Pioglitazone prevents intimal hyperplasia in experimental rabbit vein grafts

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Background: Intimal hyperplasia is a major obstacle to patency after vein grafting. Several clinical trials revealed that pioglitazone, a peroxisome proliferator-activated receptor-γ ligand, exerts beneficial actions on cardiovascular complications. We investigated whether pioglitazone modulates intimal hyperplasia in experimental rabbit autologous vein grafts. Methods: Male Japanese White rabbits were randomly divided into two groups: one group received pioglitazone as food admixture at a concentration of 0.01%, and the other did not (control). One week later, each group underwent reversed autologous vein bypass grafting of the right common carotid artery using ipsilateral external jugular vein. Pioglitazone therapy was continued after surgery and until harvest. Intimal hyperplasia of the graft vein was assessed at 28 days. Two weeks after implantation, proliferative cells in the neointima were identified by immunohistochemical staining with Ki-67 monoclonal antibody. To determine apoptotic cells, we performed terminal deoxynucleotidyl transferase-mediated deoxyuride-5′-triphosphate nick-end labeling (TUNEL) staining. Blood samples were collected at 28 days after implantation for measuring metabolic parameters such as plasma glucose and total cholesterol. Adiponectin levels were determined by Western blot analysis. Finally, we assessed adiponectin-related signaling pathway, 5′ adenosine monophosphate-activated protein kinase (AMPK), and extracellular signal-regulated kinase (ERK) in the grafted vein by Western blot analysis. Results: Treatment with pioglitazone markedly inhibited intimal hyperplasia of carotid interposition-reversed jugular vein grafts in the pioglitazone group (0.54 ± 0.04 mm2) vs control (0.93 ± 0.04 mm2; n = 7; P < .01). Pioglitazone treatment reduced the number of Ki-67-positive proliferating cells in the neointima of the vein grafts at 14 days after implantation in the pioglitazone group (4.1% ± 1.1%) vs the controls (16.8% ± 1.7%; P < .05). The frequency of TUNEL-positive apoptotic cells was enhanced by pioglitazone (3.5% ± 0.5%) vs the controls (1.2% ± 0.1%; P < .05). Pioglitazone treatment also increased plasma levels of adiponectin, a vascular protective hormone, and led to an increase in phosphorylation of AMPK and a decrease in phosphorylation of ERK in the grafted vein. Conclusions: Pioglitazone attenuates intimal hyperplasia of the vein graft after autologous bypass grafting by its ability to suppress cell proliferation and enhance apoptosis. Pioglitazone could represent a therapeutic target for the prevention of graft failure after bypass grafting. (J Vasc Surg 2011;54:1753-9.)

Clinical Relevance: Intimal hyperplasia is a major obstacle to patency after vein grafting. Various treatments to reduce neointimal hyperplasia have been examined; however, a standard clinical treatment has not yet been established. We report that pioglitazone, a peroxisome proliferator-activated receptor-γ ligand, inhibits intimal hyperplasia of autologous vein grafts. Pioglitazone also increased adiponectin, an adipose-derived hormone. Obesity-related complications, such as diabetes, are closely associated with autologous vein graft stenosis after bypass surgery. The findings reported here suggest that pharmacologic approaches aimed at increasing adiponectin production, such as pioglitazone, can contribute to the prevention of autologous vein graft stenosis in obese individuals.
Consistent with these clinical observations, a number of experimental studies show that pioglitazone attenuates neointimal thickening in response to vascular injury \(^8,9,10\) and suppresses growth factor-stimulated proliferation and migration of vascular smooth muscle cells (SMCs). \(^11,12\) These findings indicate that in addition to its glucose-lowering effect, pioglitazone acts as a biologically relevant modulator of vascular remodeling. We investigated the effect of the systemic administration of pioglitazone on autologous vein grafts using an experimental rabbit vein graft model.

