FK409, a Spontaneous Nitric Oxide Releaser, Attenuates Allograft Vasculopathy in a Rat Aortic Transplant Model

Johji Fukada, Stefano Schena, Ivan Tack, Phillip Ruiz, Yoshihiko Kurimoto, Manhui Pang, Abdelouahab Aitouche, Tomio Abe, Liliane J. Striker, Si M. Pham

Abstract—Although systemic administration of NO donors has been shown to attenuate the development of neointimal hyperplasia in the balloon injury model, this strategy has not been tested in a model of allograft vasculopathy. In this study, we investigated the effect of FK409, a spontaneous NO releaser, on the development of allograft vasculopathy, using a rat aortic transplant model. Thoracic aortas from ACI rats were transplanted heterotopically into the abdominal aorta of Wistar-Furth rats. Postoperatively, recipients received FK409 orally every 8 hours from the day of transplantation to the time of euthanization. Morphometric and immunohistochemical analyses were performed on the aortic grafts 8 weeks after transplantation. Control allografts showed severe neointimal hyperplasia, which consists mainly of α-actin–containing vascular smooth muscle cells. The FK409-treated allografts showed a dose-dependent reduction (statistically significant compared with the control) in the neointimal thickness as the dose increased from 1 to 10 mg/kg (thrice per day). However, there was no significant difference in the neointimal thickness between groups treated with 10 and with 20 mg/kg. FK409 treatment (10 mg/kg) caused a significant decrease in DNA synthesis (5-bromo-2-deoxyuridine [BrdU] uptake), an increase in DNA fragmentation (terminal deoxynucleotidyltransferase–mediated uridine nick-end labeling [TUNEL]), and upregulation of Fas expression, in the neointimal vascular smooth muscle cells. These data suggest that FK409 attenuates the allograft vasculopathy in a rat aortic transplant model. (Circ Res. 2000;87:66-72.)

Key Words: FK409 ■ allograft ■ apoptosis ■ in situ nick-end labeling ■ Fas

Advances in the past 3 decades have dramatically enhanced the early survival of cardiac transplant recipients. However, the improved early graft survival has unveiled another problem: coronary allograft vasculopathy, which is responsible for >50% of late deaths.1 Coronary allograft vasculopathy is a diffuse disease involving the entire network of epicardial coronary vessels and their intramural branches.2 A growing body of evidence has suggested that chronic immune system–mediated endothelial injury, operating in a milieu of nonimmunologic risk factors, provides the primary stimuli for the phenotypic changes (from contractile to synthetic phenotype) and the migration of vascular smooth muscle cells (VSMCs) into the intima.3 Allograft vasculopathy is thought to result from this chronic immune rejection process. The discovery of NO has brought new insights regarding the mechanisms leading to allograft vasculopathy. Recent studies have demonstrated that the cytokine-inducible isoform of NO synthase (iNOS) is upregulated in both acute and chronic rejection processes.4–6 In acute rejection, iNOS is expressed predominantly in the infiltrating inflammatory cells that invade the subendothelial and periadventitial layers.5 In chronic cardiac rejection, iNOS expression can be detected in the myocardium and the VSMCs of the medial and the neointimal layers.6 NO is known for its physiological regulation of vasomotor tone and its ability to inhibit platelet aggregation.7,8 In addition to these functions, NO suppresses T-cell proliferation and inhibits leukocyte chemotaxis,9–11 lending support to an immunomodulatory role for NO during allograft rejection. NO also plays an important role in cellular growth and apoptosis,12–14 VSMC migration, which is a critical step in the development of neointimal hyperplasia, can also be inhibited by NO.15,16

The ability of NO to inhibit VSMC proliferation and migration has been exploited in gene transfer strategies as a means to prevent neointimal hyperplasia in the balloon injury model.17 Furthermore, we showed that overexpression of iNOS gene in aortic allografts suppressed the development of allograft vasculopathy.18 Collectively, these data suggest that NO may ameliorate the vascular response to both mechanical and immunologic injuries. Although exogenous NO (via the use of NO donors) has been shown to attenuate the development of neointimal hyperplasia in arterial injury models,19,20 this form of therapy has not been used in allograft vasculopa-
thy. Because neointimal hyperplasia results both from mechanical and immunologic injury, we postulated that exogenous NO would attenuate the development of allograft vasculopathy.

