Rosiglitazone Facilitates Angiogenic Progenitor Cell Differentiation Toward Endothelial Lineage

A New Paradigm in Glitazone Pleiotropy

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Background—Peroxisome proliferator–activated receptor-γ (PPAR-γ) agonists inhibit vascular smooth muscle proliferation and migration and improve endothelial function. It is unknown whether PPAR-γ agonists favorably modulate bone marrow (BM)–derived angiogenic progenitor cells (APCs) to promote endothelial lineage differentiation and early reendothelialization after vascular intervention.

Methods and Results—C57/BL6 mice, treated with or without rosiglitazone (8 mg/kg per day), a PPAR-γ agonist, underwent femoral angioplasty. Rosiglitazone treatment attenuated neointimal formation (intima/ media ratio: 0.98 ± 0.12 [rosiglitazone] versus 3.1 ± 0.5 [control]; P < 0.001; n = 10 per group). Using a BM transplantation model, we identified that 58 ± 12% of the cells within the neointima at 4 weeks were derived from the BM. Pure endothelial marker–positive, pure α-smooth muscle actin (αSMA)–positive, or double-positive APCs could be found both in mouse BM and in human peripheral blood after culture in conditional medium enriched with vascular endothelial growth factor. Rosiglitazone caused a 6-fold (P < 0.001) increase in colony formation by human endothelial progenitor cells, promoted the differentiation of APCs toward the endothelial lineage in mouse BM in vivo (0.66 ± 0.06% [control] to 0.95 ± 0.08% [rosiglitazone]; P < 0.05) and in human peripheral blood in vitro (13.2 ± 1.5% [control] to 28.4 ± 3.3% [rosiglitazone]; P < 0.05), and inhibited the differentiation toward the smooth muscle cell lineage. Within the neointima, rosiglitazone also stimulated APCs to differentiate into mature endothelial cells and caused earlier reendothelialization compared with controls (31 ± 5 versus 8 ± 2 CD31–positive cells per millimeter of neointimal surface on day 14; P < 0.01).

Conclusions—Similar to embryonic stem cell–derived progenitors, the adult BM and peripheral blood harbor APCs that are at least bipotential and able to differentiate into endothelial and smooth muscle lineages. The PPAR-γ agonist rosiglitazone promotes the differentiation of these APCs toward the endothelial lineage and attenuates restenosis after angioplasty. (Circulation. 2004;109:1392-1400.)

Key Words: cells • endothelium, vascular • vessels • receptors, cytoplasmic and nuclear • thiazolidinediones
Peroxisome proliferator-activated receptor-\(\gamma\) (PPAR-\(\gamma\)) agonists, a new class of insulin sensitizers, are used clinically to treat diabetes. PPAR-\(\gamma\) agonists appear to improve endothelial function independent of their insulin sensitization effects.\(^{6,7}\) They also favorably limit vascular inflammation\(^8\) and decrease circulating levels of C-reactive protein,\(^9\) which serves not only as a marker but also as a mediator of atherosclerosis.\(^{10}\) PPAR-\(\gamma\) agonists inhibit intimal hyperplasia after balloon injury in both diabetic and nondiabetic animal models.\(^{11-13}\) However, it is unknown whether PPAR-\(\gamma\) agonists have effects on the bioactivity and maturity of putative EPCs. In this study we demonstrate that the PPAR-\(\gamma\) agonist rosiglitazone stimulates the differentiation of APCs toward the endothelial cell lineage both in vitro and in vivo. These data identify a novel mechanism of vascular protection by PPAR-\(\gamma\) agonists.

**Methods**

**Mouse Model of Femoral Artery Angioplasty**

Male C57/BL6 mice (Charles River, Quebec, Canada) under general anesthesia underwent transluminal mechanical injury of the femoral arteries by insertion of a straight spring wire (0.38 mm in diameter; Cook) for >5 mm toward the iliac artery, as previously described.\(^1\) All procedures involving experimental animals were approved by the institutional committee for animal research of the Toronto General Hospital and Mount Sinai Hospital.

