Prevention of Cardiac Allograft Arteriosclerosis by Protein Tyrosine Kinase Inhibitor Selective for Platelet-Derived Growth Factor Receptor

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Background—Increased immunoreactivity of platelet-derived growth factor (PDGF)-AA, -Rα, and -Rβ in intimal cells correlates with the development of cardiac allograft arteriosclerosis, a condition for which there is little or no current therapy. Therefore, we hypothesized that PDGF may have a rate-limiting role in the development of this disease.

Methods and Results—The hypothesis was tested in a rat model of heterotopic cardiac and aortic allografts using dark agouti (AG-B4, RT1<sup>d</sup>) donors and Wistar-Furth (AG-B2, RT1<sup>b</sup>) recipients. The recipients received CGP 53716, a selective PDGF-R protein tyrosine kinase inhibitor, 50 mg · kg<sup>-1</sup> · d<sup>-1</sup>, or vehicle for 60 days. Cardiac allograft recipients also received background cyclosporin A immunosuppression. Our results demonstrate that CGP 53716 significantly reduced the incidence and intensity of arteriosclerotic lesions in rat cardiac and aortic allograft recipients. When rat coronary smooth muscle cells were stimulated in vitro with PDGF-AA or -BB in the presence of interleukin-1β or tumor necrosis factor-α, CGP 53716 significantly inhibited only AA-ligand–induced but not BB-ligand–induced replication. Concomitantly, in quantitative reverse transcriptase–polymerase chain reaction, interleukin-1β or tumor necrosis factor-α stimulation specifically upregulated the expression of PDGF-Rα mRNA but not of other ligand or receptor genes in cultured smooth muscle cells.

Conclusions—We conclude that a PDGF-AA/Rα–dependent cycle is induced in the generation of allograft arteriosclerosis that may be inhibited by blocking of signaling downstream of PDGF-R. (Circulation. 1999;99:2295-2301.)

Key Words: transplantation ■ muscle, smooth ■ cells ■ proteins

Studies on platelet-derived growth factor (PDGF) ligand and receptor expression in ordinary atherosclerotic lesions have suggested a regulatory role for PDGF in the vascular wall proliferative diseases (reviewed in Reference 1). PDGF is a major mitogen for mesenchymally derived cells, such as smooth muscle cells (SMCs) and fibroblasts. The PDGF ligand consists of a disulfide-linked dimer of 2 polypeptides, the PDGF-A and PDGF-B chains, and can be expressed in the form of homodimers (PDGF-AA or -BB) or a heterodimer (PDGF-AB) (reviewed in Reference 2). Two separate PDGF receptors (PDGF-Rα and PDGF-Rβ) have been identified. These receptors exist as monomers on the cell surface, but signal transduction by PDGF requires receptor dimerization. PDGF-Rβ binds only the PDGF-B chain, whereas PDGF-Rα binds both the A and B chains. Dimerization of receptor molecules, followed by autophosphorylation of the receptor protein tyrosine kinase, initiates the signaling cascades and leads to the biological responses of PDGF.

Using a panel of antibodies, we found that the expression of PDGF-AA, -Rα, and -Rβ in intimal cells and PDGF-BB in macrophages correlated with the development of cardiac allograft arteriosclerosis. Therefore, we hypothesized that PDGF may have a rate-limiting role in the development of this disease. To test this hypothesis, rats with heterotopic cardiac and aortic allografts received CGP 53716, a protein tyrosine kinase inhibitor selective for PDGF-R. The compound shows selectivity for inhibition of PDGF-mediated events, such as PDGF-R autophosphorylation, cellular tyrosine phosphorylation, and c-fos mRNA induction in response to PDGF stimulation of intact cells. In contrast, ligand-induced autophosphorylation of epidermal growth factor-R, insulin receptor, and insulin-like growth factor-1R, as well as c-fos mRNA expression induced by epidermal growth factor, fibroblast growth factor, and phorbol ester, was insensitive to inhibition by CGP 53716. In addition, we demonstrate that the macrophage-derived cytokines interleukin (IL)-1β and tu-
mor necrosis factor (TNF)-α regulate PDGF-R expression at the mRNA level in SMCs.

