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PPARγ Promotes Monocyte/Macrophage Differentiation and Uptake of Oxidized LDL

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Summary

The formation of foam cells from macrophages in the arterial wall is characterized by dramatic changes in lipid metabolism, including increased expression of scavenger receptors and the uptake of oxidized low-density lipoprotein (oxLDL). We demonstrate here that the nuclear receptor PPARγ is induced in human monocytes following exposure to oxLDL and is expressed at high levels in the foam cells of atherosclerotic lesions. Ligand activation of the PPARγ:RXRα heterodimer in myelomonocytic cell lines induces changes characteristic of monocytic differentiation and promotes uptake of oxLDL through transcriptional induction of the scavenger receptor CD36. These results reveal a novel signaling pathway controlling differentiation and lipid metabolism in monocytic cells, and suggest that endogenous PPARγ ligands may be important regulators of gene expression during atherogenesis.

Introduction

The deposition of cholesterol ester within cells of the artery wall is fundamental to the pathogenesis of atherosclerosis (Brown and Goldstein, 1983; Steinberg et al., 1989). Atherogenic cholesterol-rich diets lead to an increase in monocyte adherence to arterial endothelium and their subsequent migration into the subendothelial space, where they take up large amounts of cholesterol ester. These cholesterol-laden macrophages are known as foam cells, and it is the accumulation of these cells beneath the endothelium that forms the earliest grossly recognizable atherosclerotic lesion, the fatty streak (Stary et al., 1994). An understanding of the mechanisms by which monocytes accumulate LDL cholesterol is central to an understanding of the early stages of atherogenesis.

The observation that patients lacking functional LDL receptors still develop atherosclerotic lesions rich in foam cells led to the discovery of the "scavenger pathway" for LDL uptake (Goldstein et al., 1979; Buja et al., 1983). Macrophages express relatively few receptors for native LDL, and these receptors are down-regulated in the presence of high concentrations of LDL (Brown and Goldstein, 1983). It is now clear that uptake of LDL by foam cells is mediated by a group of cell surface proteins termed scavenger receptors. Brown and Goldstein and colleagues were the first to demonstrate that these receptors have virtually no affinity for native LDL and that chemical modification of LDL is required for recognition (Goldstein et al., 1979). While these initial studies utilized acetylated LDL as a ligand, the relevant chemical modification in vivo is likely to be oxidation (Steinberg et al., 1989, and references therein).

A number of macrophage cell surface proteins have been identified that can specifically bind oxLDL. These include the class A scavenger receptor (SR-A; Kodama et al., 1988; Matsumoto et al., 1990), SR-BI (Acton et al., 1994), CD36 (Endemann et al., 1993), CD68 (Ramprasad et al., 1996), and CD32 (FcγRII; Stanton et al., 1992). While the in vivo relevance of some of these proteins remains to be determined, recent data support an important role for both SR-A and CD36 in the pathogenesis of atherosclerosis. Monocytes derived from mice deficient in SR-A (Suzuki et al., 1997) or from humans lacking functional CD36 (Nozaki et al., 1995) exhibit significantly decreased uptake of oxLDL. While expression of SR-A is restricted to macrophages, CD36 is also known to be expressed in platelets, adipose tissue, and mammary epithelium. Indeed, CD36 has been proposed to function as an adhesion molecule in platelets (Asch et al., 1987) and as a fatty acid transporter in adipose tissue (Abumrad et al., 1993).

PPARγ is a member of the nuclear hormone receptor superfamily that heterodimerizes with the retinoid X receptor (RXR) and functions as a transcriptional regulator of genes linked to lipid metabolism. Known targets of PPARγ include the genes encoding the adipocyte fatty acid binding protein aP2 (Tontonoz et al., 1994a), phosphoenolpyruvate carboxykinase (Tontonoz et al., 1995), lipoprotein lipase (Schoonjans et al., 1996), and the brown fat uncoupling protein UCP1 (Sears et al., 1996). The thiazolidinedione class of antidiabetic drugs and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) have been identified as ligands for this receptor, facilitating analysis of its biologic function (Forman et al., 1995a; Kliewer et al., 1995; Lehmann et al., 1995). PPARγ is expressed at highest levels in adipose tissue and serves a central regulator of the process of adipocyte differentiation (Chawla et al., 1994; Tontonoz et al. 1994b). However, mounting evidence suggests that this transcription factor may also play an important role in the regulation of lipid metabolism in other cell types, such as mammary and colonic epithelium (Mueller et al., 1998).

