Effects of Immunosuppressants on Platelet-Derived Growth Factor-A Chain mRNA Expression and Coronary Arteriosclerosis in Rat Cardiac Allografts

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Graft coronary arteriosclerosis (GCA) that results in proliferative and obstructive lesions limits the long-term success of cardiac transplantation. Despite extensive study, the pathogenic mechanisms underlying GCA are still unclear and therapeutic strategies for this condition have been inadequate. In this study, we compared the therapeutic effectiveness of cyclosporine A (CsA), 15-deoxyspergualin (DSG), and Multiglycosidum tripterygii (MT) on GCA. In addition, we studied the correlation between the extent of GCA and the degree of platelet-derived growth factor (PDGF)-A chain mRNA expression in cardiac grafts. Lewis rats receiving heterotopic heart transplants from Wistar King donors were treated with $10\text{ mg kg}^{-1}\text{ day}^{-1}$ of CsA ($n=7$), $5\text{ mg kg}^{-1}\text{ day}^{-1}$ of DSG ($n=7$) or $30\text{ mg kg}^{-1}\text{ day}^{-1}$ of MT ($n=7$) respectively. Histological evaluation of coronary arteriosclerosis and Northern blot analysis of cardiac allograft PDGF-A chain mRNA expression were conducted on day 60 after transplantation. Varying levels of GCA were observed in the 21 transplanted hearts. Significant differences in both the degree of PDGF-A mRNA expression and the extent of GCA were found among the 3 groups. GCA was significantly reduced in allografts treated with MT or DSG in comparison with the level seen in CsA-treated grafts. A significant correlation was found between PDGF-A chain mRNA expression and the grade of arterial intimal thickening ($r=0.76$, $p<0.05$) as well as with the incidence of diseased vessels ($r=0.82$, $p<0.01$). Our results indicate that both MT and DSG are more effective in the treatment of GCA than CsA. In our cardiac allografts, the degree of PDGF-A chain mRNA expression correlated well with the extent of GCA, suggesting that PDGF-A may play an important role in the development of transplant-related GCA. (*Jpn Circ J* 1999; 63: 303–308)

Key Words: Coronary arteriosclerosis; Heart transplantation; Immunosuppressant; Platelet-derived growth factor

Graft coronary arteriosclerosis (GCA) is the major determinant of long-term survival after heart transplantation. Despite extensive research, the etiological and pathogenic mechanisms underlying the development of GCA are still unclear. Therapeutic strategies for this condition are still inadequate. Cyclosporine (CsA) has greatly improved the actuarial survival rate of heart transplant recipients. However, it has not been shown to decrease the incidence or the extent of GCA.¹ Moreover, it has been suggested that longterm treatment with CsA may be a risk factor for GCA.^{2,3} There is therefore a need for promising new immunosuppressants.

Intimal and medial proliferation of smooth muscle cells (SMCs) plays an important role in the development of GCA. Enhanced expression of platelet-derived growth factor (PDGF) has been observed in cardiac allografts, and an involvement of PDGF in the occurrence of GCA has been speculated.^{4,5} PDGF is a potent mitogen for vascular SMCs, and has been implicated as a cofactor in the pathogenesis of native atherosclerosis.^{6,7} It is composed of 2 closely related but nonidentical protein subunits, a PDGF-

A chain and a PDGF-B chain, which form 3 possible configurations: AA, AB, and BB. It has been suggested recently that PDGF-AA may play a major role in the development of intimal lesions in GCA.⁸

In the present study, 2 new immunosuppressants, Multiglycosidum tripterygii (MT) and 15-deoxyspergualin (DSG) were evaluated for their effects on GCA in comparison to CsA. To clarify the contribution of PDGF to the development of GCA, we also investigated the correlation between the extent of GCA and the degree of PDGF-A chain mRNA expression in cardiac grafts.

Materials and Methods

Animals and Treatment

Male Wistar (RT-1^k) and Lewis (RT1^l) rats, weighing 200–240 g, were used as donors and recipients, respectively. All animals received care in compliance with the “Principles of Laboratory Animal Care” formulated by the Institute of Animal Resources, and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). Heterotopic heart transplantation was performed with a modification of the procedure described by Ono and Lindsey.⁹ After transplantation, the recipient rats received 60 days of immunosuppressive therapy after being randomly assigned to one of 3 groups of 7 rats each: (1) CsA group: CsA $10\text{ mg kg}^{-1}\text{ day}^{-1}$; (2) MT group: MT $30\text{ mg kg}^{-1}\text{ day}^{-1}$;

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Table 1 Morphometric Parameters

	<i>MT group</i>	<i>DSG group</i>	<i>CsA group</i>
<i>Rejection grade</i>	<i>1.1±0.4</i>	<i>1.1±0.4</i>	<i>1.2±0.3</i>
<i>Incidence of diseased vessels (%)</i>	<i>29.5±7.8*#</i>	<i>48.5±12*</i>	<i>69.1±11</i>
<i>Grade of arterial intimal thickening</i>	<i>1.12±0.31*</i>	<i>1.21±0.22*</i>	<i>1.72±0.28</i>

MT, Multiglycosidorum tripterygii; *DSG*, 15-deoxyspergualin; *CsA*, Cyclosporine A; * $p<0.05$ vs *CsA* group; # $p<0.05$ vs *DSG* group.