Methods

Heterotopic Cardiac and Aortic Allografts

Inbred dark agouti (AG-B4, RT1a) and Wistar-Furth (AG-B2, RT1b) rat strains (200 to 300 g) (Laboratory Animal Center, University of Helsinki, Finland) were used as donors and recipients, respectively. Heterotopic cardiac and aortic transplantsations were performed as described.6,8 Heart allograft function was evaluated by daily palpation, and the grafts were removed when the heart rate was >30 bpm or 60 days after transplantation. Aortic allografts were harvested at 60 days.

Drug Regimens

One group of cardiac allograft recipients was treated with cyclosporin A (CsA) 1 mg·kg⁻¹·d⁻¹ and CGP 53716, and the other received CsA 1 mg·kg⁻¹·d⁻¹ and vehicle. When the treatment with CGP 53716 was observed to elevate CsA blood trough levels, different CsA regimens were tested to achieve CsA blood trough levels equal to those in rats treated with CsA 1 mg·kg⁻¹·d⁻¹ and CGP 53716. The administration of CsA 2 mg·kg⁻¹·d⁻¹ for the first week, followed by CsA 1.5 mg·kg⁻¹·d⁻¹, was found to yield CsA blood trough levels in vehicle-treated rats matched to those in rats treated with CsA 1 mg·kg⁻¹·d⁻¹ and CGP 53716. A group of aortic allograft recipients was given CGP 53716 50 mg·kg⁻¹·d⁻¹·IP, and another group received vehicle only. Whole-blood CsA 24-hour trough levels were determined once a week by radioimmunoassay (Sandimmun-Kit, Novartis), and the rats were weighed weekly.

CGP 53716, a protein tyrosine kinase inhibitor selective for PDGF receptor (Novartis), was dissolved in DMSO to a concentration of 200 mg/kg, diluted 1:20 with 1% Tween in 0.9% NaCl, and sonicated.7 Rats received CGP 53716 50 mg·kg⁻¹·d⁻¹·IP by single injection starting 24 hours before transplantation. This dose has previously been shown to be well tolerated and effective in inhibition of tumor activity against the tumors derived from v-sis- and c-sis-transformed BALB/c cells in BALB/c nude mice.6 Cardiac allograft controls received an equal amount of vehicle prepared and administered like the drug.

Histology

Allografts were fixed in 3% paraformaldehyde overnight, processed routinely, and embedded in paraffin. Cross sections of allografts (4 μm thick) were stained with Mayer’s hematoxylin-eosin and with Masson’s trichrome for fibrosis. In cardiac allografts, only epicardial arteries and intramyocardial arterioles were evaluated for histological changes attributable to chronic rejection, and changes in intimal thickness were scored according to Billingham’s criteria (Reference 9; Figure 4).

In aortic allografts, histological changes were quantified by use of a microscope ocular grid at ×400 magnification and are expressed as mean intimal thickness (μm) ±SEM. To compare the size of intima to media, corresponding areas were quantified with a Macintosh NIH image software. The picture from the microscope was transferred to the screen with an Olympus video microscope with ×25 magnification. The areas inside the internal elastic lamina and external elastic lamina and that of the aortic lumen were measured, and the intima/media area ratios were calculated from these values. The number of medial cells per cross section was quantified to evaluate the degree of media necrosis.

Immunostaining

Acetone-fixed 4- to 6-μm cryosections were treated with 1.5% nonimmune horse serum and sequentially incubated with a primary antibody (10 μg/mL), a biotinylated secondary horse anti-mouse antibody, avidin–biotinylated horseradish complex (Vectorstain Elite ABC Kit, Vector Laboratories), and then chromogen 3-amino-9-ethylcarbazole (Sigma). The mouse anti-rat monoclonal antibodies were W3/25 (Sera Laboratory) to T helper cells (CD4 equivalent); OX8 (Sera Laboratory) to CD8+ T cells; and E3 (Serocte) to macrophages. Immunoreactivity was quantified as the total number of positive cells at high-power magnification in 20 separate visual fields per allograft cross section.