PPARγ has been reported to be expressed in several myeloid leukemia cell lines (Greene et al., 1995), although its biologic function in hematopoetic cells has not been defined. Given the established role of this nuclear receptor as a regulator of adipocyte differentiation, we explored whether PPARγ might serve a similar function in myelomonocytic cells. In the following work, we outline a novel signaling pathway for the regulation of monocyte differentiation and lipid metabolism mediated

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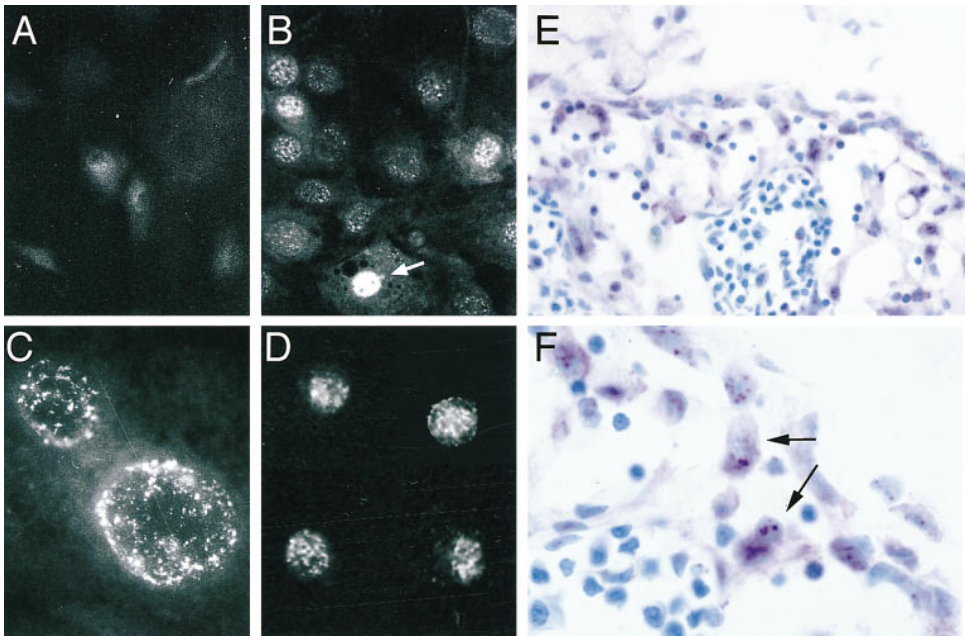

Figure 1. Expression of PPARγ in Peripheral Blood Monocytes and Tissue Macrophages

(A–C) Analysis of PPARγ protein expression in 3T3-L1 cells by immunofluorescence microscopy. (A) Preimmune serum, (B and C) anti-PPARγ.
(D) Analysis of PPARγ protein expression in human peripheral blood monocytes.
(E and F) Localization of PPARγ protein expression in murine lymph node by immunohistochemistry. Peroxidase activity was visualized by Vector VIP substrate and sections were counterstained with 0.4% methylgreen.
Objective magnification 40× for (A), (B), (D), and (E) and 100× for (C) and (F).

by the PPARγ:RXR heterodimer. These findings suggest an unexpected role for nuclear receptors in the pathogenesis of atherosclerosis.