Fig 1. (A) Section of intramyocardial coronary artery in a CsA-treated graft, showing marked myointimal proliferation and luminal narrowing. (B) Section of intramyocardial coronary artery in a DSG-treated graft, showing smooth muscle proliferation and minor intimal thickening and luminal stenosis. (C) Section of intramyocardial coronary artery in a MT-treated graft with mild smooth muscle proliferation and minor intimal thickening and luminal stenosis.

and (3) DSG group: DSG 5 mg kg⁻¹ day⁻¹. CsA and DSG were administered intraperitoneally and MT was given by gavage. Post-transplant graft function was evaluated daily by palpation. Recipients with nonbeating heterografts were excluded from the study. The transplanted allografts were harvested on day 60 after transplantation.

Histopathologic Processing

Harvested grafts were bisected through the midportion of both ventricles before fixation in 10% formalin. The specimens were then embedded in paraffin and stained with hematoxylin and eosin to assess the severity of rejection, or with the Elastica Masson stain to assess coronary arterial intimal thickening. Rejection severity was graded on a scale of 0 (none) to 3 (severe), according to the grading system of Lurie et al.¹⁰ In each section, all coronary arteries with a diameter of more than 50 μm were studied, and the extent of coronary arterial intimal thickening was classified on a scale of 0 (normal) to 4 (occluded). The grade of arter-

ial intimal thickening was then calculated by the following formula:

Grade of arterial intimal thickening =

$$\frac{(1 \times N_1) + (2 \times N_2) + (3 \times N_3) + (4 \times N_4)}{N_0 + N_1 + N_2 + N_3 + N_4}$$

where N_n is the number of vessels of classification n .¹¹

PDGF-A mRNA Analysis

The analysis of PDGF-A mRNA expression in graft myocardia was conducted by Northern blotting, using the technique described by Saiki and Oana et al.^{12,13}

RNA Isolation

After harvesting, the transplanted and native hearts were quickly frozen in liquid nitrogen and then stored at -80°C until required for RNA isolation. Total RNA was isolated

Fig 2. Northern blot analysis of myocardial PDGF-A chain mRNA expression from transplanted hearts. (Lane A) MT-treated group; (Lane B) DSG-treated group; (Lane C) CsA-treated group. Ten micrograms of total RNA from the myocardium was size-fractionated on a 0.8% denaturing agarose gel, transferred to a nylon membrane filter, and hybridized to an [32 P]-dCTP-labeled cDNA probe (A). The arrows indicate the position of PDGF-A chain mRNA. The intensities of the hybridization signals (A) were adjusted by estimating and normalizing to the OD of the 28S ribosomal RNA using an Image Master. (B). The adjusted results showed a significant decrease in PDGF-A chain mRNA expression in the MT- and DSG-treated groups in comparison to the CsA-treated group (25.4 ± 6.2 and 39.8 ± 9.4 vs 62.2 ± 12.9 ; $p < 0.05$).

and purified using RNA zol according to the manufacturer's protocol (Biotex Laboratories, Houston, TX).

cDNA Probes and Polymerase Chain Reaction (PCR)

To detect PDGF-A mRNA, an 826 bp cDNA fragment was generated by PCR from the total RNA isolated from rat heart grafts using 2 oligonucleotide primers. The primers were based on the rat PDGF-A sequence:

1 (forward):

5'-GACCGCGCCTCGCTCCCTGCCGAGCTTCC-3'

2 (reverse):

5'-GACCGCGGTCTCGAGTGCTACAGTACTGC-3'

Ten micrograms of total RNA was used for double-strand cDNA synthesis using cDNA synthesis kits (Amersham, Bucks, UK). PCR reactions were performed at pH 8.3 in 10 mmol/L Tris/HCl, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTP, and 2U Taq polymerase. PCR amplification products were generated using a DNA thermal cycler for 35 cycles, comprising denaturing at 94°C for 45 s, annealing at 55°C for 1 min, and extension at

72°C for 90 s each cycle. The resulting 826 bp cDNA fragment was subcloned into the polylinker region of pBlue-script II SK(+). The nucleotide sequence was determined by the dideoxy chain termination method, analyzed by Genetics Gene Analysis System software (Genetics, Tokyo, Japan) and found to be identical to the previously reported rat PDGF-A sequence.