Cell Cultures

CGP 53716 was initially diluted to DMSO at a 10 mmol/L concentration, and then in cell culture medium at concentrations of 0.1 to 10 μmol/L (0.05 to 5 μg/mL). A rat coronary SMC line from Dr C.A. Diglio (Wayne State University, Detroit, Mich) was kindly provided by Dariusz Leszczynski (Finnish Center for Radiation and Nuclear Safety, Helsinki, Finland). Recombinant human PDGF-AA, PDGF-BB, IL-1β, and TNF-α were purchased from Upstate Biotechnology Inc. For the experiments, the cells were trypsinized and seeded on 0.32-cm² microwells in culture medium supplemented with 10% FCS at a concentration of 25 000 cells/mL and allowed to adhere to the wells for 24 hours. After 72 hours of serum starvation (DMEM with 0.1% BSA and 0.5% FCS), the quiescent cells were preincubated with IL-1β or TNF-α (10 ng/mL in serum-free medium). After 24 hours, PDGF-AA or PDGF-BB (60 ng/mL in serum-free medium) was added to the cultures for 48 hours. Tritated thymidine ([³H]Tdr; Amershamb) incorporation was measured during the last 24 hours of this 48-hour incubation. CGP 53716 was added to the cultures at a concentration of 1.3 μmol/L.¹⁰ [³H]Tdr (1 μCi/mL) was added to each well, and after 24 hours, the wells were washed 3 times with PBS, detached with 1.25% trypsin, and mixed with OptiPhase Hisafe 3 (LKB-Wallac). Radioactivity was measured with a Rackbeta liquid scintillation counter (LKB-Wallac).

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Levels of PDGF-A, PDGF-B, PDGF-Rα, and PDGF-Rβ gene transcripts were measured with quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR) as described.¹¹ Briefly, rat coronary SMCs were challenged with either IL-1β or TNF-α or vehicle for 4 hours (see above), and total RNA was extracted with the guanidine-isothiocyanate method.¹² Six serial dilutions were made with appropriate amounts of total RNA from each preparation, mixed with either 10³ or 10⁴ molecules of synthetic template RNA containing a 46-nucleotide insert, and reverse transcribed to cDNA in a mixture containing 1× RT buffer, 300 mmol/L dNTP, 10 pmol antisense primer (PDGF-A, TACAAGTTGTCGGCCGTA; PDGF-B, CACTACTGTCACACMTGCGG; PDGF-Rα, CA- CACTGAGGTTGCGTGAAG; PDGF-Rβ, CGACCAAGCGTACCATCCA; PDGF-Rα, GACAACTGCTGAATGAT; PDGF-Rβ, GACACAGGCGTACCATCAG) (Reference 3; Figure 4). The samples were heated to 95°C for 5 minutes and cycled 40 times (94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute). Two milliliters of cDNA mixtures was supplemented with 10× PCR buffer, radioactive tracer, 10 pmol antisense primer, 10 pmol of sense primer (PDGF-A, GACAACTGCTGAATGAT; PDGF-B, TACAGTTGTCGGCCGTA; PDGF-Rα, GACACAGGCGTACCATCAG; PDGF-Rβ, GACAACTGCTGAATGAT; PDGF-Rβ, GACACAGGCGTACCATCAG) (Reference 3; Figure 4). The PCR samples were electrophoresed through 2% agarose gel, the fragments of interest were cut off the gel, and incorporated radioactivity was counted by Cherenkov counting protocol. RNA quantification was performed as originally described.¹¹

Statistical Analyses

Data are mean±SEM. A nonparametric Mann-Whitney U test, z corrected for ties (StatView 4.1 program; Abacus Concepts), was used to evaluate the significances between 2 groups. For multiple comparison, Kruskal-Wallis and Dunn tests were applied. Graft survival between the groups was analyzed by log-rank test (Medstat, Astra Group A/S). A value of P<0.05 was regarded as statistically significant.
Results