Results

Expression of PPARγ Protein in Peripheral Blood Monocytes and Tissue Macrophages

The expression of PPARγ in spleen and myelomonocytic leukemia cells (Greene et al., 1995), led us to search for its expression in normal peripheral blood monocytes and tissue macrophages. To address this issue, we performed immunofluorescence microscopy using immunoaffinity-purified antisera raised against the N terminus of human PPARγ. The ability of this antisera to detect PPARγ protein in cells was verified using cultured 3T3-L1 preadipocytes, which have been shown to express PPARγ (Chawla et al., 1994; Tontonoz et al., 1994b). A low level of background cytoplasmic staining but no nuclear staining was seen with preimmune sera (Figure 1A). Distinct punctate nuclear staining of 3T3-L1 cells was seen with the PPARγ antiserum (Figures 1B and 1C). Most of the cells in Figure 1B are undifferentiated preadipocytes and stain weakly for PPARγ; however, a single differentiated adipocyte with obvious cytoplasmic lipid accumulation stains very strongly (arrow). This is consistent with the known differentiation-dependent expression of PPARγ in 3T3-L1 cells. A similar pattern of punctate nuclear staining is observed in human peripheral blood monocytes with anti-PPARγ antisera (Figure 1D). To determine whether PPARγ was also expressed in tissue macrophages, we performed immunohistochemistry on sections of murine lymph node. As shown in Figures 1E and 1F, the nuclei of the macrophages in these sections show strong staining (purple) for PPARγ (arrows). By contrast, no staining is seen in the lymphocyte nuclei.

Regulation of PPARγ Expression during Myelomonocytic Differentiation

The human leukemia cell line HL60 was used to begin to characterize the role of PPARγ in monocytic cells. HL60 cells display controlled differentiation along the granulocyte or the monocyte lineage in response to various inductive signals. For example, retinoic acid receptor (RAR) ligands such as 9-*cis* retinoic acid and the synthetic retinoid AM580 are inducers of HL60 granulocytic differentiation, while 1,25-dihydroxyvitamin D3 and TPA promote monocytic differentiation. Expression of *PPARγ* mRNA during HL60 cell differentiation was analyzed by Northern blotting. As shown in Figure 2A, a 3- to 5-fold increase in PPARγ expression was observed when HL60 cells were treated for 5 days with either 40 nM 1,25-dihydroxyvitamin D3 or for 24 hr with 40 ng/ml TPA. No change in PPARγ expression was seen after 5 days of treatment with 40 nM AM580. Thus, expression of PPARγ in this cell line is induced by agents known to trigger monocytic differentiation.

PPARγ and RXR Ligands Promote the Differentiation of Myelomonocytic Cells

Next we asked whether ligand activation of the PPARγ pathway could influence maturation of HL60 cells. Cells

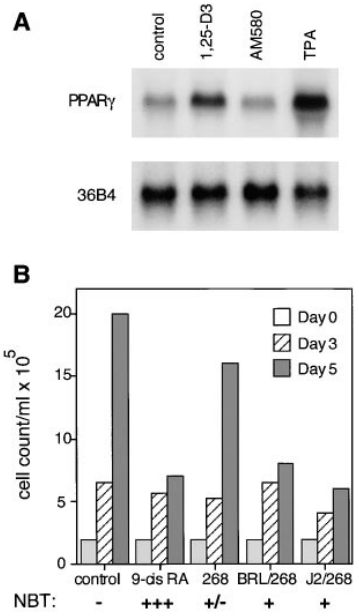

Figure 2. Expression and Function of PPARγin HL60 Cells

(A) Expression of PPARγ is regulated during HL60 cell differentiation. HL60 cells were cultured for 5 days in the presence of 30 nM 1,25-dihydroxyvitamin D3, 30 nM AM580 or for 24 hr in the presence of 40 ng/ml TPA. Total RNA (15 μg per lane) was analyzed by Northern blotting using ^{32}P-labeled cDNA probes. Equivalent amounts of intact RNA were run in each lane as indicated by hybridization to a *36B4* cDNA probe.

(B) PPARγ and RXR ligands induce growth arrest and oxidative burst capacity in HL60 cells. HL60 cells were plated at a density of 2×10^5 cells/ml and cultured for 5 days in the presence of one or more of the following activators as indicated: 100 nM 9-*cis* retinoic acid, 100 nM LG268, 3 μM 15d-PGJ2, or 5 μM BRL49653. On day 3 and day 5, cell number was quantitated by hemocytometer, and on day 5 an NBT reduction assay was performed.

