Antisense Bcl-x oligonucleotide induces apoptosis and prevents arterial neointimal formation in murine cardiac allografts

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Abstract

Objective: Cardiac allograft arteriosclerosis, which limits long-term survival of recipients, cannot be prevented by conservative therapies. The arteriopathy is characterized by diffuse intimal thickening comprised of proliferative smooth muscle cells (SMCs). Cell death is a prominent feature of atherosclerosis; Bcl-x is one of the anti-apoptotic mediators. Methods: To test the hypothesis that antisense bcl-x oligodeoxynucleotide (ODN) is effective in preventing intimal hyperplasia through enhancing apoptosis after cardiac transplantation, we performed single intraluminal delivery of antisense bcl-x ODN into murine cardiac allografts (n=9). DBA/2 (H-2\(^d\)) hearts were transplanted into B10.D2 (H-2\(^b\)) mice. Sense bcl-x ODN (n=8) and no treatment (n=8) studies were also performed. Results: Allografts were harvested at 4 weeks after transplantation; all allografts kept beating throughout the period. Coronary intimal thickening had developed in nontreated and sense ODN transfected allografts at 4 weeks after transplantation with enhanced expression of Bcl-x and cell adhesion molecules, and suppressed apoptosis. However, antisense bcl-x ODN prevented neointimal formation through enhanced apoptosis. Conclusion: These results indicate that apoptosis of vascular SMCs induced by Bcl-x is associated with initial hyperplasia after heart transplantation. Antisense bcl-x ODN inhibits SMC proliferation by inducing apoptosis in graft coronary arteries.

Keywords: Apoptosis; Transplantation

1. Introduction

Cardiac allograft arteriopathy is now the primary factor limiting long-term survival of organ recipients [1]. Several therapeutic trials have been performed in search of methods to prevent this arteriopathy, which is characterized by diffuse intimal thickening comprised of proliferative smooth muscle cells (SMCs), without significant success [2,3]. Recent studies have documented that cell death is a prominent feature of atherosclerosis [4], however the role of apoptosis as a determinant of cardiac allograft neointimal formation remains to be demonstrated. It is postulated that the regulation of apoptosis involves the balance between pro-apoptotic mediators such as Bax, and anti-apoptotic mediators such as Bcl-x [5]. Although the precise mechanisms by which Bcl-x prevents cell death remain to be further defined, recent studies indicate that Bcl-x inhibits caspase activation and alterations in mitochondrial function associated with cell execution [6]. However, the role of Bcl-x in the pathophysiology of cardiac allograft arteriopathy remains unelucidated. Recently, it was reported that antisense bcl-x ODN is an efficient therapeutic strategy for prevention of neointimal formation after balloon injury of rat carotid arteries [7]. We therefore hypothesized that graft arteriopathy after cardiac

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transplantation could also be prevented by antisense bcl-x ODN.

In the present study, we revealed that Bcl-x is enhanced in murine cardiac allograft arteriopathy, and that antisense bcl-x ODN inhibits arterial neointimal formation through the induction of apoptosis.

2. Methods

2.1. DNA sequences and preparation of HVJ liposome

Antisense phosphorothioate ODN sequence to the murine bcl-x sequence is 5’-GTT-GCT-CTG-AGA-CAT-TTT-3’. Sense bcl-x ODN sequence is 5’-AAA-ATG-TCT-CAG-AGC-AAC-3’ [7]. Phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4:8: 2. Dried lipid was hydrated in 200 μl of balanced salt solution (BSS) containing sense or antisense ODNs. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg/mm²/s) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg of lipids) was mixed with HVJ (10 000 hemagglutinating units) in a total volume of 4 ml BSS. The mixture was incubated at 4°C for 5 min and then for 30 min at 37°C while gently shaking. Free HVJ was removed from the HVJ liposomes by sucrose density gradient centrifugation [8–10].

2.2. Murine cardiac transplantation and ex vivo gene transfer

Male DBA/2 (H-2<sup>a</sup>) and B10.D2 (H-2<sup>b</sup>) mice (age 4–6 weeks, 20–25 g) were obtained from Japan Charles River Laboratories (Tokyo, Japan). The phosphorothioate ODN (15 μM) was injected (0.2 ml) into the descending aorta of donor hearts just before transplantation and incubated for 10 min on ice. Efficiency of ex vivo transfection of ODN is more than 90% of graft cells [10]. After transfection, donor hearts were immediately transplanted into recipient B10.D2 mice. Briefly, this technique involves anastomosing the end of the donor aorta to the side of the recipient abdominal aorta, after which the donor pulmonary artery is connected to the inferior vena cava of the recipient animal to return the myocardial blood flow. Ischemic time averaged 60 min and the overall success rate was greater than 90% [10–12]. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by Shinshu University.