**MATERIALS AND METHODS**

Antibodies to 5′ adenosine monophosphate-activated protein kinase (AMPK) \(a_1\), phosphorylated AMPK \(a_1\), and phosphorylated extracellular signal-regulated kinase (ERK) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Total ERK antibody was purchased from Cell Signaling (Beverly, Mass). Adiponectin antibody for detecting all forms of adiponectin was purchased from R&D Systems (Minneapolis, Minn). Pioglitazone was a gift from Takeda Pharmaceutical Company Limited (Tokyo, Japan).

**Experimental protocol.** The study protocol was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. The study also conformed with the requirements in *Guiding Principles for the Care and Use of Laboratory Animals* (National Institutes of Health publication 80-23, revised 1985). Male Japanese White rabbits (2.5-3.0 kg) were randomly divided into two groups: one group was treated with pioglitazone, and one group was not (controls). Treatment with pioglitazone was initiated 1 week before surgery and continued for 4 weeks as a food admixture (3 mg/kg/d), consistent with previous reports. \(^13,14\)

**Vein graft implantation.** Rabbits underwent reversed autologous vein bypass grafting of the right common carotid artery using the external jugular vein, as described previously. \(^15-17\) In brief, anesthesia was induced intramuscularly with ketamine hydrochloride (10 mg/kg) and xylazine (10 mg/kg). The right jugular vein and the right common carotid artery were exposed, and the branches of the jugular vein were ligated. Approximately 2 cm of the vein was taken for being implanted. In brief, the graft was isolated and harvested after systemic heparinization (200 IU/kg intravenously), and the rabbits were euthanized by an intravenous overdose of pentobarbital (50 mg/kg).

The harvested graft was fixed 4% formaldehyde at 100 mm Hg for 30 minutes. The perfused vein graft was immersed in the same fixative overnight at room temperature. Four sections were obtained from each vein graft. Each sample was embedded in paraffin and cut into 5-μm sections.

**Measurement of metabolic parameters.** Blood samples were collected from rabbits after an overnight fast at 28 days after implantation. Plasma glucose, total cholesterol, triglyceride, and low-density and high-density lipoprotein

**Assessment of intimal hyperplasia.** Intimal hyperplasia was assessed in the harvested vein grafts in each group \((n = 7\), respectively). Four sections were obtained from each vein graft and processed as described in the previous section. Each section was deparaffinized in a xylene/ethanol series and stained with hematoxylin and cosin or with elasta van Gieson. The intimal area, medial area, and total lumen area were measured by MACSCOPE (Mitani Co, Fukui, Japan) for each section. The average of the four sections was considered to represent the intimal hyperplasia of the vein graft. These values were used for statistical analysis.

**Immunohistochemical staining.** Vein grafts \((n = 4\), in each group) were harvested 14 days after implantation, processed as described previously, and then deparaffinized in a xylene/ethanol series. Proliferative cells in the neointima were identified by immunohistochemical staining with Ki-67 monoclonal antibody (DAKO Cytomation Inc, Carpenteria, Calif). To determine apoptotic cells, we also performed terminal deoxynucleotidyl transferase-mediated deoxyuridine-5′-triphosphate nick-end labeling (TUNEL) staining (Roche, Mannheim, Germany), as previously described. \(^16,17\) The total, Ki-67-positive, and TUNEL-positive nucleated cells in the neointima were counted (original magnification of ×400) by three observers who were blinded to the groups. The values obtained by the three observers were averaged in each section. The quantitative analysis was performed in eight independent sections in each rabbit. The number of Ki-67-positive and TUNEL-positive cells/total number of cells counted was defined as the Ki-67 index and TUNEL index of the vein graft.

**Western blot analysis.** The harvested vein samples obtained at 14 days after implantation were excised and washed with ice-cold phosphate-buffered saline, cut into 3-mm-wide strips, and immediately frozen in dry ice/acetone. Frozen strips were homogenized in ice-cold lysis buffer containing 20 mM Tris-HCl (hydrochloride) (pH 8.0), 1% NP-40, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma, St. Louis, Mo). Identical amounts of protein were separated with denaturing sodium dodecylsulfate 10% polyacrylamide gels. The membranes were immunoblotted with the primary antibodies at a 1:1000 dilution, followed by secondary antibody at a 1:5000 dilution. Bands were visualized using an enhanced chemiluminescence Western Blotting Detection Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Western blot analysis was examined among control, treatment, and sham-operated rabbits.