were cultured for 5 days in the presence of either 9-*cis* retinoic acid (9-*cis* RA; 100 μM), the natural PPARγ ligand 15d-PGJ2 (3 μM), the synthetic PPARγ ligand BRL49653 (5 μM), the RXR-specific retinoid LG268 (100 nM; Boehm et al., 1995), or the combination of a PPARγ ligand and LG268. As anticipated, HL60 cells treated with 9-*cis* RA exhibited a reduction in cell growth, an induction of oxidative burst capacity as measured by NBT reduction assay, and distinct changes in nuclear morphology (Figure 2B and data not shown). Unexpectedly, the combination of 15d-PGJ2 and LG268 or BRL49653 and LG268 led to similar effects. Treatment with either 15d-PGJ2, BRL49653, or LG268 alone had a lesser effect (Figure 2B and data not shown). No effect was observed with the PPARα-specific activator Wy14,643 (5 μM) or the PPARδ ligand carbaprostacyclin (1 μM), either when used alone or in combination with LG268 (data not shown). These observations suggested that activation of the PPARγ:RXR heterodimer represents a novel regulatory pathway for HL60 maturation.

To further characterize the effect of PPARγ and RXR ligands on HL60 cells, we examined differentiation-dependent surface marker expression. Expression of the integrins CD18 and CD11b is up-regulated during differentiation to either granulocytes or monocytes, while expression of the lipopolysaccharide receptor CD14 is selectively induced during monocytic differentiation (Lubbert et al., 1991). Following culture for 5 days in the presence of various combinations of receptor-specific ligands, surface antigen expression was analyzed by flow cytometry. As expected, the RAR-specific retinoid AM580 was an effective inducer of CD18 and CD11b, but had only a small effect on CD14 expression (Figures 3A and 3B). In contrast, BRL49653, 15d-PGJ2, or LG268 alone induced CD18, CD11b, and CD14 expression, and simultaneous treatment with PPARγ and RXR ligands had a synergistic effect on all three markers (Figure 3 and data not shown). The magnitude of the change in surface CD14 expression is underscored by the distinct shift in the fluorescence intensity of the counted population (Figure 3B, insert).

To determine whether the expression of PPARγ was required for induction of monocyte markers by these agonists, we examined their effect on the HL60-derived leukemia cell line CDM-1. This cell line has an intact RXR signaling pathway (Nagy et al., 1995); however, it does not express PPARγ (Figure 3C, insert). Treatment of CDM-1 cells with 15d-PGJ2 and LG268 had no effect on CD11b, CD18, or CD14 expression (Figure 3C and data not shown), suggesting that PPARγ is required for the differentiation response to these compounds in HL60 and related cell lines. Similarly, no effect on expression of these antigens was observed in KG-1 leukemia cells, which also lack PPARγ expression (data not shown).

The effects of PPARγ:RXR activation were also examined in the monocytic leukemia cell line THP-1, which can be induced to differentiate terminally into macrophage-like cells by treatment with TPA or 1,25-dihydroxyvitamin D3. Again, treatment of THP-1 cells with PPARγ and RXR-specific ligands resulted in a synergistic induction of surface CD14 expression (Figure 3D). Note that this induction is equivalent in magnitude to that seen with TPA, a known inducer of monocytic differentiation. As expected, an induction of CD14 expression was also seen following treatment with 1,25-dihydroxyvitamin D3.

We also examined the ability of PPARγ ligands to modulate surface marker expression in primary cultures of human peripheral blood monocytes. As shown in Figure 3E, treatment of monocytes with PPARγ activators from each of three different chemical classes, troglitazone, 15d-PGJ2, or oxLDL (Nagy et al., 1998, this issue of *Cell*) resulted in similar changes in CD14 expression. These findings indicate that ligand activation of PPARγ in monocytic cells promotes changes in surface marker expression characteristic of monocytic differentiation.

Expression of PPARγ Is Induced by Exposure to Oxidized LDL

Treatment of THP-1 cells with PPARγ and RXR ligands resulted in characteristic morphological changes, including a tendency to adhere to the culture dish and to accumulate cytoplasmic lipid droplets (Figures 4A and 4B). This observation suggested that PPARγ might function in monocytic cells to regulate genes involved in lipid metabolism. Since the foam cells of atherosclerotic