**Rosiglitazone Therapy**

Animals received rosiglitazone (8 mg/kg per day; GlaxoSmithKline)\(^{14}\) or saline by oral gavage (n = 10 for each group) 2 weeks before surgery. Treatments were continued until the mice were killed (at 1, 2, 3, or 4 weeks).

**BM Transplantation Model**

Recipient 129S6 mice at 8 weeks of age were lethally irradiated with a total dose of 950 rad, 9.5 Gy. TgN(ActbEYFP) (or eYFP) transgenic mice (129S6 background) that ubiquitously express enhanced YFP were used as the donors.\(^{15}\) After irradiation, the recipient mice received unfractionated BM cells (3 \(\times\) 10\(^6\)) from eYFP mice by tail vein injection. At 8 weeks after injection, angioplasty was performed. Repopulation by eYFP-positive BM cells was measured by flow cytometry to be 74\%. The chicken \(\alpha\)-actin and CMV enhancer that drives eYFP in the TgN(ActbEYFP) mice demonstrates some silencing in BM-derived cells (N. Anderson, MSc, W. L. Stanford, PhD, unpublished data, 2003), suggesting that the contribution of BM cells to neointimal formation is greater than observed.

**Mouse Progenitor Cell Purification and Culture**

A SpinSep kit (Stem Cell Technologies) was used to enrich murine hematopoietic progenitor cells. After the enriched murine hematopoietic progenitor cells were resuspended in EGM-2 medium (Clonetics), 1 \(\times\) 10\(^6\) cells were plated on 60-mm plates coated with fibronectin and treated with or without rosiglitazone (1 \(\mu\)mol/L). After 4 weeks, cells were evaluated by morphology and immunofluorescence expression analysis.

**Human Mononuclear Cell Isolation and Culture**

Mononuclear cells were isolated from the blood of healthy young volunteers by density gradient centrifugation with Ficoll separating solution (Becton Dickinson). Mononuclear cells were isolated from the blood of healthy young volunteers by density gradient centrifugation with Ficoll separating solution (Becton Dickinson). After resuspension in EGM-2 medium (containing vascular endothelial growth factor [VEGF] 10 ng/mL), 10\(^5\) mononuclear cells/cm\(^2\) were plated on fibronectin-coated 60-mm dishes and separated into subgroups treated with or without rosiglitazone (1 \(\mu\)mol/L), 15d-PGJ2 (1 and 10 \(\mu\)mol/L; Calbiochem), or platelet-derived growth factor (PDGF) (10 ng/mL; Sigma). In the PDGF subgroup, PDGF was added 4 days after cell plating. Cells were evaluated by flow cytometry, colony formation, or immunofluorescence expression analysis at the indicated time points.

**Colonies Formation Assay**

After 4 days in culture with or without rosiglitazone (1 \(\mu\)mol/L), adherent mononuclear cells were gently detached with cell dissociation solution (Sigma). Cells (1 \(\times\) 10\(^5\)) were seeded in methylcellulose plates (Stem Cell Technologies) containing 100 ng/mL human recombinant VEGF with or without rosiglitazone (1 \(\mu\)mol/L). Plates were studied under phase-contrast microscopy, and colonies were counted after 9 days of incubation by 2 independent investigators. A colony included at least 50 cells.

**Fluorescence-Activated Cell Sorter Analysis**

In both human and murine cells, fluorescence-activated cell sorting (FACS) (FacScan, Becton Dickinson) was performed to identify both cell-surface and intracellular antigens. Intracellular antigens were exposed with the use of the Cytofix/Cytoperm kit (Pharminogen). For cultured human mononuclear cells, Cy3-conjugated anti-\(\alpha\)-smooth muscle actin (\(\alpha\)SMA) (Sigma) and FITC-conjugated anti-human VE-cadherin antibodies (Serotec) were used. In mouse experiments, FACS was performed on both peripheral blood and BM cells collected from mice treated with or without rosiglitazone (8 mg/kg per day) for 16 days (n = 7 to 8 for each group). Cy3-conjugated anti-\(\alpha\)SMA, PE-conjugated anti-mouse Sca-1 (Pharminogen), and biotin-conjugated anti-mouse KDR antibodies (eBioscience) with secondary detection by FITC-conjugated streptavidin were used.

