Cellular and Molecular Changes Associated With Inhibitory Effect of Pioglitazone on Neointimal Growth in Patients With Type 2 Diabetes After Zotarolimus-Eluting Stent Implantation

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Objective—To investigate the mechanistic basis underlying antirestenosis and the antiatherogenic effect of pioglitazone in patients with type 2 diabetes mellitus who were undergoing zotarolimus-eluting stent implantation.

Methods and Results—Recent studies highlight the beneficial effect of pioglitazone in attenuating neointimal growth after stent implantation. Patients with coronary artery diseases were randomly assigned to pioglitazone (n=47) or placebo (n=47) after stent implantation. Pioglitazone significantly reduced neointimal hyperplasia within the stented lesion and attenuated total plaque burden in the in-segment regions of the stent, as assessed by intravascular ultrasonography at the 8-month follow-up. These changes were preceded by reduced circulating natural killer (NK) cells, diminished interleukin 6 and monocyte chemoattractant protein-1 levels, and downregulation of chemokine receptor 2 at 2 days after stent implantation; and an elevated interleukin 10 level at 10 days after implantation. Furthermore, the proliferation and migration of vascular smooth muscle cells were inhibited in the presence of pioglitazone-treated patient serum, demonstrating that the antiproliferative effects of pioglitazone occurred concurrently with its antiinflammatory action.

Conclusion—Our data present early cellular and immunologic changes by pioglitazone that might have been associated with antirestenotic and antiatherogenic effects in diabetic patients. Inhibiting proinflammatory responses while promoting antiinflammatory circuits, together with an antiproliferative action, may, in part, account for the antirestenotic effect of pioglitazone by altering vascular remodeling processes in the early phase. (Arterioscler Thromb Vasc Biol. 2010;30:2655-2665.)

Key Words: pioglitazone ■ restenosis ■ diabetes ■ inflammation ■ zotarolimus-eluting stent

In-stent restenosis remains a significant clinical problem, especially in patients with type 2 diabetes mellitus.1,2 Diabetic patients have demonstrated poor prognosis in terms of long-term clinical and angiographic outcomes after percutaneous coronary intervention with bare-metal stents.3,4 With the introduction of drug-eluting stents, which contain methotrexate, rapamycin, or paclitaxel, a considerable reduction in the in-stent restenosis rates and target lesion revascularization have been achieved.5 Nevertheless, patients with type 2 diabetes continued to show increased rates of restenosis and late lumen loss compared with nondiabetic patients.6 Previous reports suggest that a combination of local and systemic pharmacological approaches could provide a synergistic inhibitory effect on restenosis.7 Thus, the development of pharmacological therapies is warranted to prevent restenosis in diabetic patients with coronary artery diseases (CADs).

Thus, recent studies underscore the beneficial effects of peroxisome proliferator–activated receptor (PPAR)γ agonists, thiazolidinedione and its derivatives, rosiglitazone, and pioglitazone, in the prevention of atherogenesis and in-stent restenosis.8 Consistent with these data, recent clinical trials9,10 have shown a positive effect of pioglitazone on the prevention of restenosis after coronary intervention, both in nondiabetic and diabetic patients. Although the antiinflammatory and antiproliferative functions of PPARγ agonists have accounted for a reduction in inflammatory changes and a proliferation of vascular cell lines in vitro, it remains unclear how pioglitazone controls neointimal proliferation in diabetic patients with CAD undergoing drug-eluting stent implantation.11,12 Therefore, we conducted a prospective, randomized, single-blinded, placebo-controlled trial to delineate the mechanisms underlying antirestenosis and the antiatherogenic effects of pioglitazone.

Methods

Study Patients
A randomized single-blinded clinical trial, in which the individual subjects were blinded, was performed on 298 patients, aged 40 to 75...
years, with type 2 diabetes and underlying CAD. Both investigators and clinicians were aware that particular patients had been prescribed either pioglitazone or placebo. All patients were Korean and recruited to the Cardiovascular Center, Korea University Anam Hospital, Seoul. Patients who did not fulfill the inclusion criteria (n = 52) or who had exclusion criteria (n = 152) were excluded. The inclusion criteria for this study were as follows: (1) patients with previously treated diabetes, (2) patients with a fasting plasma glucose level of 126 mg/dL or greater, and (3) patients with a plasma glucose level of 200 mg/dL or greater at 2 hours after a 75-g oral glucose load. The exclusion criteria for this study were as follows: (1) patients with liver or renal dysfunction, (2) patients with unsuccessful reperfusion after coronary stent implantation, and (3) patients with cardiogenic shock or congestive heart failure. Eligible patients (n = 94 [33 women and 61 men]) were randomly assigned prospectively to receive either pioglitazone, 30 mg/d (titrated over 4 weeks) (n = 47), or placebo (n = 47), in addition to standard diabetic management (Figure 1).