**Harvest of implanted vein grafts.** The autologous vein grafts were harvested under general anesthesia 4 weeks after being implanted. In brief, the graft was isolated and harvested after systemic heparinization (200 IU/kg intravenously), and the rabbits were euthanized by an intravenous overdose of pentobarbital (50 mg/kg).
Table I. Metabolic parameters in rabbits treated with and without pioglitazone

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (mg/dL)</th>
<th>Pioglitazone (mg/dL)</th>
<th>P</th>
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<tbody>
<tr>
<td>Plasma glucose</td>
<td>214 ± 19</td>
<td>196 ± 19</td>
<td>.50</td>
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<tr>
<td>Triglyceride</td>
<td>37.4 ± 6.3</td>
<td>24.9 ± 4.3</td>
<td>.13</td>
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<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>26.0 ± 3.7</td>
<td>30.4 ± 3.5</td>
<td>.40</td>
</tr>
<tr>
<td>LDL</td>
<td>5.9 ± 1.2</td>
<td>6.9 ± 1.7</td>
<td>.64</td>
</tr>
<tr>
<td>HDL</td>
<td>14.6 ± 2.2</td>
<td>20.3 ± 2.3</td>
<td>.09</td>
</tr>
</tbody>
</table>

LDL, Low-density lipoprotein; HDL, high-density lipoprotein.

*a Blood samples were collected from rabbits treated with or without pioglitazone (control) for 28 days after surgery.

*b Data are shown as mean ± standard error of the mean.

cholersterol levels were measured with enzymatic kits (Wako Chemicals, Shiga, Japan). Adiponectin levels were determined by Western blot analysis. After centrifugation, the supernatant (0.7 μL) was diluted into nonreducing sample buffer and separated with denaturing sodium dodecylsulfate 10% polyacrylamide gels, as described previously.18

Sample size calculation and statistical analysis. We assumed a mean difference of 30 μm with a standard deviation of 10 μm, referring to a previous report.16 The study was designed with 5% level of significance (α = 0.05) and 90% power to reject the null hypothesis of equivalence between the two treatment groups. To achieve this objective, five rabbits were required for each group. Considering that some of the grafted veins would be occluded, we examined intimal hyperplasia of grafted veins of seven in each group. StatView 5.0 software (SAS Institute, Cary, NC) was used for statistical calculations. Data are presented as means ± standard error of the mean. All of the data were subjected to one-way analysis of variance (ANOVA), followed by Scheffé analysis for comparison between any two means. Statistical significance was also evaluated using ANOVA for comparison among the three groups (control, treatment, and sham operation groups). A value of P < .05 was considered significant.

RESULTS

Effect of pioglitazone on intimal hyperplasia of the vein graft. All rabbits survived the implantation procedure and appeared healthy during the follow-up period. All of the vein grafts were patent at harvest. Pioglitazone treatment had no effects on heart rate, blood pressure, or body weight during the observation period. There were no statistically significant differences between rabbits treated with pioglitazone for 28 days and the controls in levels of plasma glucose, total cholesterol, triglycerides, and low-density and high-density lipoprotein cholesterol (Table I).

To assess the effect of pioglitazone on intimal hyperplasia of vein grafts, rabbits underwent reversed autologous vein graft surgery with the external jugular vein into the common carotid artery. Intimal hyperplasia developed in the grafted vein at 4 weeks after implantation in both groups (n = 7, respectively; Fig 1). The degree of intima area in the pioglitazone-treated rabbits at 2 and 4 weeks after implantation was markedly suppressed compared with the untreated controls (P < .01; Table II). The intima/media index at 2 and 4 weeks after implantation was also significantly smaller in the pioglitazone-treated rabbits than in the untreated rabbits (P < .01) (Table II). The differences in medial area and total lumen area were not significant (Table II).