2.3. Histological examination and morphometry

Mice were killed on day 28 after transplantation and the grafts were sectioned transversely at the maximal circumference of the ventricle and stored in Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Finetecnical Co. Tokyo). Serial sections (6–8 μm) were cut and stained with Elastaic van Gieson (EvG) to highlight the internal elastic lamina (IEL). The area encompassed by the lumen and IEL was traced and the area of luminal stenosis in each cross section was calculated according to the formula: luminal occlusion=(IEL area−luminal area)/IEL area [13,14].

2.4. TUNEL assay

Free 3’-OH ends were assessed using digoxigenin-conjugated dUTP labeling with terminal deoxynucleotidyltransferase according to the manufacturer’s instructions; color development was achieved with Fast Red solution (In Situ Cell Death Detection Kit, Boehringer Mannheim, Indianapolis, IN) and the tissues were counterstained with hematoxylin. Normal murine testes were used as positive controls. The negative control involved the substitute of distilled sterile water for the TdT enzyme [15].

2.5. Immunohistochemistry

Serial sections (6–8 μm) were cut and dipped in cold acetone for 10 min. Anti-Bcl-x (Transduction Lab. Lexington, KY), anti-Bax (Santa Cruz Biochem. Santa Cruz, CA), anti-proliferating cell nuclear antigen (PCNA) (Santa Cruz), anti-ICAM-1 (YN1/1.7) and anti-VCAM-1 (MK/2) monoclonal antibodies (mAbs) were used as primary antibodies in this study; the sections were incubated with each antibody for 12 h at 4°C. Antibody–biotin conjugate was detected with Vectastain ABC Kit (Vector Lab. Inc., Burlingame, CA). Enzyme activity was detected with diaminobenzidine (0.5 mg/ml) with 0.05% NiCl in 50 mM Tris buffer.

2.6. Statistical analysis

All data are expressed as mean±S.D. Scoring of TUNEL assay and immunohistochemistry was as follows: 0, no visible staining; 1, few cells with faint staining; 2, moderate staining; and 3, intense diffuse staining [13,14]. Percentages of intimal thickening and the scores were compared using a Scheffe’s ANOVA. Values of P<0.05 were considered significant.

3. Results

3.1. Histological findings of the graft arteries and myocardium

All allografts kept beating throughout the observation period. Coronary arteries of native hearts and isografts did not develop intimal thickening at any time during this study. At 28 days after transplantation, heavy neointimal
Fig. 1. Representative histopathological findings of graft coronary arteries. Upper panels show allograft arteries stained with Elastica van Gieson; lower panels show the arteries with TUNEL assay. Left panels show allograft arteries without ODN transfection; center panels show the arteries with antisense \textit{bcl}-x ODN. Right panels show the arteries of native hearts. No ODN transfection resulted in heavy arterial neointimal formation (1) with faint TUNEL positive cells (4), while antisense \textit{bcl}-x ODN suppressed neointimal development (2) with enhanced TUNEL staining; arrows indicate TUNEL positive cells (5). Neither neointimal formation (3) nor TUNEL positive cells (6) were seen in native heart arteries (original magnification: X400).

Fig. 2. Representative immunohistochemical findings of graft coronary arteries; upper panels show Bcl-x and lower show Bax expression. Left panels show allograft arteries without ODN transfection; center show the arteries transfected with antisense \textit{bcl}-x ODN. Right panels show the native hearts. Bcl-x was strongly and diffusely expressed in the thickened intima of the allograft arteries from recipients without ODN transfection (1), while Bax expression was weak (4). Both were suppressed in the allografts transfected with antisense \textit{bcl}-x ODN (2, 5) and native hearts (3, 6) (original magnification: X400).
thickening had formed in the coronary arteries of sense ODN transfected or untreated allograft recipients, however in recipients treated with antisense bcl-x ODN, arterial neointimal formation was dramatically suppressed. The scores between the antisense bcl-x ODN transfected and other groups were statistically different, however the scores between the sense bcl-x ODN transfected and no treatment groups were not (Table 1, Fig. 1).

### 3.2. TUNEL assay

Limited TUNEL positive cells were observed in the arterial thickened intima of allografts from nontreated or sense bcl-x ODN-treated recipients. However, increased TUNEL positive cells were seen in the mildly thickened intima of the allografts treated with antisense bcl-x ODN. Scores of TUNEL positive cells were significantly different between the antisense bcl-x ODN transfected group and other groups (Fig. 1, Table 1). We also transfected antisense bcl-x ODN into isografts to confirm the specificity. Medial vascular SMCs of the isografts absorbed the antisense bcl-x ODN, yet did not undergo apoptosis, and were as free from TUNEL positive cells in the apoptotic mediators such as Bax, and anti-apoptotic mediators such as Bcl-x [6–8]. In this study, we examined whether the downregulation of Bcl-x is a determinant of cardiac allograft neointimal formation, as a prognostic feature involved in the process of graft arteriopathy.