**Confocal Immunofluorescent and Histological Analysis**

For in vitro studies, cells were analyzed by immunofluorescent staining for von Willebrand factor (vWF), \(\alpha\)SMA, caldesmon, and calponin. For in vivo studies, frozen sections of femoral arteries were stained with primary antibodies (\(\alpha\)SMA, Sigma; vWF, Dako; VE-cadherin, CD31, Mac-3, Pharmigen; caldesmon, calponin, Santa Cruz) followed by incubation with FITC-, PE-, or Alexa Fluor 647–conjugated secondary antibodies. The ratio of intimal area to medial area (I/M ratio) was calculated. Cells positive for endothelial markers were counted in at least 10 different cross sections from different animals and expressed as the average number of positive cells per luminal surface length (in millimeters).

**Figure 1. Rosiglitazone attenuates intimal hyperplasia.** C57/BL6 mice were treated with rosiglitazone (Rosi) (8 mg/kg per day) or saline (control [Con]) 2 weeks before angioplasty and for 4 weeks after vessel injury. The I/M ratio at 4 weeks after angioplasty was significantly lower in the rosiglitazone group than in controls (n = 10 for each group; \(P<0.001\)). Arrows indicate vascular medial layer.
Statistical Analysis

Data were compared by unpaired t tests. A probability value of <0.05 was considered significant. All data presented in the text and figures are expressed as mean±SEM.

Results

Rosiglitazone Attenuates Neointimal Formation

Femoral arteries were harvested 4 weeks after angioplasty. Compared with the control group (treated with saline), there was a 3-fold decrease in I/M ratio in the rosiglitazone-treated group (3.1±0.5 [control] versus 0.98±0.12 [rosiglitazone]; P<0.001; Figure 1).

Angiogenic Progenitor Cells From Mouse BM Are Multipotential Cells

In 129S6 mice after BM reconstitution from eYFP mice, most of the cells in the neointima that formed 2 weeks after angioplasty were eYFP+αSMA+ (Figure 2A), suggesting that they were BM-derived vascular smooth muscle cells. Four weeks after angioplasty, 58±12% of the cells within the neointima were eYFP+αSMA+. To investigate the contribution of BM to neointimal formation, lineage-negative progenitor cells were purified from mouse BM and cultured in EGM-2 medium. Although 1×10^6 cells were initially plated in each 60-mm dish, only 2 to 3 colonies formed after culture for 2 weeks. In the colonies, cells could be generally sorted into 2 different morphologies: oval or spindle shaped (Figure 2B and 2C). After the cells were cultured for a total of 4 weeks in EGM-2, immunofluorescent analysis with anti-αSMA and anti-vWF (markers of vascular smooth muscle and endothelial populations, respectively) was performed, which revealed 4 different cell groups: pure αSMA+ (vWF-; Figure 2D), pure vWF+αSMA+caldesmon- (F), vWF+αSMA+calponin- (G), and differentiated smooth muscle-like cells (H and I) that expressed αSMA, calponin, and H-caldesmon.

Human Circulating APCs

Ten days after culture in EGM-2 medium, human mononuclear cells transformed into either spindle- or polygonal-shaped cells (Figure 3A and 3B). In general, the cells in the rosiglitazone group appeared to undergo a more dramatic transformation compared with the control group. Via the
colony formation assay assessed on day 9, the number of colonies that formed in the rosiglitazone group was 6-fold over that in the control group (Figure 3C). After the cells were cultured for 4 weeks in EGM-2 medium alone, mononuclear cells in a few colonies transformed into endothelial cells (Figure 3D). In cells supplemented with PDGF (10 ng/mL) 4 days after being cultured in EGM-2, mononuclear cells proliferated greatly over the next 4 weeks (Figure 3E). However, these cells did not transform. In plates treated with 15d-PGJ2, a natural PPAR-γ agonist, a great deal of cell detachment was observed, and the remaining cells did not transform (Figure 3F).