FK409, (±)-(E)-4-ethyl-2-hydroxyimino-5-nitro-3-hexamide, is a newly discovered NO donor obtained from fermentation products.21 Similar to other NO donors, FK409 causes vasodilatation via the activation of soluble guanylate cyclase and a resultant increase in intracellular cGMP.22 One of the unique properties of FK409 is that it releases NO spontaneously from the compound itself without the need for metabolic bioconversion, which may account for its potent pharmacological actions.23 Indeed, FK409 is 300 times more potent than isosorbide dinitrate (ISDN) as a vasodilator of isolated rat arteries in vitro.23 Another advantage of FK409 is that it does not lead to the development of drug tolerance, whereas other organic NO donors, such as ISDN and nitroglycerine, do.22 The aim of this study was to determine whether FK409 inhibits neointimal hyperplasia in a transplantation setting, using computer-assisted image analysis in the well-characterized chronic rejection model of rat aortic transplantation. Because previous studies only explored the neointimal hyperplasia formation resulting from balloon injury, the mechanism(s) by which NO may affect transplant-associated vasculopathy was not investigated. Thus, an additional aim of this study was to examine how NO regulates VSMC accumulation in the neointima. In this regard, we evaluated the relative contribution of apoptotic cell death and proliferation of neointimal VSMCs.

Materials and Methods

Aortic Transplantation

Male ACI (RT1A⁺) and Wistar-Furth (WF, RT1A⁻) rats weighing 200 to 300 g (Harlan Sprague Dawley) were used. Aortic transplantation was performed as described.18,24 After transplantation, recipient animals received FK409 (Fujisawa Pharmaceutical Co), which was dissolved in 0.5% methylcellulose solution, orally every 8 hours from the day of transplantation to the time of euthanization (Table 1).19 Control animals received ISDN (Sigma; 100 mg/kg, thrice per day). Grafts were explanted 8 weeks after transplantation. Animals received humane care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (86-23, revised 1985).

Quantitative Morphometry

Three segments from each graft were fixed in 10% buffered formalin and embedded in glycol-methacrylate. Five-micrometer sections were stained with periodic acid-Schiff. Microscopic pictures of these sections were recorded using a digital charge-coupled device video camera mounted on an Olympus BH-2 light microscope. Images were processed using Photoshop version 5.0 software (Adobe). On the basis of their morphological characteristics, lumen, intima, and media were individually selected and their area was measured. The thickness of the intima (Qint) and that of the media (Qmed) were expressed as a fraction of the total area as follows: Qint = intima/(lumen + intima + media) ×100% and Qmed = media/(lumen + intima + media) ×100%, respectively.25

<table>
<thead>
<tr>
<th>TABLE 1. Quantitative Morphometry of Intima and Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg, Thrice Per Day)</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td><strong>Allografts</strong></td>
</tr>
<tr>
<td>Vehicle</td>
</tr>
<tr>
<td>ISDN</td>
</tr>
<tr>
<td>FK409</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Isografts</strong></td>
</tr>
<tr>
<td>Vehicle</td>
</tr>
<tr>
<td>FK409</td>
</tr>
<tr>
<td><strong>Native grafts</strong></td>
</tr>
<tr>
<td>Vehicle</td>
</tr>
</tbody>
</table>

All values are mean±SD.