Northern Blot Analysis

Total RNA (10 μ g) was denatured at 65°C for 2 min, fractionated by electrophoresis in 1% agarose gels containing formaldehyde, and transferred to nylon membranes (Zeta-Probe Blotting Membranes, Bio-Rad) by blotting. Hybridization was performed at 65°C in a buffer containing 0.5% sodium dodecyl sulfate (SDS), 5 $\times$ Denhardt's solution, 10% dextran sulfate, 5 $\times$ SSC, and 50 μ g of salmon sperm DNA per ml, followed by serial washing with a final wash in 0.1 $\times$ SSC/0.1% SDS at 65°C. The probe was labeled with [32 P]-dCTP (Amersham) using a nick translation labeling kit (Boehringer Mannheim, Germany). The intensity of the hybridization signal was determined by photostimulated luminescence using an image analyzer (BAS 2000, Fuji, Tokyo).

Statistical Analysis

All numerical data are expressed as mean $\pm$ SE. The Kruskal-Wallis test (Z corrected for ties) was used to evaluate difference between the 3 groups. A p value of less than 0.05 was regarded as statistically significant. Correlation coefficients were calculated by Spearman's nonparametric rank test in which "rs" means the correlation coefficient.

Results

Histopathologic examination revealed prominent luminal narrowing in most of the grafts in the CsA-treated group, with intimal and myointimal proliferation (Fig 1A). In contrast, only moderate narrowing was found in the DSG-treated group (Fig 1B), and narrowing was seen rarely in the MT-treated group (Fig 1C).

The morphometric results from all 21 rats are summarized in Table 1. There were no significant differences in the grade of rejection between the 3 groups. The calculated incidence of diseased vessels (IDV) was found to be lowest in the MT group and highest in the CsA group. There was a significant difference in IDV between the CsA and MT groups, and the CsA and DSG groups. Furthermore, we found a significant difference in IDV between the MT and DSG groups. The grade of arterial intimal thickening was found to be significantly less in the MT and DSG groups than in the CsA group. No significant difference in the grade of arterial intimal thickening was found between the MT- and DSG-treated grafts.

PDGF-A chain mRNA expression is shown in Fig 2. The level of PDGF-A mRNA in cardiac grafts was significantly lessened by treatment with MT or DSG compared to treatment with CsA.

According to correlation analysis, the degree of PDGF-A mRNA expression was found to correlate significantly with IDV (Fig 3). In addition, a positive correlation was found between the degree of PDGF-A mRNA expression and the grade of arterial intimal thickening (Fig 4). No correlation was demonstrated between rejection grade and IDV, the grade of arterial intimal thickening, or the degree of PDGF-A mRNA expression. These results suggest that

Fig 3. Correlation of PDGF-A mRNA expression with the incidence of diseased coronary vessels in cardiac allografts. PDGF-A mRNA expression was measured by Northern blot analysis. OD $\times$ mm² indicates the adjusted signal intensity of PDGF-A mRNA to the OD of the 28S ribosomal RNA. (●) CsA-treated group; (□) DSG-treated group; (▲) MT-treated group.

PDGF-A mRNA expression is associated with the extent of GCA in cardiac grafts.

Discussion

Transplant-derived GCA represents one of the major barriers to longterm survival of cardiac transplant recipients. The incidence of this condition varies from 25% to 80% within the first 5 years¹⁴ The pathogenic mechanism of GCA is still unknown. Multiple factors are believed to be involved. Growth factors including PDGF, insulin-like growth factor-1, and fibroblast growth factor have been suggested to play a causal role in the development of GCA.¹⁴ In the present study we focused on the correlation between PDGF-A chain mRNA expression and the extent of GCA in cardiac grafts, the therapeutic effects of 3 immunosuppressants and the effect of these immunosuppressants on the level of PDGF-A mRNA expression after heart transplantation.

The MT and DSG compounds used in this study are relatively new immunosuppressants. MT, also termed *Tripterygium wilfordii*, is extracted from the plant *Tripterygium wilfordii* Hook F and refined. It contains chiefly stable glycosides and has relatively few side effects. Li et al¹⁵ demonstrated that MT is capable of inhibiting several afferent immune functions including the response of human peripheral blood mononuclear cells to mitogens and alloantigens, and the generation of cytotoxic T cells. They suggest that this inhibition is likely mediated via a reduction in both IL-2 production and responsiveness by blocking DNA replication. We demonstrated previously that MT inhibits the extent of GCA significantly more than CsA.¹⁶ However, the exact mechanism underlying the effect of MT on GCA inhibition remains to be determined. Recently, our *in vitro* study showed that MT inhibited mixed lymphocyte reactions with a 50% inhibition concentration 10 times lower than that of CsA. The mechanism underlying the immunosuppressive effect of MT appears to relate to its inhibition of the T-cell response to IL-2. However this needs to be confirmed. The effect of DSG on