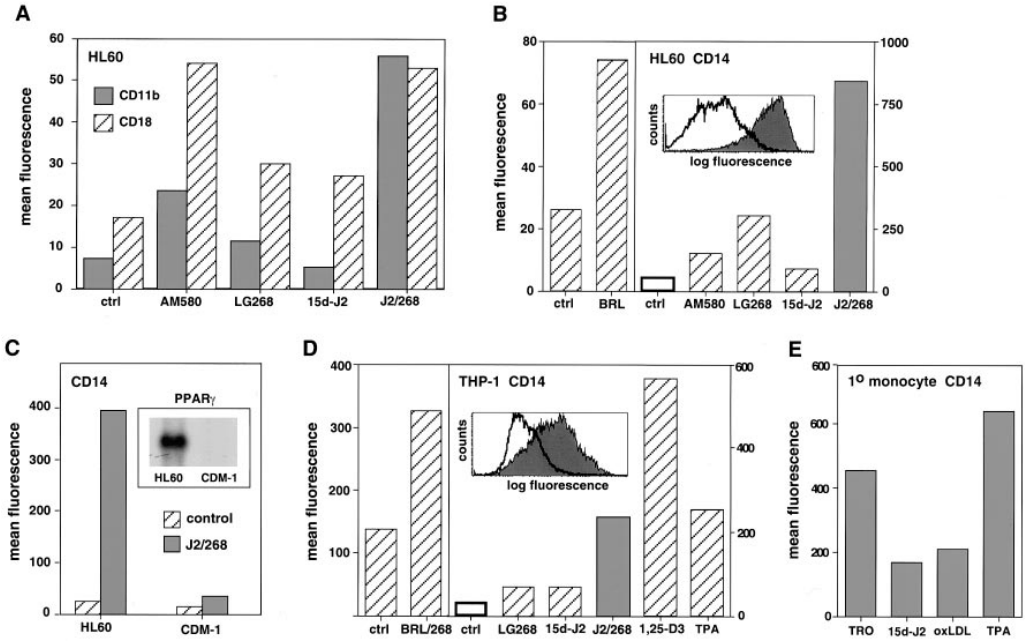

Figure 3. Induction of Differentiation-Linked Surface Marker Expression in HL60 and THP-1 Cells by PPARγ and RXR Ligands

HL60, CDM-1, and THP-1 cells were cultured for 6 days in the presence of one or more of the following activators as indicated: 100 nM LG268, 5 μM BRL49653, 3 μM 15d-PGJ2, 30 nM AM580, or 30 nM 1,25-dihydroxyvitamin D3. THP-1 cells were treated for 48 hr with 40 ng/ml TPA as indicated. Cells were incubated with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry as described in the Experimental Procedures. A population of 10,000 viable cells was analyzed for each data point. The data are presented as the mean fluorescence intensity of the counted population (A–D) or the change in fluorescence over the control population (E). For (C), expression of *PPAR*γ mRNA in HL60 and CDM-1 cells was determined by Northern blotting. (A) Expression of CD11b and CD18 expression on HL60 cells. (B) Expression of CD14 on HL60 cells. (C) Differential induction of CD14 and expression of PPARγ in HL60 and CDM-1 cells. (D) Expression of CD14 on THP-1 cells. (E) Expression of CD14 on primary human monocytes.

lesions are derived primarily from macrophages, the lipid metabolism of these cells is of particular relevance to human disease. Foam cells arise in a microenvironment characterized by high local concentrations of oxidized lipoproteins. Accordingly, we considered the possibility that expression of PPARγ might be modulated in response to such environmental conditions. THP-1 cells were cultured for 5 days in the presence of LG268 and 50 μg/ml native or modified human LDL, and the expression of *PPAR*γ mRNA analyzed by Northern blotting. While treatment of THP-1 cells with LG268 alone had no effect on expression of *PPAR*γ mRNA, simultaneous treatment with oxLDL and LG268 resulted in a significant induction (Figure 4C). A smaller increase in PPARγ expression was observed when THP-1 cells were treated with oxLDL alone (data not shown). Surprisingly, the ability to induce PPARγ expression was specific for oxidatively modified LDL and was not observed with either native or acetylated LDL (acLDL). We also examined PPARγ expression in primary human peripheral blood monocytes cultured in the presence or absence of 50 μg/ml oxLDL. Figure 4C (right) shows that exposure of human monocytes to oxLDL strongly induced PPARγ expression. Primary monocytes were more responsive than THP-1 cells to oxLDL and did not require LG268 for maximal induction of PPARγ. These results established a link between oxLDL and the PPARγ signaling pathway, and suggested that PPARγ may play a role in the macrophage response to high extracellular concentrations of oxLDL.