Effect of CGP 53716 on Cardiac Allograft Arteriosclerosis

The effect of CGP 53716 was first investigated in the in vivo cardiac allograft model using an equivalent dose of CsA 1 mg · kg⁻¹ · d⁻¹ for baseline immunosuppression. As shown in Figure 1A, there was a possible interaction between CGP 53716 and CsA, causing increased blood CsA levels in CGP 53716–treated rats. Treatment with vehicle and CsA 1 mg · kg⁻¹ · d⁻¹ led to irreversible episodes of acute rejections and poor graft survival (mean survival, 25±7 days), whereas treatment with CGP 53716 and CsA 1 mg · kg⁻¹ · d⁻¹ was associated with a graft survival >60 days (P<0.001; Figure 1B). The total number of arteries and arterioles in cardiac cross sections analyzed was 31±2 in allografts of vehicle-treated rats and 49±4 in allografts of CGP 53716–treated rats. The smaller numbers of vessels observed in the vehicle group was probably a result of acute rejection and graft destruction. In cardiac allografts of vehicle-treated rats, 95±3% of vessels were affected by intimal thickening, and the mean grade of intimal thickening was 2.6±0.4. Treatment with CGP 53716 reduced the percentage of affected vessels significantly, to 51±7% (P<0.01; Figure 1C), and the mean grade of intimal thickening to 0.7±0.1 (P<0.01; Figure 1D), compared with vehicle-treated rats.

To rule out the possibility that the inhibition of cardiac allograft arteriosclerosis in the CGP 53716 group was not due to elevated blood CsA trough levels, new groups were formed, and the CsA dose was adjusted to equivalent blood CsA concentrations in the vehicle- and CGP 53716–treated groups (P=NS; Figure 2A). In the new vehicle group, the rats received CsA 2 mg · kg⁻¹ · d⁻¹ for the first week, followed by 1.5 mg · kg⁻¹ · d⁻¹. Recipients treated with CGP 53716 were given CsA 1 mg · kg⁻¹ · d⁻¹ for the whole experiment. The graft survival was now >60 days in both groups (Figure 2B).

The number of arteries and arterioles in cardiac cross sections analyzed was 45±4 in allografts of vehicle-treated rats and 49±5 in allografts of CGP 53716–treated rats. In the new vehicle group, 83±4% of vessels were affected by intimal thickening, and the mean grade of intimal thickening was 1.2±0.1. Treatment with CGP 53716 reduced the percentage of affected vessels significantly, to 46±6% (P<0.01; Figure 2C) and the mean grade of intimal thickening to 0.6±0.1 (P<0.01; Figures 2D, 4, and 5A and B), compared with vehicle-treated allografts with equal blood CsA levels.

The impact of CGP 53716 on the intragraft inflammatory cell response was analyzed by immunohistochemistry, revealing a 30% reduction in the graft-infiltrating ED3⁺ activated macrophages, but not in CD4⁺ and CD8⁺ T cells, compared with the vehicle group (P=NS).

Effect of CGP 53716 on Aortic Allograft Arteriosclerosis

To study in vivo effects of CGP 53716 on allograft arteriosclerosis without immunosuppression, aortic transplantsations were performed. CGP 53716 treatment reduced intimal thickening significantly, from 80±16 to 35±12 μm (P<0.05; Figures 3A and 5C and D), and the intima/media ratio from 80±10% to 30±10% (P<0.05; Figure 3B), compared with vehicle-treated rats. The intima/media ratio was not affected by media necrosis, because the number of medial cells per aortic cross section was 265±78 in vehicle-treated and 239±43 in CGP 53716–treated rats (P=NS; Figure 3C).
Effect of Cytokine Treatment and CGP 53716 on PDGF-Mediated SMC Proliferation

On stimulation with PDGF-AA alone, there was no increase in the SMC [3H]TdR incorporation, whereas after PDGF-BB stimulation, the SMC [3H]TdR incorporation was increased to 12-fold and was inhibited by CGP 53716 (P<0.05; Figure 6B and C). When SMCs were preincubated with TNF-α, the response to PDGF-AA increased to levels comparable to those with PDGF-BB stimulation, and this increase was strongly inhibited by CGP 53716 (Figure 6B). A similar pattern of response to PDGF-AA, but of lesser magnitude, was seen when SMCs were preincubated with IL-1β. PDGF-BB–dependent [3H]TdR incorporation was only slightly increased on prestimulation with either IL-1β or TNF-α, and CGP 53716 showed no inhibitory effect in this constellation.