Aspirin and clopidogrel were maintained in all patients during the 8-month follow-up. Atorvastatin, 10 mg, was administered unless contraindicated (43 patients [91.5%] in the pioglitazone group versus 44 patients [93.6%] in the placebo group; P = 0.69). Hypertensive medications, such as angiotensin receptor blockers (16 patients [34.0%] versus 14 patients [29.8%]; P = 0.66), angiotensin converting enzyme inhibitors (8 patients [17.0%] versus 11 patients [23.4%]; P = 0.44), β-blockers (2 patients [4.3%] versus 1 patient [2.1%]; P > 0.99), calcium channel blockers (9 patients [19.1%] versus 8 patients [17.0%]; P = 0.79), and diuretics (3 patients [6.4%] versus 4 patients [8.5%]; P > 0.99), were similar between the pioglitazone and placebo groups, respectively, at enrollment. Oral glucose-lowering agents, such as biguanides, α-glucosidase inhibitors, and sulfonylureas, were given as needed.

A complete clinical workup was performed at 1, 4, and 8 months after the procedure. A follow-up angiogram was performed after 8 months of procedure. The primary end point of the study was to assess neo-intima volume and atherosclerosis progression on follow-up intravascular ultrasonography (IVUS). The study was approved by the institutional review board at Korea University Anam Hospital Cardiovascular Center.

**Stent Implantation, Angiographic Analysis, and IVUS Measurements**

All participating patients received a zotarolimus-eluting stent (ZES) (Endeavor Sprint; Medtronic Inc, Minneapolis, Minn) according to the study protocol. Procedural success was defined as residual stenosis of less than 15% in the absence of closure during the first 48 hours after the procedure. Coronary angiograms were obtained at baseline, immediately after stent implantation, and at the 8-month follow-up. All angiographic, IVUS, and clinical data were analyzed by individuals unaware of the patients’ treatment assignments. End-diastolic frames were chosen for quantitative analysis, which was performed using a computer-based transducer communication systems system, Version 2.02 (Medcon Inc, Tel-Aviv, Israel). The reference diameter, minimal luminal diameter, percentage of stenosis, and lesion length were calculated as the average value of the 2 orthogonal views. The same views and calibration were used at follow-up angiography. The average diameter of normal segments proximal and distal to the treated lesion was used as the reference diameter. Lesions were characterized according to the modified American College of Cardiology/American Heart Association classification. Restenosis was defined as stenosis of greater than 50% of the luminal diameter. The Mehran classification was used for the description of angiographic patterns of in-stent restenosis. Balloon angioplasty and stent implantation were performed according to the standard clinical practice, as previously described. IVUS examination (GalaxyII 3.6F, 40 MHz; Boston Scientific SCIMED, Maple Grove, Minn) was performed after ZES implantation at baseline and 8 months. The neo-intima was calculated per millimeter of stent length for the stented segment and per millimeter of segment length for the proximal and distal 10-mm segment. Plaque volume was calculated by subtracting lumen volume from vessel volume, and neo-intima volume was calculated by subtracting lumen volume from stent volume, as previously described.

**Measurement of Metabolic Parameters and Inflammatory Biomarkers**

Venous blood samples were drawn from each patient after overnight fasting. Fresh blood samples were centrifuged to obtain serum and used immediately for the following measurements. The fasting plasma glucose level was measured by the glucose oxidase method with a biochemical analyzer (Tba 200FR/NEO; Toshiba Medical System Co, Tokyo, Japan). The fasting insulin level was measured by ELISA (ALPCO Diagnostics, Salem, NH). Glycosylated hemoglobin A1c was measured with an analyzer (G7 HPLC Analyzer; Tosoh Bioscience, Tokyo). Total cholesterol and triglyceride levels were measured with the enzymatic method using standard biochemical procedures on an automated clinical chemistry analyzer (model B.M. Hitachi; Hitachi, Tokyo). High-density lipoprotein (HDL) cholesterol levels were measured in plasma after the precipitation of low-density lipoprotein (LDL) and very-LDL. The LDL cholesterol concentrations were calculated with the following formula: LDL Cholesterol = Total Cholesterol − (HDL Cholesterol + Triglyceride).

The high-sensitivity C-reactive protein (hsCRP) concentrations were quantified using a latex nephelometer (model II: Dade Behring Inc, Newark, Del), and adiponectin concentration was assessed by radioimmunoassay (Linco Research, Inc, St Charles, Mo).