After the APCs were cultured for 4 weeks in EGM-2, cells were supplied with PDGF to stimulate transformation into smooth muscle cells. After 4 additional weeks, the initially oval and endothelial-like cells followed different fates. Some cells maintained their oval shape (Figure 3G), while others became elongated (Figure 3H) and transformed into smooth muscle-like cells (Figure 3I). Immunofluorescence double staining showed that some of the oval-shaped cells were pure vWF+ (Figure 3J) and others were double positive (vWF+αSMA+; Figure 3K). Some elongated cells developed filament-shaped αSMA (Figure 3L and 3M).

Rosiglitazone Promotes APC Differentiation Toward the Endothelial Lineage

FACS was performed 7 and 28 days after the plating of mononuclear cells. The percentages of pure αSMA+, pure VE-cadherin+, and αSMA+VE-cadherin+ cells were 0.33±0.05%, 5.78±0.61%, and 0.81±0.1%, respectively, on day 7 (Figure 4A) and increased to 1.27±0.23%, 13.16±1.5%, and 8.92±2.11%, respectively, on day 28 (Figure 4B). Rosiglitazone treatment markedly increased pure VE-cadherin+ cells to 28.39±3.28% and double-positive cells up to 18.97±2.32% on day 28 (Figure 4C) but decreased pure αSMA+ cells to 0.24±0.01% (all P<0.01 compared with groups without rosiglitazone).

In the mouse in vivo study, rosiglitazone treatment significantly increased the percentage of Sca-1+KDR+ cells (putative EPCs) in the BM (0.66±0.06% [control] to 0.95±0.08% [rosiglitazone]; P<0.05) and also had a tendency to increase Sca-1+KDR+ cells in peripheral blood (1.19±0.39% [control] to 2.36±0.37% [rosiglitazone]; P=NS). Rosiglitazone did not decrease the percentage of αSMA+ cells in BM (1.46±0.32% [control] to 0.4±0.24% [rosiglitazone]; P=NS) but significantly decreased αSMA+ cells in peripheral blood (1.2±0.34% [control] to 0.26±0.09% [rosiglitazone]; P<0.05).

Rosiglitazone Promotes APC Maturation to Endothelial Cells

As shown in Figure 5A and 5C, the majority of the cells in the neointima on day 14 expressed vWF, αSMA, and eYFP, although some neointimal cells expressed αSMA only. However, on day 28 the majority of the neointimal cells were αSMA+ (Figure 5B). Only cells on the surface of the neointima were vWF+, suggesting the phenotypic maturation of these 2 cell populations. Additional staining with other endothelial markers was done to investigate the process of endothelial cell maturation. Vessels were stained with anti-VE-cadherin, an early marker for endothelial cells, on days 8 and 21 (Figure 5D), and stained with anti-CD31, a late and mature endothelial marker, on days 14 and 21 (Figure 6). On day 8, the injured vessel surface was covered with a monolayer of cells that expressed both VE-cadherin and αSMA, supporting that they are APCs rather than pure EPCs. With neointimal formation, most of the APCs in the neointima turned off the expression of endothelial markers and maintained the expression of αSMA. However, gradually, more and more cells on the neointimal surface expressed more mature endothelial markers and differentiated into mature
endothelial cells. On day 21, rosiglitazone treatment significantly increased the cells expressing VE-cadherin on the surface of the neointima compared with the control group ($43 \pm 5$ versus $17 \pm 3$ cells/mm; $P<0.01$; $n=8$). In addition, rosiglitazone treatment increased the number of cells expressing CD31 on the surface of injured vessels compared with controls on both day 14 ($31 \pm 5$ versus $8 \pm 2$ cells/mm; $P<0.01$; $n=7$) and day 21 ($36 \pm 6$ versus $11 \pm 3$ cells/mm; $P<0.01$; $n=8$). The majority of these BM-derived cells within the neointima expressed not only $\alpha$SMA but also calponin and caldesmon (Figure 7A, 7B, 7C). The $\alpha$SMA$^+$ cells within the neointima were negative for Mac-3, a marker for macrophages, further suggesting they were not inflammatory cells (Figure 7D).