*P<0.05 vs vehicle-treated allografts.
†P<0.05 vs allografts with FK409 at 1 or 5 mg/kg, thrice a day.
Immunohistochemical Staining, Terminal Deoxynucleotidyltransferase–Mediated Uridine Nick-End Labeling (TUNEL) Assay, and 5-Bromo-2-Deoxyuridine (BrdU) Incorporation Analysis

Immunohistochemical staining was performed using 5-μm serial paraffin-embedded or frozen sections as described. The polyclonal antibodies against Fas (1:100) and FasL (1:80; Santa Cruz) and the monoclonal antibodies against monocyte-derived macrophages (Mo/Mφ; 1:2400; Serotec), α-actin–containing smooth muscle cells (1:1400; DAKO), CD8+ T cells (1:400; PharMingen), and CD4+ T cells (1:200; PharMingen) were used. For BrdU analysis, rats were injected with BrdU at 30 mg/kg through the penile vein 24 and 12 hours before euthanization. The TUNEL assay and BrdU labeling assay were performed as described. For double-labeling assays, anti-α-actin or anti-Fas antibodies were used along with TUNEL or BrdU labeling.

Quantification of Immunostainings and TUNEL- and BrdU-Positive Nuclei

For quantification of α-actin–positive cells, the immunopositive components from 3 microscopic images were dissected using the property of color recognition of PhotoShop version 5.0 software. The α-actin–positive area was expressed as a fraction of the total area as follows: (α-actin–positive area/intimal area)×100%. For quantification of the stainings with other antibodies, the number of immunopositive cells was scored on a scale ranging from 0 to 3. Essentially, the proportion of cells was estimated in a blinded fashion and scored using 0 = no staining, 1 = mild percentage of staining, 2 = moderate percentage of staining, and 3 = significant percentage of staining. The apoptotic index and BrdU labeling index were calculated as (TUNEL-positive nuclei/total nuclei)×100% and (BrdU-labeled nuclei/total nuclei)×100%, respectively.

Transmission Electron Microscopy (TEM)

Tissue samples were processed for TEM as described and were examined using a JEM-100CX II electron microscope (JOEL Ltd).

Statistical Analysis

Data are presented as mean±SD. For comparison between 2 groups, we used an unpaired t test. For comparison of >2 groups, an ANOVA followed by a Bonferroni post hoc test was used. A χ2 test was applied to compare mortality. Correlation analysis was performed by using a Spearman rank correlation. P<0.05 was considered significant.

Results

Clinical Data

Total ischemic time, which was defined as the interval between transection of the donor descending aorta and release of both proximal and distal clamps on the recipient aorta, ranged from 46 to 69 minutes. There was no significant difference in the mean ischemic times among groups. After FK409 administration, there was a transient drop in carotid arterial blood pressure in anesthetized rats if the dose of FK409 was >1 mg/kg (Figure 1). The blood pressure dropped to a minimum at 10 minutes and returned to its baseline value at 30 minutes. One ACI recipient (syngeneic group) treated with vehicle, 3 ACI recipients (syngeneic group) receiving FK409 at a dose of 10 mg/kg, and 1 WF recipient (allogeneic group) receiving 5 mg/kg FK409 died 1 week after transplantation; these deaths were attributed to viral infection. There was no significant difference in mortality among the groups. In the allogeneic groups, recipients that were treated with vehicle, ISDN at 100 mg/kg; and FK409 at 1.5, or 10 mg/kg had similar weight gain during the 8-week follow-up (57.9±32.4, 73.7±28.1, 43.8±9.1, 56.3±10.7, and 61.9±18.3 g, respectively), whereas the weight increase in the recipients that were treated with FK409 at 20 mg/kg thrice per day (5.0±7.0 g) was significantly lower compared with that of the others (P<0.05). In the syngeneic groups, there was no difference in body weight change between the recipients that received vehicle and those receiving 10 mg/kg thrice per day of FK409 (39.3±18.2 and 21.2±9.8 g, respectively).