Effect of pioglitazone on cell proliferation and apoptosis in the vein graft. We next assessed the effect of pioglitazone on cell proliferation and apoptosis at day 14 after implantation, because maximum numbers of Ki-67-positive proliferating or TUNEL-positive apoptotic cells were observed.16,17 In the neointimal lesions, pioglitazone treatment significantly reduced the frequency of Ki-67-positive cells by 53% compared with non-treatment (Fig 2). In contrast, pioglitazone treatment increased the number of TUNEL-positive cells in the neointimal lesions by a factor of 2.2 compared with non-treatment (Fig 3).

Effect of pioglitazone on circulating adiponectin levels. Plasma adiponectin levels in each group were assessed by Western blot analysis. The vein graft surgery did not affect adiponectin protein levels in the plasma. Treatment with pioglitazone resulted in a 1.8-fold increase in the plasma adiponectin level independent of the vein graft surgery (Fig 4).
Effects of pioglitazone on AMPK and ERK activities in the vein graft. Phosphorylation of AMPK and ERK in the grafted vein was assessed by Western blot analysis because adiponectin modulates vascular remodeling by directly affecting these signaling pathways in vascular cells. The level of AMPK phosphorylation in the grafted vein was significantly higher than that in sham-operated jugular vein. The magnitude of this induction was greater in the pioglitazone-treated rabbits than in the untreated rabbits (Fig 5, A). The phosphorylation of ERK in the grafted vein was markedly increased by the vein graft surgery, but this induction was significantly less in the pioglitazone-treated rabbits than in the untreated rabbits (Fig 5, B).

DISCUSSION

The present study provides evidence that pioglitazone can suppress the intimal hyperplasia of vein grafts implanted in the arterial circulation. Treatment of rabbits with pioglitazone attenuated intimal thickening of carotid interposition-reversed jugular vein grafts through suppression of cell proliferation and induction of apoptosis.

A major process that leads to neointima formation in arterialized vein grafts is the SMC accumulation in the neointima, which is the sum of cell migration, cell proliferation, and apoptotic cell loss.19-21 In our experimental rabbit vein graft model, SMC replication and apoptosis were transiently and coincidentally observed after implantation in the neointima with a peak at day 14, declining to lower levels at day 28, consistent with previous studies.16,17 Treatment with pioglitazone significantly decreased cell proliferation and increased apoptosis in the neointima at day 14 after implantation. Accordingly, inhibition of neointima formation by pioglitazone appears to be attributable to suppression of SMC proliferation and induction of SMC apoptosis.

The beneficial actions of pioglitazone on vascular remodeling are likely to be at least partly due to its ability to activate AMPK signaling. Activation of AMPK was reported to attenuate angiotensin II-stimulated ERK phosphorylation and growth of SMCs.22,23 The AMPK activator, 5-aminoimidazole-4-carboxamide ribonucleoside, also suppressed neointimal formation in a wire injury model of the rat femoral artery when administered by subcutaneous injection for 14 days.22 In the present study, pioglitazone treatment promoted AMPK activation and attenuated ERK activity in the grafted vein after implantation.

Table II. Morphometric analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 4)</td>
<td>Pioglitazone (n = 4)</td>
</tr>
<tr>
<td>Intima area, mm²</td>
<td>0.66 ± 0.04</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>Media area, mm²</td>
<td>0.91 ± 0.05</td>
<td>0.99 ± 0.04</td>
</tr>
<tr>
<td>Lumen area, mm²</td>
<td>11.5 ± 0.65</td>
<td>12.36 ± 0.67</td>
</tr>
<tr>
<td>Intima/media index</td>
<td>0.730 ± 0.002</td>
<td>0.459 ± 0.001</td>
</tr>
</tbody>
</table>

*The degree of intima area in the pioglitazone-treated rabbits at 2 and 4 weeks after implantation was markedly suppressed compared with untreated control rabbits (P < .01). The intima/media index at 2 and 4 weeks after implantation was also significantly smaller in the pioglitazone-treated rabbits than in the untreated rabbits (P < .01). There were no significant differences in medial area and total lumen area.