**Discussion**

We demonstrate that, similar to embryonic stem cell–derived progenitors, the adult BM and peripheral blood contain APCs that are at least bipotential and able to differentiate into endothelial and smooth muscle lineages. These APCs may be mobilized and contribute to neointimal formation after vascular interventions. In this study we show that rosiglitazone, a PPAR-$\gamma$ agonist, promotes the differentiation of APCs toward the endothelial lineage, in vitro and in vivo, both before and after mobilization from the BM or after homing to injured vascular sites. Furthermore, we demonstrate that the PPAR-$\gamma$ agonist rosiglitazone attenuates restenosis after angioplasty, possibly as a result of its favorable effect on APCs.

Early reendothelialization plays an important role in preventing complications and attenuating intimal hyperplasia after vascular interventions. Reports demonstrate that PPAR-$\gamma$ ligands inhibit smooth muscle proliferation and migration, improve endothelial dysfunction, and attenuate intimal hyperplasia after balloon injury in diabetic and nondiabetic animal models. Alternatively, the beneficial effect of PPAR-$\gamma$ agonists may be ascribed to their ability to increase the release of nitric oxide by endothelial cells. However, direct effects of rosiglitazone on progenitor cell differentiation and mobilization may also play a key role in inhibiting neointimal formation after vascular injury. It is unknown whether PPAR-$\gamma$ ligands can enhance BM-derived APC-mediated reendothelialization. In the present study we define APCs as a population of progenitor cells that have the multipotential to differentiate into either endothelial or smooth muscle cells, and we highlight the role of rosiglitazone to favorably promote the former.

By using embryonic stem cell differentiation as a developmental model, we previously demonstrated that manipulation...
of cell signaling parameters can regulate the cell fate in early development toward hematopoietic, endothelial, and smooth muscle lineages. Embryonic stem cell–derived APCs differentiate into distinct vascular lineages in response to different signaling cues. In the present study we show that bipotential APCs are also present in adult tissues, although the markers of APCs are still not well defined. The relationship between adult APCs and embryonic primitive mesodermal progenitors, often termed hemangioblasts, capable of generating hematopoietic progenitors as well as APCs, is currently unclear. In progenitor cells purified from adult mouse BM, APCs could be easily cultured, although their numbers are few. In addition to cells expressing markers specific for either endothelial or smooth muscle lineages, there is a population of double-positive progenitor cells that were also found in human peripheral blood. Our report suggests that double-positive progenitor cells hold dual potential to develop into either endothelial or smooth muscle cells in response to different stimuli. However, the timing for programming of this differentiation response is not clear. For example, when VEGF was added to selectively guide cells toward both endothelial and smooth muscle lineages, early stimulation with PDGF promoted only cell proliferation rather than differentiation. Interestingly, in the in vitro experiments, rosiglitazone was shown to stimulate the differentiation of human mononuclear cells toward the endothelial lineage. In the in vivo experiments, in mice without surgical intervention, rosiglitazone increased the amount of EPCs in the BM and decreased the number of smooth muscle progenitor cells in the circulation. An increase in EPCs (versus smooth muscle progenitors) by rosiglitazone treatment may serve to facilitate early reendothelialization.