Analysis of Morphological Changes

Table 1 summarizes the quantitative morphometric analysis of allografts (ACI→WF) and isografts (ACI→ACI) 8 weeks after transplantation and of native aortas (ACI). In the vehicle-treated allografts, there was a pronounced neointimal thickening, which was significantly greater than the neointimal thickness of allografts in the FK409 treatment groups.
Among the FK409-treated allografts, there was a dose-dependent reduction in neointimal thickening when the dosage was increased from 1 to 10 mg/kg thrice per day. However, there was no significant difference between rats receiving 10 and 20 mg/kg thrice per day. The neointimal thickness of the allograft recipients treated with ISDN 100 mg/kg thrice per day was similar to that of the controls. The isografts in FK409- and vehicle-treated groups continued to be devoid of neointimal hyperplasia 8 weeks after transplantation. Averages of $Q_{med}$ in the FK409-treated allografts at 10 and 20 mg/kg were significantly greater than those in the rest of the allogeneic groups ($P<0.005$). $Q_{med}$ in the isografts of both vehicle- and FK409-treated animals remained at the pretransplant control level (native aorta).

**Effect on Cellular Composition Detected by Immunohistochemistry**

In the allografts, the neointima contained mostly $\alpha$-actin–positive VSMCs (Figure 2D). FK409 treatment significantly decreased the contribution of the $\alpha$-actin–positive cell area within the neointima as compared with the vehicle-treated group (24.7 ± 16.1 versus 48.8 ± 12.1%; Figures 2D and 3). Monocytes/macrophages were rarely present; however, CD4$^+$ and CD8$^+$ T lymphocytes were occasionally identified in the neointima of allografts both in the FK409- and the vehicle-treated group (Table 2). In the media of allografts, residual VSMCs decreased in parallel to the accumulation of neointimal VSMCs. However, there was a larger $\alpha$-actin–positive area in the media of FK409-treated allografts compared with the vehicle-treated grafts (16.9 ± 8.1 versus 5.9 ± 8.4%; Figure 3). CD4$^+$ and CD8$^+$ T cells were occasionally observed in the media of allografts from both treatment groups (Table 2).

**BrdU Uptake in Neointima**

In the neointima of vehicle-treated allografts, a notable amount of DNA synthesis was observed, mostly in the basal

---

**TABLE 2. Immunohistochemical Stainings of Aortic Grafts**

<table>
<thead>
<tr>
<th>Layer Antibody</th>
<th>Isograft</th>
<th>Allograft</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>FK409*</td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
<td>(n=6)</td>
</tr>
<tr>
<td>Intima M-20 (Fas)</td>
<td>1.8±0.8</td>
<td>2.3±0.5</td>
</tr>
<tr>
<td>Q-20 (FasL)</td>
<td>1.5±0.5</td>
<td>1.3±0.5</td>
</tr>
<tr>
<td>ED1 (Mo/Mφ)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OX-8 (CD8$^+$ T cells)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OX-35 (CD4$^+$ T cells)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Media M-20 (Fas)</td>
<td>1.2±0.4</td>
<td>1.3±0.5</td>
</tr>
<tr>
<td>O-20 (FasL)</td>
<td>1.5±0.5</td>
<td>1.3±0.5</td>
</tr>
<tr>
<td>ED1 (Mo/Mφ)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OX-8 (CD8$^+$ T cells)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OX-35 (CD4$^+$ T cells)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adventitia M-20 (Fas)</td>
<td>2.5±0.5</td>
<td>2.7±0.5</td>
</tr>
<tr>
<td>Q-20 (FasL)</td>
<td>1.8±0.8</td>
<td>2.0±0.6</td>
</tr>
<tr>
<td>ED1 (Mo/Mφ)</td>
<td>0.7±0.5</td>
<td>0.3±0.5</td>
</tr>
<tr>
<td>OX-8 (CD8$^+$ T cells)</td>
<td>0.3±0.5</td>
<td>0.8±0.4</td>
</tr>
<tr>
<td>OX-35 (CD4$^+$ T cells)</td>
<td>0.2±0.4</td>
<td>0.3±0.5</td>
</tr>
</tbody>
</table>