bData are shown as mean ± standard error of the mean.
Pioglitazone increased the expression and secretion of adiponectin by its ability to activate PPARγ in adipocytes.24,25 Adiponectin is downregulated in obesity-linked diseases, including ischemic heart disease and peripheral arterial disease.26,27 Adiponectin exerts protective actions on a variety of metabolic and cardiovascular disorders, including insulin resistance,28 atherosclerosis,29,30 vascular dysfunction,31 angiogenesis,32 and cardiac injury, partly through activating AMPK signaling in target cells.33,34 We and other groups have recently shown that the protective effects of pioglitazone on cardiovascular systems largely depend on its ability to increase adiponectin production.14 In addition, pioglitazone treatment led to increased AMPK activation and reduced ERK phosphorylation in the heart, which largely depend on its ability to enhance adiponectin levels.14 Lack of adiponectin causes increased intimal hyperplasia in response to vascular injury in mice.30 Conversely, overexpression of adiponectin reduces atherosclerotic lesions in a mouse model of atherosclerosis.29

We observed here that pioglitazone significantly increased circulating adiponectin levels in rabbits and also attenuated ERK activation and stimulated AMPK phosphorylation in the grafted vein. These data suggest that the inhibition of neointima formation by pioglitazone is attributed to modulation of ERK and AMPK signaling pathways by adiponectin and the prevention of autologous vein graft stenosis.

Obesity-related complications are closely associated with autologous vein graft stenosis after bypass surgery.35,36 The findings reported here suggest that elevated adiponectin can contribute to the prevention of the autologous vein graft stenosis in obese individuals. Pharmacologic approaches aimed at increasing adiponectin production, such as pioglitazone treatment, could be useful for prevention of late graft failure after bypass grafting.

The oral administration of pioglitazone caused no adverse effects in this study. Pioglitazone is widely used as an insulin sensitizer in the treatment of type 2 diabetes.
mellitus. Therefore, we believe the use of pioglitazone as a therapeutic approach could be safe and beneficial for the treatment of cardiovascular disease in humans.

The present study has several limitations. First, we did not measure PPARγ activities and pioglitazone concentrations. In the present study, rabbits were fed an average of ~80 grams of food per day; therefore, the daily dosage of pioglitazone was about 3 mg/kg/d. This dose was determined from the recent report by Shiomi et al. demonstrating that the dose of pioglitazone for treatment of mice and rabbits is 3 mg/kg, which is similar to the dosage of oral administration in humans. Under these conditions, we confirmed the effect of pioglitazone on messenger RNA levels of PPAR-regulated genes, aP2 in the grafted vein, by using real-time polymerase chain reaction. Pioglitazone upregulated aP2 transcript levels in the grafted vein compared with controls (data not shown). A recent report indicated that the genetic ablation of adiponectin did not affect the pioglitazone-induced expression of other PPAR-responsive genes, including aP2, in various tissue. Collectively, pioglitazone might exert both direct and indirect actions on the grafted vein.

Second, we did not assess the effect of pioglitazone on reendothelialization after bypass grafting. Treatment with pioglitazone was reported to accelerate reendothelialization after vascular injury in rabbits. Thus, pioglitazone might affect endothelial cells after autologous bypass grafting.

CONCLUSIONS

Our present study clearly demonstrates that the long-term administration of pioglitazone induces suppression of intimal hyperplasia development in experimental autologous vein grafts by its ability to suppress cell proliferation and enhance apoptosis. This novel finding may facilitate the clinical use of PPARγ agonists, such as pioglitazone, in postoperative patients to prevent late graft failure after bypass grafting.

We gratefully acknowledge the technical assistance of Megumi Kondo and Rie Miura.

AUTHOR CONTRIBUTIONS

Conception and design: KM, RS, KK
Analysis and interpretation: KM, RS, NT, KK
Data collection: KM, RS, NT
Writing the article: KM, RS, NT
Critical revision of the article: KM, RS, NO, KK
Statistical analysis: KM, RS
Obtained funding: Not applicable
Overall responsibility: KK

REFERENCES


Submitted Feb 6, 2011; accepted Jun 23, 2011.