Our in vivo studies lend further support to this notion. Mouse femoral angioplasty causes local vascular smooth muscle apoptosis and mobilizes BM cells to repair the resultant injury. In accord with the report by Sata et al, our data showed a significant amount of BM-derived cells contributing to neointimal formation. Although the first monolayer of cells that formed on the injured vascular surface may have been smooth muscle cells migrating from the media.
layer, our confocal figures demonstrated that this was unlikely because these cells expressed both α-SMA and VE-cadherin, an early marker of endothelial cells. The location of VE-cadherin was observed right at the tight junctions between cells, excluding the possibility of false-positive staining. These findings suggest that these αSMA+ cells were not derived from the local vessel medial wall but may have been APCs from the circulation. As expected, these cells gradually expressed vWF, which is a marker expressed later than VE-cadherin in the maturation course of EPCs. However, coinciding with this reendothelialization was neointimal growth, in which the majority of the participating APCs differentiated into smooth muscle–like cells and extinguished expression of endothelial markers. Although the molecular controls of APC differentiation are not well characterized, our report shows that rosiglitazone accelerates the development of cells with the mature endothelial marker CD31 that cover the neointimal surface.

It is interesting to speculate why the PPAR-γ ligand 15d-PGJ2 did not have the same effects as rosiglitazone. Rosiglitazone is approximately 20-fold more efficacious than 15d-PGJ2 or troglitazone in binding to PPAR-γ and in increasing transcriptional activity of PPAR-γ, which may be one reason for this discrepancy.

In addition, the biological effects of 15d-PGJ2 are complex because of its potential to activate prostaglandin receptors, induce apoptosis and Waf1 gene expression,18,19 and inhibit aromatase,20 which might be detrimental for growing APCs, as observed by the increased cell detachment and absent differentiation after 15d-PGJ2 administration. Although PPAR-γ ligands inhibit endothelial proliferation in vitro, inhibit retinal angiogenesis in vivo, and promote endothelial apoptosis in vitro,21 these effects may be concentration specific12 and may be attributed to pharmacological activity independent of PPAR-γ,22 the potency of PPAR-γ receptors,17 or the differing transcriptional activation in different cell types,15 such as BM-derived progenitor cells.

The present study reinforces the role of VEGF and PDGF as critical mediators that guide the fate of APCs.23–26 VEGF increases in both blood and local tissues after vascular injury.27 Previous reports showed that PPAR-γ agonists increase VEGF expression in human vascular smooth muscle,23 increase nitric oxide production by endothelial cells, upregulate endothelial nitric oxide synthase expression by APCs (S. Verma, MD, PhD, unpublished data, 2003), and increase blood VEGF concentrations in patients with diabetes.28 On the other hand, PPAR-γ ligands, through a variety of

Figure 6. Rosiglitazone promotes early reendothelialization by APCs. A and B, Double-immunofluorescent labeling of mouse femoral artery at 14 and 21 days after angioplasty. Red indicates αSMA; green, CD31 (arrows). Con indicates control group; Rosi, rosiglitazone-treated group.
pathways have been shown to attenuate the effects of PDGF, a factor that has long been known to stimulate smooth muscle proliferation and neointimal growth. The increased cellular and systemic VEGF levels may facilitate the differentiation of APCs to the endothelial lineage. In contrast, the attenuated effect of PDGF and upregulated expression of endothelial nitric oxide synthase in APCs may minimize the number of progenitor cells pursuing the path toward the smooth muscle lineage and also contribute to slower proliferation and migration of local smooth muscle cells.

Currently, the most commonly used method for culturing EPCs from peripheral blood is to isolate mononuclear cells by gradient centrifugation and to stimulate EPC maturation by conditional culture medium. However, these putative EPCs have been successfully transformed into cells with different phenotypes, such as smooth muscle cells and cardiomyocytes. Accompanied with our findings, we suggest that cells isolated by this method include a variety of progenitor cells with multiple potentials. Although it is the direction of the future to transplant these putative EPCs for therapeutic purposes, the present information suggests that biochemical and molecular manipulations may be required to guide the fate of these cells before using them for regeneration therapy or to attenuate restenosis.

In summary, in addition to hematopoietic stem and progenitor cells, the adult BM and peripheral blood harbor bipotential progenitors capable of developing endothelial and smooth muscle lineages. The PPAR-γ agonist rosiglitazone promotes the differentiation of these APCs toward the endothelial lineage and attenuates restenosis after angioplasty.

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