To explain the differences observed in the response patterns of cytokine-primed and nonprimed SMCs, we challenged rat coronary SMCs with IL-1β and TNF-α for 4 hours and quantified the mRNA transcript levels of PDGF ligands and receptors. As shown in Figure 7, IL-1β induced a 60-fold and TNF-α a 100-fold upregulation of PDGF-Rα mRNA expression in SMCs, whereas the levels of PDGF-A, -B, and -Rβ remained unchanged.

Discussion

In the present study, we demonstrate the inhibitory effect of CGP 53716, a selective PDGF-R protein tyrosine kinase inhibitor, on the development of rat cardiac and aortic allograft arteriosclerosis, both in regard to the incidence of allograft coronary artery lesions and the intensity of allograft coronary artery and aortic lesions. These are the first observations to demonstrate a functional role for PDGF in the pathophysiology of this disorder, and they open an entirely new therapeutic strategy for prevention of chronic rejection.

In regard to the inhibitory effect in vivo, an unexpected finding was that CGP 53716 had an effect on CsA metabolism. First, CGP 53716, compared with controls, increased the blood CsA trough levels in rat allograft recipients receiv-
ing the same CsA dose/weight. Conversely, CGP 53716 reduced the dose of CsA needed to maintain target level by 30%. To the best of our knowledge, the interaction of tyrphostins and CsA is not established. CsA is metabolized by the cytochrome P450 enzyme system, and competition or inhibition of the same enzyme between CGP 53716 and CsA may have caused increased CsA blood levels. Whether this is the case remains to be shown in future studies. On the other hand, inhibition of CsA metabolism by ketoconazole or diltiazem has been demonstrated in humans, with a reduction in the dose of CsA after cardiac transplantation.13,14

On the basis of our previous results, we hypothesized that continuous low-grade perivascular and subendothelial inflammatory response may be related to release of cytokines and growth factors from endothelial and inflammatory cells, resulting in the migration of SMCs from the media and their proliferation in the intima.15 Of the cells present during the lesion formation, macrophages, SMCs, and injured endothelial cells are capable of producing PDGF.16–18 Although T cells themselves do not produce PDGF, they may induce the secretion of a PDGF-like protein from arterial endothelial cells.19 The presence of PDGF-BB in macrophages of intimal lesions during all phases of ordinary atherosclerosis has been observed.20 Macrophages can penetrate into the subendothelial space of arteries, and this may be facilitated by concomitant enhanced expression of adhesion molecules, such as P-selectin and vascular cell adhesion molecule-1, as observed in chronically rejected cardiac allografts.21,22 Furthermore, macrophages are able to produce other proinflammatory factors, such as transforming growth factor-β, TNF-α, IL-1, and IL-6, which either induce PDGF-AA secretion from SMCs or act as a direct mitogen for SMCs.23–26

The in vitro results presented in this study show that prestimulation with IL-1β and TNF-α renders SMCs responsive to PDGF-AA up to levels comparable to PDGF-BB stimulation, whereas prestimulation does not significantly alter the response to PDGF-BB. Because the concentration of CGP 53716 used was suboptimal (50% inhibition) in inhibiting PDGF-Rβ but optimal (total inhibition) in inhibiting PDGF-Rα,10 the results suggest that the TNF-α–induced response to PDGF-AA is mediated by PDGF-Rα. Our next observations revealed that when rat coronary artery SMCs were challenged with IL-1β or TNF-α, IL-1β induced a 60-fold and TNF-α a 100-fold increase in the PDGF-Rα mRNA expression in these cells, whereas the expression of other PDGF ligands and receptors remained unchanged. In our previous study in rat cardiac allografts, a prominent induction of PDGF ligand and receptor protein occurred in macrophages during acute rejection, whereas PDGF-AA, PDGF-Rα, and PDGF-Rβ were observed in intimal cells and PDGF-BB in macrophages during the development of cardiac allograft arteriosclerosis.6 In a recent study, chronic treatment with IL-1β induced coronary intimal lesions in native vessels, and the lesion formation was inhibited with a neutralizing antibody to either IL-1β or PDGF.27 Thus, these in vitro and in vivo findings support the hypothesis that TNF-α and IL-1β produced by activated macrophages during alloimmune re-

