplantation. Lanes 1, 4, and 7, normal control heart; lanes 2 and 3, heart treated with AxCAhBclxL; lanes 5 and 6, heart treated with saline; lanes 8 and 9, heart treated with AxCAZ3; lanes 2, 5, and 8, heart after 24 hour of cold preservation; lanes 3, 6, and 9, heart after 24 hours of cold preservation and 15 minutes after heart transplantation. B, Densitometry of Bcl-xL expression in heart after 24 hours of cold preservation and 15 minutes after heart transplantation. Value represent mean±SD, n=3, *P<0.05.

Figure 7. A, Representative Western blot analysis of Bax and cyto-c in mitochondrial and cytosolic fractions after 24 hours of cold preservation and 15 minutes after heart transplantation. Left, mitochondrial fraction; right, cytosolic fraction. 1, Normal control heart; 2, heart treated with saline; 3, heart treated with AxCAZ3; 4, heart treated with AxCAhBclxL. COX-V was used as mitochondrial marker; α-tubulin was used as internal control of cytosolic fraction. B, Densitometry of cytosolic Bax. Results are representative of 3 independent experiments.
ical settings, apoptosis frequently induces inflammation because of the onset of secondary necrosis and stimulation of cytokine and chemokine expression, which inevitably result in the demise of adjacent cells that are not directly damaged by the original insult. Necrosis is another form of cardiac cell death after cold preservation and reperfusion. The cardiac necrosis that was evidenced by infarct size after ex vivo perfusion (Figure 2A) and inflammatory response after heart transplantation (Figure 5d and 5h) were decreased in the AxCAhBclXL-treated group, which indicated that Bcl-XL may play a role in antinecrosis. Recently, it has been suggested that cyto-c release potentially induces necrosis by depleting cellular ATP levels; thus, the pathway leading to apoptosis or necrosis can be shared. The heterogeneity and extent of cyto-c release are critical for regulating the switch between alternative development of apoptosis or necrosis.

Thus, before irreversible changes occur in the mitochondria, inhibition of cyto-c release and protection of mitochondrial integrity would be important in preventing heart injury caused by prolonged cold preservation and warm reperfusion. Cyclosporin A, an inhibitor of mitochondrial permeability transition, was proven to inhibit the apoptosis of cultured neonate cardiac myocytes by decreasing the release of cyto-c.

In the present study, we showed that prolonged cold expression and warm reperfusion decreased Bcl-XL level of the heart, but in Bcl-XL gene-transduced hearts, even after 24 hours of cold preservation followed by 15 minutes of heart transplantation, Bcl-XL still had strong expression. TUNEL staining showed that after a long period of cold preservation, cardiac cell apoptosis during the early stage of reperfusion in the control group was significantly higher than in the AxCAhBclXL-treated group. We found that overexpression of Bcl-XL inhibited the translocation of Bax to the mitochondrial and decreased the release of cyto-c from the mitochondria to the cytosol. This is in accordance with results in a transgenic mouse model in which overexpression of Bcl-XL prevented retinal cell apoptosis by inhibiting the translocation of Bax. Therefore, inhibition of Bax translocation may be one of the mechanisms through which Bcl-XL protects the heart against prolonged cold preservation and warm reperfusion injury.

Our finding validated the feasibility of use of the Bcl-XL gene in heart transplantation. This may contribute to the development of a novel method aimed at prolongation of cardiac cold preservation time.

Acknowledgments
This work was supported in part by a grant to Drs Hamada and Ito from the Ministry of Education, Science, Japan.

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_Circulation_. 2005;112:76-83; originally published online June 27, 2005;

doi: 10.1161/CIRCULATIONAHA.105.535740

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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