**Analysis of Lymphocyte, Monocyte, and Granulocyte Populations in Peripheral Blood Mononuclear Cells of Patients**

Blood samples were obtained and collected into test tubes containing heparin before stenting at baseline and at days 2, 10, and 240 after stenting. The numbers of lymphocytes, monocytes, and neutrophils in peripheral blood mononuclear cells (PBMCs) were measured using automated hematology analyzers (Coulter LH 755; Beckman Coulter Inc, Fullerton, Calif). Erythrocytes were lysed by incubating in 1 milliliter of lysing solution (FACS; Becton Dickinson Pharmingen, San Diego, Calif), and leukocytes in pellet were subjected to flow cytometric analysis (Becton Dickinson Bioscience, San Diego, Calif). The lymphocyte subsets analyzed were as follows: T cells (CD3+CD4+ or CD3+CD8+ cells), B cells (CD3-CD20+), and NK cells (CD3-CD56+). Cells were stained with fluorochrome-conjugated monoclonal antibodies against CD20 (2H7), CD56 (MEM188), CD8 (OKT8), CD14 (MEM14), CD3 (SK7), CD4 (555346), and C-C chemokine receptor 1 (CCR2) (558406); and their expression levels were analyzed by flow cytometer and using computer software (CellQuest Pro software; Becton Dickinson Bioscience, San Diego, Calif).
Flow Cytometric Detection of Cytokines and Chemokine

Interleukin (IL) 6, IL-10, tumor necrosis factor (TNF) α, and monocyte chemoattractant protein-1 (MCP-1) in each serum sample were measured using cytometric bead array (CBA) kits (Becton Dickinson PharMingen) and software (BD Cytometric Bead Array Software; Becton Dickinson Bioscience).

Proliferation and Migration Assay of Smooth Muscle Cells

Human aortic smooth muscle cells (SMCs) (Cascade Biologics, Portland, Ore) were cultured and maintained in medium 231 (Cascade Biologics, Portland, Ore) with smooth muscle growth supplement (Cascade Biologics, PO). Before the assay, SMCs were seeded onto a 24-well plate at 1×10^5 cells per well. Twenty-four hours later, 500 μL of patient serum was obtained at day 0, 2, 10, and 240; or the indicated concentration of pioglitazone (Jeil-Pharm Co LTD, Seoul, Korea) was added to each well. To decrease experimental errors that might have occurred from running multiple experiments at different days, patient serum samples were stored at −80°C before the analysis. Proliferation was measured at 2 days after the addition of thiazoyl blue tetrazolium bromide, as previously described (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay).29 For the migration assay, SMCs were seeded onto a 24-well plate at 1×10^5 cells per well 24 hours before the experiment. On the day of the experiment, subconfluent SMC monolayers were scratched with sterile Cell Scraper (Costar Corning, New York, NY) and cultured in the presence or absence of patient serum. Cells were allowed to migrate onto the plastic surface for 10 hours, and photographs were taken using an inverted microscope (model CKX41-DP20; Olympus, Tokyo).

Statistical Analysis

Data are expressed as mean±SD for continuous variables, and data are expressed as number (percentage) of patients for categorical variables. The Fisher exact test or a χ² test was used for categorical variables. The change from baseline was calculated as the value obtained at the end of treatment subtracted from the value obtained at the beginning of the intervention. The results between 2 groups were compared by an unpaired t test, and the comparisons between before and after treatment were analyzed by a paired t test. Angiographic analyses were performed according to the number of patients available for each analysis. Multivariate nonparametric methods for Mann-Whitney statistics were performed to investigate the possible contribution of multiple factors (ie, sex, age, total cholesterol, triglyceride, HDL, LDL, hsCRP, and number of white blood cells to the antirestenosis effect of pioglitazone. SPSS software, version 10.0 (SPSS Inc, Chicago, Ill), was used for analyses.

Results

Clinical and Angiographic Data

Ninety-four diabetic patients after coronary ZES implantation were randomly assigned to receive either placebo or pioglitazone, 30 mg/d, in addition to standard therapy (Figure 1). As can be seen in Table 1, no significant difference was found in the baseline characteristics between the 2 groups. Risk factors, such as hypertension, dyslipidemia, and current smoking, were similar in both groups. The use of various hypoglycemic medications at baseline and after randomization did not show significant differences between the 2 groups. The ratio of patients who took medications for hypertension and hypercholesterolemia was comparable between the pioglitazone and the placebo groups after stent implantation. Similarly, no significant difference was found in the angiographic lesion morphology and target vessels (Table 2). Reference diameter, minimal lumen diameter, and percentage of stenosis were similar between the 2 groups at baseline and after the procedure. The 8-month angiographic follow-up showed no significant changes in the reference diameter between the 2 groups. However, the lumen diameter was larger, suggesting lower late lumen loss in the pioglitazone group versus the placebo group (0.41±0.40 versus 0.65±0.54 mm; P=0.04). As a consequence, the resulting stenosis was lower in the pioglitazone group versus the placebo group (20±14 versus 28±17; P=0.02). These data demonstrate a beneficial role of pioglitazone in preventing late lumen loss and protection from restenosis after ZES implantation in patients with type 2 diabetes.