All values are mean±SD.
*10 mg/kg FK409.
†$P<0.05$ vs vehicle-treated allografts.
‡$P<0.05$ vs FK409 or vehicle-treated isografts.
FK409 treatment significantly decreased the BrdU labeling index in the neointima when compared with vehicle treatment (12.9 ± 6.6% versus 21.6 ± 7.6%, *P* = 0.02; Figure 4D). A combination of immunohistochemical staining and BrdU labeling suggested that most of the proliferating cells in the neointima were VSMCs (Figure 5C).

**Apoptosis of VSMCs**

In the neointima, Fas protein expression in FK409-treated allografts was significantly increased when compared with that in the vehicle-treated control allografts (Table 2). The apoptotic index in the neointima was also higher in FK409-treated allografts than in control allografts (15.7 ± 5.5 versus 9.7 ± 6.1%, *P* = 0.04; Figure 6A). There was a weak correlation between the apoptotic index and the degree of Fas expression in the neointima (*r* = 0.5, *P* = 0.03) and in the media (*r* = 0.6, *P* = 0.03; Figures 6C and 6D). Double labeling with TUNEL and anti-Fas antibody demonstrated that TUNEL-positive nuclei were mostly located in the area of Fas expression (Figure 5B). The principal apoptotic cell population in the neointima was composed of VSMCs, as...
shown by double staining with TUNEL and anti-α-actin antibody (Figure 5A). TEM confirmed that the majority of VSMCs in the neointima of FK409–treated allografts underwent apoptosis, characterized by nuclear lobulation or fragmentation, margination and condensation of chromatin, cytoplasmic condensation, and membrane budding (Figure 5D). There were no significant differences in intimal FasL expression detected by immunohistochemistry between FK409- and vehicle-treated allografts (Table 2). Similarly, no significant differences were observed in Fas expression and apoptotic index between the residual VSMCs in vehicle- and FK409-treated allografts in the media (Table 2, Figure 6B).

Discussion
This study provides the first evidence that FK409 attenuates the development of allograft vasculopathy in the rat aortic allograft model. The reduction in neointimal thickness was dose-dependent, with significant reduction (40%) in neointimal hyperplasia observed at 1 mg/kg (thrice per day). The effect of FK409 was maximal at a dose of 10 mg/kg (thrice per day), which resulted in a 76% reduction in neointimal thickness (Table 1). Furthermore, FK409 treatment inhibited VSMC accumulation and induced apoptosis of VSMCs, which resulted in a decreased number in the neointima.

FK409, a spontaneous NO releaser, is a compound isolated from the fermentation broth of Streptomyces griseopurpureus.21 FK409 has been shown to inhibit platelet aggregation and noradrenaline-induced contraction of rat aorta in vitro.21 Isono et al28 reported that the vasorelaxant effect of FK409 was due to the activation of soluble guanylyl cyclase and increase in cGMP level. Subsequently, other investigators demonstrated that the NO, released spontaneously from the compound itself, was responsible for the antiplatelet and vasorelaxant effects of FK409.23,29

NO, a short-lived, highly diffusible free radical, is synthesized endogenously by a family of NO synthase enzymes that catalyze the oxidation of 1 of the 2 chemically equivalent guanidino nitrogens of l-arginine. NO is critical to the maintenance of vascular homeostasis. In the blood vessel, NO is produced in the endothelium by a constitutively expressed endothelial isoform of NO synthase (eNOS). NO has important vasodilatory and antithrombotic properties.30 In addition, NO is a potent inhibitor of VSMC proliferation.17,19,31–33