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**Figure 4.** Changes in intimal thickness were quantified according toBillingham’s criteria9 in arterioles (left; magnification ×250) and arteries (right; magnification ×100): grade 0, normal artery with intact internal elastic lamina; grade 1, <10% occlusion of lumen by intimal thickening and proliferation, disruption of internal elastic lamina, with some foam or vacuolated endothelial cells present; grade 2, <50% occlusion of lumen; grade 3, >50% but <100% occlusion of lumen; and grade 4, 100% occlusion of vessel lumen. Mayer’s hematoxylin–eosin and resorcin fuchsin for internal elastic lamina.

**Figure 5.** Photomicrographs from coronary arteries of (A) vehicle + CsA–treated and (B) CGP 53716 + CsA–treated rat cardiac allografts with equal blood CsA trough levels, and aortic allografts in (C) vehicle–treated and (D) CGP–treated recipients. Magnification ×100 (A and B) and ×50 (C and D). Internal elastic lamina is indicated by arrows. L indicates lumen; I, intima; M, media; and A, adventitia. Mayer’s hematoxylin and eosin and resorcin fuchsin for internal elastic lamina.
response have a pivotal role in the regulation of PDGF-mediated SMC proliferation, in particular PDGF-AA/R_α, during cardiac allograft arteriosclerosis.

Recent data show that CGP 53716 also inhibits c-kit tyrosine kinase receptor (Buchdunger et al, unpublished observations) for stem cells. We did not find any immunoreactivity for c-kit in our cardiac allografts during acute or chronic rejection (data not shown). Our data showed that CGP 53716 somewhat but not significantly reduced the number of graft-infiltrating ED3⁺ macrophages, but not that of CD4⁺ and CD8⁺ T cells. In addition, CGP 53716 did not affect graft survival in an acute rejection model (data not shown). Thus, it seems that CGP 53716 selectively and directly inhibited PDGF-R protein tyrosine kinase and SMC migration and proliferation in our model and did not mediate its effect by downregulating alloimmune response.

Low-molecular-weight tyrosine kinase inhibitors, tyrphostins, have been shown to inhibit PDGF-dependent SMC proliferation and chemotaxis in vitro. At the dose given (50 mg · kg⁻¹ · d⁻¹), CGP 53716 is selective for PDGF receptors. We showed in vitro that at a dose of 1.3 μmol/L, CGP 53716 did not have any effect on cytokine-induced [³H]TdR incorporation, indicating that the inhibition seen in vivo is due to specific inhibition of the PDGF-R. A dose of 100 mg · kg⁻¹ · d⁻¹ yields CGP 53716 levels of 2 μmol/L in rats (E. Buchdunger, unpublished observations). The IC₅₀ for inhibition of PDGF-R by CGP 53716 is ≈0.1 μmol/L, whereas IC₅₀ for other growth factor receptor tyrosine kinases is 10 to 500 μmol/L, demonstrating high selectivity to PDGF-R tyrosine kinase. It is suggested that SMC migration is a PDGF-R_β-mediated event, whereas proliferation may be due to PDGF-R_α triggering.

To conclude, the results of this study indicate that a PDGF-AA/R_α-dependent cycle is induced in the generation of allograft arteriosclerosis that may be inhibited by blocking of signaling downstream of PDGF-R. Thus, selective nontoxic protein tyrosine kinase inhibitors may provide entirely new tools for the prevention of cardiac allograft arteriosclerosis. The findings also support the hypothesis that cytokines produced by activated macrophages during alloimmune response may have a pivotal role in the regulation of PDGF-mediated events on target cells by receptor regulation.

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