Metabolic Parameters and IVUS Measurements at Baseline and the 8-Month Follow-Up

Next, we investigated the effect of pioglitazone on the patient’s metabolic parameters and the degree of in-stent
and placebo groups. Nonetheless, patients receiving pioglitazone demonstrated an additional decrease of triglyceride concentration and increased HDL without changing the concentration of LDL and total cholesterol, when compared with the placebo group. Furthermore, pioglitazone versus placebo significantly increased the serum levels of adiponectin (7.7±3.9 versus 6.4±3.3 µg/mL; *P*<0.05) without affecting the levels of hsCRP. Although the level of hsCRP in the pioglitazone group at the 8-month follow-up was significantly decreased from baseline (1.8±2.9% versus 3.5±2.2%, a 51.4% reduction), a similar level of reduction was also apparent in the control group (1.2±1.4% versus 2.8±3.0%, a 53.6% reduction). The difference between the pioglitazone and placebo groups was not statistically significant (*P*>0.05). These data suggest that pioglitazone improves patients’ multiple metabolic parameters in addition to that observed from ZES implantation in diabetic patients with CAD.

Furthermore, a significantly lower neointima volume was detected within the stented segment compared with the placebo group at 8 months (1.3±0.7 versus 2.5±1.4 mm³/mm, as measured by IVUS; *P*<0.001) (Figure 2). Measurement of proximal and distal 10-mm segments of the stent revealed that patients receiving pioglitazone showed less plaque volume in the adjacent segments compared with that in the placebo group (7.2±3.2 versus 8.6±3.2 for the proximal segment, and 5.0±2.6 versus 5.8±3.7 for the distal segment). Probability values calculated from multivariate nonparametric methods for Mann-Whitney statistics demonstrated that none of the variables measured in the placebo and pioglitazone groups at baseline appeared to significantly modulate the antirestenotic effect of pioglitazone: sex (*P*=0.24), age (*P*=0.87), total cholesterol (*P*=0.72), triglyceride (*P*=0.72), HDL (*P*=0.86), LDL (*P*=0.58), hsCRP (*P*=0.27), and number of white blood cells (*P*=0.94). Therefore, the antirestenotic effect of pioglitazone appeared to be because of its intrinsic antiatherogenic property, independent of other individual factors.

### Comparison of the Cellular and Immunologic Profiles Between the Pioglitazone and Placebo Groups

The antirestenosis effects seen in the pioglitazone group might have been attributed to alterations of the cellular and/or immunologic profile, leading to early inflammatory changes and consequently affecting the proliferation of vascular SMCs within the stented area in the patients. To test this hypothesis, we monitored the blood leukocyte populations before and after pioglitazone administration after stent implantation (Figure 3A). The number of circulating neutrophils, monocytes, and lymphocytes was similar immediately after pioglitazone administration (day 0); however, the lymphocyte number decreased within 48 hours of pioglitazone administration (2320±415 cells/µL for the pioglitazone group versus 2741±493 cells/µL for the placebo group; *P*=0.02). This decrease was transient and returned to normal by 10 days after pioglitazone administration. In contrast, the number of monocytes and neutrophils stayed constant for up...
To identify the lymphocyte populations responsible for the pioglitazone-induced reduction in the peripheral blood compartment, we performed flow cytometric analysis to detect population changes of T, B, and NK cells within PBMCs. The absolute number of these lymphocytes in the patients was calculated by multiplying the percentage of each population obtained from flow cytometric analysis by the number of lymphocytes obtained on a given day (Figure 3B). As can be seen in Figure 3B, the total number of CD4$^+$ T, CD8$^+$ T, or CD20$^+$ B cells did not undergo drastic changes during pioglitazone administration compared with the control group. However, circulating NK cell numbers were significantly reduced by up to 42% within 48 hours after pioglitazone administration (326 ± 100 cells/µL in the pioglitazone group versus 563 ± 134 cells/µL in the placebo group; \( P = 0.008 \)). Reduced NK cell numbers correlated well with those seen with the whole lymphocyte population in Figure 3A; thus, these numbers largely accounted for the reduction of lymphocytes in the peripheral blood of patients receiving pioglitazone.