### 3.3. Immunohistochemistry

Bcl-x was strongly and diffusely expressed in the thickened intima of allograft arteries from nontreated or sense ODN-treated recipients, while Bax expression was not enhanced. Antisense bcl-x ODN suppressed Bcl-x expression in the endothelial cells in the mildly thickened allograft intima, while Bax expression was not affected by antisense bcl-x ODN. Expression scores of Bcl-x were significantly different between the antisense bcl-x ODN transfected group and other groups, while the Bax scores were not statistically different among the groups. PCNA was enhanced in the thickened intima of nontreated or sense ODN-treated allografts, while antisense bcl-x ODN treatment limited PCNA expression. ICAM-1 and VCAM-1 were expressed in the arterial thickened intima of the allografts from nontreated or sense ODN-treated recipients, while antisense bcl-x ODN treatment suppressed these expression in the endothelial cells in the mildly thickened allograft intima. Expression scores of PCNA and adhesion molecules were significantly different between the antisense bcl-x ODN transfected group and other groups, however, were not statistically different between sense ODN and nontreated groups (Table 1, Fig. 2). Intensity of ICAM-1 or VCAM-1 expression in the myocardial interstitium did not differ among the groups. Isografts were as free from Bax, Bcl-x, PCNA, ICAM-1 and VCAM-1 expression in the coronary arterial endothelium as native hearts.

### 4. Discussion

#### 4.1. Expression of Bcl-x in graft coronary arteries

Recent studies have documented that cell death is a prominent feature of atherosclerosis [4], however, the role of apoptosis as a determinant of cardiac allograft neointimal formation remains to be demonstrated. It is postulated that the regulation of apoptosis involves the balance between pro-apoptotic mediators such as Bax, and anti-apoptotic mediators such as Bcl-x [6–8]. In this study, we revealed that Bax is faintly expressed throughout the arterial thickened intima and media of nontreated allografts, while Bcl-x is more abundantly expressed within the arterial thickened intima, with enhanced PCNA expression. This observation is consistent with previous studies reporting that intimal SMCs follow a proliferating pattern of gene expression in association with alterations in the regulation of cell growth [16]. Alterations in the genetic program regulating intimal cell apoptosis may be a newly defined pathogenic feature involved in the process of graft arteriopathy.

#### 4.2. Antisense bcl-x ODN prevents neointimal formation

To clarify the pathophysiological role of Bcl-x as a determinant of intimal cell death and neointimal formation, we examined whether the downregulation of Bcl-x is sufficient to selectively induce intimal cell apoptosis. Recently, antisense bcl-x ODN has been shown to be an efficient therapeutic strategy for prevention of neointimal formation after balloon injury of rat carotid arteries [7]. We
demonstrated the feasibility of modifying gene expression within cardiac allografts by transfection of ODN using the HVJ liposome method [10]. We therefore hypothesized that graft arteriopathy could also be prevented by antisense bcl-x ODN administered using the HVJ liposome method. The efficacy of transfecting antisense bcl-x ODN on the inhibition of vascular Bcl-x expression was confirmed by immunohistochemistry. Transfection of antisense bcl-x ODN into the allografts resulted in a marked inhibition of the anti-apoptotic gene Bcl-x, while transfection with sense bcl-x ODN had no significant effects. Although the antisense bcl-x ODN was transfected throughout the allografts, the induction of apoptosis was limited to the transplanted allograft arteries expressing Bcl-x. To confirm this specificity, we transfected the antisense bcl-x ODN into isografts, which do not express Bcl-x. We revealed that medial vascular SMCs of the isografts absorbed the ODN with similar efficiency, yet did not undergo apoptosis. These findings suggest that the proliferating SMC, recognized by its PCNA expression, is essential to the process of apoptosis induced by a reduction in Bcl-x expression. Although no direct relationship between Bcl-x and adhesion molecules was reported, apoptosis of proliferating SMCs decreases adhesion molecule expression because the number of proliferating cells was decreased.

This study demonstrated that the anti-apoptotic gene Bcl-x is necessary to maintain cell viability within the intima and to promote lesion formation. Therefore, down-regulation of this gene selectively suppresses graft arterial neointimal formation via induction of apoptosis of proliferating SMCs. In this report, we clearly demonstrate that a single intraluminal administration of antisense bcl-x ODN dramatically prevents arterial neointimal formation in cardiac allografts.

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