Although systemic treatment (by continuous infusion) with organic NO donors has been shown to attenuate the development of intimal hyperplasia in the balloon injury model,20,31 this form of therapy has never been utilized in the treatment of allograft vasculopathy. The present data suggest that an orally active NO donor may be a treatment of choice to prevent the development of neointimal hyperplasia in allograft vasculopathy. Our data are in agreement with those of Seki et al.19 These investigators showed that FK409 at a dose of 10 mg/kg twice per day caused a 48% reduction in the neointimal thickness in balloon-injured rat carotidots, whereas ISDN at a dose of 200 mg · kg⁻¹ · d⁻¹ failed to improve the lesions. FK409 has been developed as an antianginal drug in Japan, and the clinical dose that is recommended is ≈2 mg · kg⁻¹ · d⁻¹.34 Our findings that a low dose of FK409 (1 mg/kg, thrice per day) did not cause significant hypotension (Figure 1) but resulted in a significant attenuation (40% reduction in thickness) in neointimal hyperplasia suggest that this compound may have promising clinical application.

Similar to the findings in the balloon-injured rat carotid artery,19 we also found that FK409 treatment reduced DNA synthesis in neointimal VSMCs by BrdU uptake assay. Because it has been established that NO can limit DNA synthesis of VSMCs,17,19 a direct effect through the inhibition of VSMC proliferation is likely, as suggested by the in vitro study of FK409 by Seki et al.19 However, it is still possible that decreased DNA synthesis of neointimal cells merely reflects the decreased number of migrating VSMCs into neointima.

Finally, there was an increase in the apoptotic activity and an upregulation of Fas protein expression in neointimal VSMCs of the FK409-treated grafts. However, contrary to the increase in apoptosis observed in the neointimal VSMCs, there were no significant differences in the apoptosis rates of medial VSMCs between vehicle- and FK409-treated allografts. These findings suggest that the neointimal VSMCs, which are of the synthetic phenotype, are more sensitive to NO-mediated apoptosis than the medial VSMCs. This observation is in agreement with the finding by Bennet et al.35 who demonstrated that human VSMCs cultured from atherosclerotic plaques were more susceptible to p53-mediated apoptosis than those from the media of normal coronary artery.

Although the rat aortic allograft model has been widely used as a model of allograft vasculopathy, and the histochemical changes seen in this model are comparable with those observed clinically,24,36 this model has several limitations. First, the aortic graft is not truly a solid organ. It is not surrounded by the parenchyma of a solid organ and therefore is not affected by a cytokine milieu generated by immune and nonimmune injuries to the parenchyma of the organ. Another limitation is related to the use of immunosuppression. Commonly used immunosuppressive agents such as cyclosporine A (CsA) and tacrolimus have been shown to modulate the degree of neointimal hyperplasia in this model of allograft vasculopathy.18,24,37–40 CsA also inhibits the induction of FasL expression,41 which indicates that CsA might prevent the apoptotic process in neointimal formation. Because this model requires no immunosuppression, the effect of immunosuppressive agents on the development of allograft vasculopathy cannot be assessed.

In summary, we have demonstrated for the first time that FK409, a spontaneous NO donor, attenuates the development of allograft vasculopathy in the rat aortic allograft model. FK409 suppresses DNA synthesis, upregulates Fas protein expression, and increases apoptosis, of the VSMCs in the neointima. One of the major side effects of FK409 is the transient hypotension developed shortly after oral administration of a dose higher than 1 mg/kg in rat. Other spontaneous NO releasers of the same family that have fewer side effects may show promise in clinical applications.

Acknowledgments
This work was supported in part by NIH grant R01 HL63426–01 (to S.M.P.). We thank Carlos Fajardo for his invaluable technical support.
References
FK409, a Spontaneous Nitric Oxide Releaser, Attenuates Allograft Vasculopathy in a Rat Aortic Transplant Model
Johji Fukada, Stefano Schena, Ivan Tack, Phillip Ruiz, Yoshihiko Kurimoto, Manhui Pang, Abdelouahab Aitouche, Tomio Abe, Liliane J. Striker and Si M. Pham

Circ Res. 2000;87:66-72
doi: 10.1161/01.RES.87.1.66

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/87/1/66