### Table 3  Inflammatory Cytokines, Insulin Resistance, and Lipid Profile During the 8 Month Follow-Up

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pioglitazone Group (n=47)</th>
<th>Placebo Group (n=47)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>8-Month Follow-Up</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>148.3 ± 46.7</td>
<td>113.8 ± 22.0*</td>
</tr>
<tr>
<td>Changes from baseline (mg/dL)</td>
<td>-39.4 ± 36.5</td>
<td>-28.4 ± 46.1</td>
</tr>
<tr>
<td>Fasting insulin (µU/mL)</td>
<td>12.8 ± 4.3</td>
<td>8.4 ± 1.7*†</td>
</tr>
<tr>
<td>Changes from baseline (µU/mL)</td>
<td>-4.3 ± 2.2†</td>
<td>-2.8 ± 1.9</td>
</tr>
<tr>
<td>HOMA index (mU/mL)/(mmol/L)</td>
<td>4.6 ± 2.4</td>
<td>2.4 ± 1.7*†</td>
</tr>
<tr>
<td>Changes from baseline (%)</td>
<td>-2.2 ± 1.6†</td>
<td>-1.5 ± 1.1</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.3 ± 1.3</td>
<td>6.8 ± 0.8*</td>
</tr>
<tr>
<td>Changes from baseline (%)</td>
<td>-0.6 ± 1.0</td>
<td>-0.5 ± 0.6</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>186.5 ± 37.1</td>
<td>152.3 ± 32.5*</td>
</tr>
<tr>
<td>Changes from baseline (mg/dL)</td>
<td>-33.5 ± 37.2</td>
<td>-30.5 ± 40.1</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>120.1 ± 35.0</td>
<td>87.5 ± 24.3*</td>
</tr>
<tr>
<td>Changes from baseline (mg/dL)</td>
<td>-32.5 ± 29.8</td>
<td>-31.5 ± 31.3</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>42.1 ± 10.3</td>
<td>43.4 ± 11.1</td>
</tr>
<tr>
<td>Changes from baseline (mg/dL)</td>
<td>2.3 ± 7.8</td>
<td>1.3 ± 12.2</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>136.5 ± 50.9</td>
<td>112.7 ± 44.5</td>
</tr>
<tr>
<td>Changes from baseline (mg/dL)</td>
<td>-24.0 ± 44.9</td>
<td>-20.0 ± 68.0</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>3.5 ± 2.2</td>
<td>1.8 ± 2.9*†</td>
</tr>
<tr>
<td>Changes from baseline (mg/L)</td>
<td>-1.8 ± 3.1</td>
<td>-1.5 ± 2.8</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>5.7 ± 5.2</td>
<td>7.7 ± 3.9*†</td>
</tr>
<tr>
<td>Changes from baseline (µg/ml)</td>
<td>1.9 ± 2.7†</td>
<td>0.4 ± 2.0</td>
</tr>
</tbody>
</table>

*p < 0.05 compared with baseline.
†p < 0.05 compared with placebo group.

Figure 2. IVUS measurements at the 8-month follow-up. The IVUS measurements were performed as described in the “Methods” section. Pioglitazone reduced the neointima volume and the total plaque volume at the 8-month follow-up after ZES implantation in patients with type 2 diabetes, as assessed by IVUS. *P < 0.05 vs placebo.
Changes in Inflammatory Cytokines and Chemokines During the 8-Month Follow-Up

We further investigated if the reduction of NK cells in the peripheral blood was associated with changes in the inflammatory cytokines or chemokines. The Cytokine Bead Assay (CBA) was performed because it allowed the detection of multiple cytokines in one patient’s PBMC sample. IL-6 and TNF-α were chosen for their role in promoting inflammation in CAD while IL-10 was chosen for its antiinflammatory role. MCP-1 is a chemoattractant for monocytes and has been shown to play an important role in initiating local inflammation at the damaged site. We found that the levels of IL-6 and MCP-1, but not TNF-α, were increased within 48 hours of stent implantation and then returned to the basal level by day 10 in both the pioglitazone and placebo groups. The increase in IL-6 and MCP-1 at the 48-hour point was significantly attenuated by pioglitazone treatment compared with the placebo group (16.1±6.1 versus 22.6±3.7 pg/mL; P<0.001) for IL-6 and 374±140 versus 429±264 pg/mL; P=0.04) for MCP-1. Different from IL-6 and MCP-1, the serum level of TNF-α was constant at baseline, after implantation, and at the 8-month follow-up in both groups (Figure 4A). Nevertheless, pioglitazone induced a small but statistically significant decrease in the serum TNF-α level (8.1±2.4 pg/mL in the pioglitazone group versus 9.3±2.7 pg/mL in the placebo group; P=0.03) at 2 days after stent implantation. On the contrary, we found that the serum level of the anti-inflammatory cytokine, IL-10, was significantly increased at 10 days after stent implantation only in the pioglitazone group (15.3±8.0 versus 10.0±2.7 pg/mL; P=0.01) (Figure 4A). These data demonstrate that pioglitazone-induced suppression of proinflammatory responses was followed by induction of antiinflammatory IL-10 cytokines.