Fig 4. Correlation of PDGF-A mRNA expression with the grade of coronary arterial intimal thickening. PDGF-A mRNA expression was measured by Northern blot analysis. OD $\times$ mm² indicates the adjusted signal intensity of PDGF-A mRNA to the OD of the 28S ribosomal RNA. (●) CsA-treated group; (□) DSG-treated group; (▲) MT-treated group.

the inhibition of GCA was first reported by Nagamine et al¹¹ Zhang et al¹⁷ further showed that DSG may inhibit PDGF-A chain mRNA expression in rat cardiac allografts. The protective effects against GCA of DSG over those of CsA may result from its different immunosuppressive actions. CsA is known to suppress the immune system by inhibiting interleukin-2 production!⁸ Its role in directly suppressing macrophage function and humoral immune responses remains controversial!^{9,20} In contrast, DSG directly suppresses macrophage function, inhibiting the expression of HLA-DR antigens and the production of antibody!^{21,22}

PDGF is one of the best characterized mitogens for vessel wall cells. It is secreted by platelets, monocytes, macrophages, endothelial cells, vascular SMCs and other cell types!²³ PDGF has been implicated as an important growth factor contributing to the pathophysiology of arteriosclerosis and chronic inflammation-derived tissue remodeling. Shaddy et al⁵ reported that PDGF expression was increased during vascular rejection, suggesting a potential correlation between vascular rejection and increased PDGF synthesis. Transplant-derived immune injury to endothelial cells may increase the expression of adhesion molecules by T-lymphocytes and monocytes/macrophages, causing activation of cytokine cascades and the release of growth factors including PDGF. These growth factors, expressed in SMCs and endothelial cells, act as autocrine and/or paracrine growth regulators to accelerate the proliferation of blood vessel SMCs!²⁴ In cardiac grafts undergoing chronic rejection, Lemstrom et al⁸ suggested that continuous low-grade immunological damage and inflammatory responses may also be related to the release of cytokines and growth factors from these cells, resulting in the migration of SMCs from the media and their proliferation in the intima followed by intimal thickening. Furthermore, they demonstrated that high-dose CsA treatment concomitantly results in a significant reduction in PDGF-AA and PDGF receptor- expression in intimal cells and intimal thickening compared with low-dose CsA treatment. In transplanted hearts, Saito et al²⁵ demonstrated that PDGF is

localized mainly in the intimal layer of graft vessels.

GCA may result in severe obstruction in the allograft vessels that could eventually lead to deterioration in graft function. GCA differs in its pathohistological characteristics from age-related coronary atherosclerosis. In GCA, the lesion appears to be diffuse and concentric, affecting the entire length of the vessel of all coronary arteries in the transplanted heart. The internal elastic lamina also remains intact, calcification is rare, and the disease tends to develop rapidly.^{26,27} Studies of GCA have been focused on (1) a better understanding of pathologic mechanisms underlying the development of GCA, (2) an effective therapeutic strategy for this irreversible condition, and (3) attempting to determine some predictors which can be used to monitor the development of GCA or the efficacy of treatment.

In the present study, we used cDNA probes complementary to the PDGF-A chain mRNA to identify the corresponding steady-state expression of PDGF-A mRNA in rat cardiac grafts. We found that there is a significant association between PDGF-A mRNA expression and the extent of GCA. This finding supports the theory that PDGF-A plays an important role in the pathogenesis of GCA. It also suggests that the level of PDGF-A chain mRNA may serve as a predictor in monitoring GCA development or in judging the efficacy of GCA treatment. Consistent with our observations is the finding that after heart transplantation the increased expression of PDGF is further increased following infection with cytomegalovirus, which may also potentially accelerate GCA.^{28,29}

An important question remains whether the increase in the expression of PDGF-A chain mRNA observed in the present study is the cause of or result of GCA. The current study does not answer this question, as we did not observe the expression of PDGF-B and other growth factors that are thought to be involved in the development of GCA.³⁰ Further studies on these issues are needed to clarify possible mechanisms underlying the effects of PDGF-A and other growth factors on cardiac grafts.

In conclusion, the new immunosuppressants MT and DSG are more effective than CsA in the attenuation of post-transplantation GCA. The effects of these 2 agents on GCA correlate with their effects on the inhibition of PDGF, and PDGF-A chain mRNA expression correlates well with the extent of GCA 2 months after heart transplantation. This suggests that PDGF-A may play an important role in the development of GCA. Thus it may be of clinical importance to inhibit the expression of PDGF-A in order to prevent the subsequent occurrence of GCA.

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