Because MCP-1 was released from damaged vessels and functions as a strong chemoattractant for blood monocytes, we investigated if administration of pioglitazone resulted in alterations of its counterreceptor, CCR2, on blood monocytes. To test this, we obtained PBMCs from the pioglitazone and placebo groups 48 hours after stent implantation, and the expression of CCR2 on purified CD14+ monocytes was monitored using flow cytometry. As seen in Figure 4B, although CD14+ monocytes from the patients receiving placebo showed slight upregulation of surface CCR2, those from the pioglitazone group resulted in downregulation of CCR2 after stent implantation. When averaged, the difference in relative mean fluorescent intensity of surface CCR2 between the pioglitazone and placebo group was approximately 2-fold (Figure 4B, bar graph; P<0.05). Together, these data strongly demonstrate that pioglitazone caused attenuation of systemic inflammation and, thereby, inhibited recruitment of monocytes to the damaged stent sites within the coronary artery in the patients with type 2 diabetes.
Effect of Pioglitazone on the Proliferation and Migration of SMCs

Next, we investigated if pioglitazone-induced changes in systemic cytokines and chemokine levels could be translated into the SMC growth occurring in the process of neointima formation. Because procurement of autologous SMCs from the patients was impossible within this protocol, we used SMC cell lines as a surrogate. As can be seen in Figure 5A, the addition of control patient serum obtained from 2, 10, or 240 days after stent implantation to SMCs resulted in stimulation of cell proliferation, whereas that from the pioglitazone group caused inhibition of cell proliferation. Because the only difference between the 2 groups was the presence of pioglitazone, this inhibitory effect was likely because of the direct effect of pioglitazone on SMC proliferation. To directly test this, we cultured SMCs in the presence of various concentrations of pioglitazone, ranging from 0.1 to 10 μmol/L. As expected, the addition of pioglitazone inhibited SMC proliferation in a dose-dependent manner (supplemental material I; available online at http://atvb.ahajournals.org).

Because migration of medial SMCs is required for the formation of neointima, we next tested if serum obtained from the pioglitazone group inhibited migration of SMCs. As can be seen in Figure 5B, patients’ serum samples obtained at day 2, 10, or 240 from the pioglitazone group profoundly inhibited migration of cells within the damaged area of subconfluent SMCs, whereas those from the placebo group did not show any significant effect. This effect was also noticeable when cells were incubated with various concentrations of pioglitazone in vitro (supplemental material II). These findings strongly demonstrate the ability of pioglitazone in reducing the proliferation and migration of SMCs necessary for the formation of neointima and restenosis in diabetic patients with CAD.
Discussion

The present study aimed to understand the cellular and molecular basis underlying the beneficial effects of pioglitazone in preventing restenosis and neointima formation in patients after ZES implantation. Our data highlight the previously recognized antiatherogenic and antirestenosis effects of pioglitazone in both diabetic and nondiabetic patients with bare metal stents and further extend patients’ criteria to those implanted with ZES. Our prospective, randomized, single-blinded, placebo-controlled trial showed that treatment with pioglitazone for 8 months reduced neointima volume and atherosclerosis progression not only within the stented segment but also in the adjacent proximal and distal segments of the vessel. This effect was observed even in the presence of zotarolimus, suggesting that the pioglitazone-induced antiatherogenic effect occurred independent of local immunosuppression and likely through systemic antiinflammatory, antiproliferative, and antimigratory effects.

Changes in immunologic and cellular parameters after stent implantation in the pioglitazone-administered group were evident in the early stages, which were expected to be associated with its effect on neointima formation at the 8-month follow-up. Such changes include reduction in circulating NK cells, decreased serum IL-6 and MCP-1 levels, downregulation of CCR2 receptor expression, and increased serum IL-10 concentration. Although direct evidence linking these changes to neointimal growth is lacking, intense local inflammatory responses early after stent implantation were critical in the recruitment of neutrophils, monocytes, and macrophages to the site of vascular injury, which might have played an important role in potentiating early SMC hypertrophy and extracellular matrix deposition. Heavy local inflammatory infiltrate ensues with early detectable systemic inflammation, which can significantly potentiate the migration and proliferation of SMCs during the vascular remodeling processes in the early phase.25 The cellular and molecular
changes observed by pioglitazone in our study well support its antiinflammatory and immunomodulatory action as a PPARγ agonist, which has previously been demonstrated in vitro, in animal models of atherosclerosis, and in vivo in patients with high cardiovascular risk and impaired glucose tolerance. Furthermore, serum samples obtained from patients receiving pioglitazone at 2, 10, or 240 days after stent implantation showed significantly reduced proliferation and migration of SMCs. Therefore, these data strongly suggest that the serum pioglitazone concentration was maintained for up to 240 days to exert its antiproliferative and antimigratory effect in the patients and that the early systemic changes that occurred in the pioglitazone group might have likely been translated into the prevention of late-phase restenosis, leading to neointimal hyperplasia.

Interestingly, pioglitazone caused specific reduction of circulating lymphocyte populations after 48 hours of stent implantation. Among the lymphocyte populations, NK cells were significantly reduced. NK cells provide the body’s first line of defense against foreign pathogens. Through secretion of interferon γ and TNF-α, NK cells also stimulate T cells and macrophages, linking innate to adaptive immunity. The role of NK cells in the process of atherosclerosis in humans has not been fully understood. However, recent studies by Whitman et al demonstrated that NK cells were infiltrated into the atherosclerotic lesion and promoted atherosclerosis in the mouse model. Similarly, human NK cells stimulated proinflammatory responses at the damaged skin region of inflammatory disease, such as psoriasis. The fact that pioglitazone induced dramatic reduction of circulating NK cells within 48 hours of stent implantation suggests that it might have lowered the availability of circulating NK cells in the peripheral blood to limit their access to the injured sites within the coronary artery. A previous report has demonstrated that NK cell function could be inhibited by PPARγ agonists 15d-PGJ: (15-Deoxy-Delta-12,14-prostaglandin J2m). Therefore, pioglitazone may regulate the inflammatory and atherogenic process in part by decreasing the number and function of circulating NK cells and subsequently lowering local and systemic inflammation. This reduction of circulating NK cells was accompanied by a reduction of MCP-1, which plays a crucial role in the recruitment of monocytes and NK cells to inflammatory sites. Because an elevated serum MCP-1 level was shown to be directly correlated with the degree of restenosis, a reduced MCP-1 level by pioglitazone treatment might have lowered early recruitment of NK cells and monocytes to the damaged stent sites. The expression of CCR2, the counterreceptor for MCP-1, was downregulated on monocytes in the pioglitazone group, demonstrating that monocyte recruitment to the stented area might also have been impaired in the presence of pioglitazone. Therefore, these data suggest that multiple pathways, including recruitment of NK cells and monocytes to the damaged sites, would have been blocked by pioglitazone in the patients undergoing ZES implantation.

On stent implantation, endothelial cell injury evokes a series of repair events, including platelet aggregation, immune cell infiltration, and release of growth factors, followed by medial SMC modulation and proliferation. Dramatic upregulation of IL-6 was observed even after ZES implantation within 48 hours in the placebo group, suggesting that zotarolimus released from the stent was not sufficient to inhibit a systemic inflammatory response. An elevated IL-6 level has been associated with a higher degree of late lumen loss, possibly because of the increased plaque formation and neointima formation within the stented area. However, the administration of pioglitazone suppressed elevation of IL-6 by up to 41% within 48 hours. In contrast, the serum level of TNF-α was not substantially upregulated with stent implantation in our study. Nonetheless, pioglitazone slightly reduced the serum concentration of TNF-α within 48 hours. Pioglitazone also dramatically elevated the serum IL-10 level by 10 days after stent implantation. Likewise, pioglitazone increased the plasma adiponectin level at the 8-month follow-up. Interestingly, the level of hsCRP was not significantly affected by pioglitazone. This might have been because hsCRP was primarily controlled by the statins taken as a daily regimen in all our patients. Regardless, these data highlight the antiinflammatory immunologic changes occurring in patients receiving pioglitazone after ZES implantation.

Apart from antiinflammatory effects, thiazolidinedione derivatives have also inhibited proliferation of vascular SMCs by blocking G1 to S phase transition and suppressing the migration of SMCs, primarily by inhibiting the expression of regulatory genes, such as matrix metalloproteinase-9. Therefore, the reduction of SMC proliferation and migration, shown in our data, might have been direct causes for the antirestenosis effect observed by pioglitazone. Both antiproliferative and antimigratory effects were found even with the serum sample of the patients obtained at 2, 10, and 240 days after stent implantation. Inhibition of SMC proliferation by pioglitazone-treated samples was more dramatic than that observed from 10 μmol/L of pioglitazone added directly to SMC cultures (supplemental material I). Because the serum concentration of pioglitazone (peak concentration) was reported to be less than 5 μmol/L, its antiproliferative action appeared more potent and complex in vivo, possibly because of the combination of its antiinflammatory actions. Thus, the antiproliferative and antimigratory effects seen with patients’ serum samples might provide early signaling to suppress migration and proliferation of medial SMCs, leading to neointima formation.

In summary, our data provide the mechanistic basis underlying the beneficial effects of pioglitazone in the prevention of neointimal hyperplasia. The fact that the antiatherogenic and antirestenosis effect of pioglitazone occurred not only within the stented segment but also in the adjacent proximal and distal segments, even in the presence of zotarolimus, demonstrates its superior systemic antiinflammatory and antiproliferative actions. Because neointimal hyperplasia and coronary atherosclerosis are part of a progressive process accompanied by complex inflammatory circuits, antiinflammatory, antiproliferative, and antimigratory effects seen in the early stages of vascular injury may underlie the beneficial effects of pioglitazone in patients with type 2 diabetes with CAD.

This study has a few limitations. First, it was extremely difficult to have our patients visit the laboratory several times...
for blood sampling after stent implantation. If we could obtain blood samples more frequently, our data on the changes of immunologic profiles would have been much more accurate with respect to time. Second, although none of the medications our patients had taken showed dramatic complications, a possible interaction between pioglitazone and atorvastatin might have caused subtle changes in lipid profiles in these patients. It was shown previously that thiazolidinediones and their analogs not only lowered blood glucose but also exerted beneficial effects on inflammatory and atherogenic parameters and blood pressure.41 Nonetheless, our clinical data demonstrated only a partial effect of pioglitazone on the patients’ lipid profiles: a decreased concentration of triglycerides and an increased HDL without a change in LDL and total cholesterol (Table 3). Furthermore, coadministered antidiabetic treatments might have hindered the observance of an additional beneficial effect by pioglitazone. Third, it must be confirmed if the cellular and molecular changes caused by pioglitazone in patients with a ZES-eluting stent can be observed in patients with a bare metal stent or other drug-eluting stents.

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Disclosures
None.

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Cellular and Molecular Changes Associated With Inhibitory Effect of Pioglitazone on Neointimal Growth in Patients With Type 2 Diabetes After Zotarolimus-Eluting Stent Implantation

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Supplement Material I

![Graph showing relative proliferation (%) vs. concentration of pioglitazone (μM).](image)

<table>
<thead>
<tr>
<th>Pioglitazone (μM)</th>
<th>Relative Proliferation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 7.3</td>
</tr>
<tr>
<td>0.1</td>
<td>94.0 ± 5.9</td>
</tr>
<tr>
<td>1</td>
<td>87.0 ± 2.6</td>
</tr>
<tr>
<td>5</td>
<td>80.9 ± 6.3</td>
</tr>
<tr>
<td>10</td>
<td>72.6 ± 15.9</td>
</tr>
<tr>
<td></td>
<td>Pioglitazone Group</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>62.5 ± 9.38</td>
</tr>
<tr>
<td><strong>Glucose [mg/dL]</strong></td>
<td>120.33 ± 28.97</td>
</tr>
<tr>
<td><strong>Total cholesterol [mg/dL]</strong></td>
<td>145.6 ± 46.11</td>
</tr>
<tr>
<td><strong>Triglyceride [mg/dL]</strong></td>
<td>164.80 ± 95.18</td>
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<tr>
<td><strong>HDL [mg/dL]</strong></td>
<td>40.40 ± 4.56</td>
</tr>
<tr>
<td><strong>LDL [mg/dL]</strong></td>
<td>84.80 ± 26.73</td>
</tr>
<tr>
<td><strong>IL-6 (pg/mL)</strong></td>
<td>9.2 ± 3.1</td>
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<tr>
<td><strong>IL-10 (pg/mL)</strong></td>
<td>9.6 ± 2.6</td>
</tr>
<tr>
<td><strong>TNF-a (pg/mL)</strong></td>
<td>9.1 ± 2.2</td>
</tr>
<tr>
<td><strong>MCP-1 (pg/mL)</strong></td>
<td>331 ± 220</td>
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