PPARγ and RXR Ligands Stimulate Uptake of OxLDL by THP-1 Cells

Since oxLDL stimulated PPARγ expression, we speculated that PPARγ and RXR ligands may promote uptake of oxLDL by monocytic cells such as THP-1. Following treatment with PPARγ and RXR ligands, THP-1 cells were incubated for 4 hr with increasing concentrations of DiI-labeled oxLDL and cell-associated fluorescence quantitated by flow cytometry. As shown in Figure 5A, treatment with either 15d-PGJ2 and LG268 or troglitazone and LG268 led to a significant increase in cell association (a combination of binding and uptake) of DiI-oxLDL. The specificity of this effect for modified lipoprotein was verified using an an excess of unlabeled lipoprotein as competitor (Figure 5B). While an excess of oxLDL abolished PPARγ-induced association, native LDL did not compete. This specificity for modified LDL is characteristic of macrophage scavenger receptors.

In order to determine whether oxLDL was actually internalized by PPARγ-activated THP-1 cells, control and 15d-PGJ2/LG268-treated cultures were incubated for 4 hr with 20 μg/ml DiI-oxLDL and examined by fluorescence microscopy. As demonstrated in Figures 5C and 5D, THP-1 cells treated with these activators showed

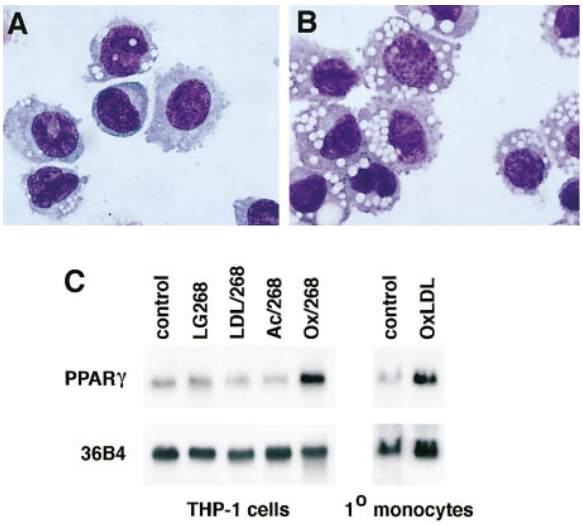

Figure 4. PPAR Stimulates Differentiation and Is Induced by OxLDL in THP-1 Cells

(A and B) PPARγ and RXR ligands stimulate morphologic differentiation of THP-1 cells. HL60 cells treated for 5 days with vehicle (A) or with 3 μM 15d-PGJ2 and 100 nM LG268 (B) were cytospun onto glass slides and stained with Diff-Quik. Objective magnification 100×. Oil red O staining demonstrated that the cytoplasmic vacuoles in B correspond to cytoplasmic lipid (not shown).

(C) OxLDL induces expression of PPARγ in THP-1 cells and human peripheral blood monocytes. Cell were cultured for 4 days in the presence of 100 nM LG268 and/or 50 μg protein/ml native LDL, acetylated LDL (acLDL), or oxidized LDL (oxLDL) as indicated. Total RNA (15 μg per lane) was analyzed by Northern blotting using ^{32}P-labeled cDNA probes.

a significant increase in cytoplasmic fluorescence compared to control cells. Thus, activation of the PPARγ:RXR pathway in these cells stimulates both binding and uptake of oxLDL.

PPARγ and RXR Ligands Regulate Expression of the Scavenger Receptor CD36

The ability of PPARγ and RXR ligands to stimulate oxLDL uptake was likely to be the result of increased surface expression of one or more macrophage scavenger receptors. The two receptors most conclusively linked to oxLDL uptake are CD36 and SR-A (type I and II). Northern analysis was used to monitor expression of the mRNAs encoding these receptors in THP-1 cells following treatment with various nuclear receptor ligands. Unstimulated THP-1 cells do not express significant amounts of mRNA for either CD36 or SR-A. TPA has been reported previously to be an effective inducer of both CD36 and SR-A type I expression in THP-1 cells (Moulton et al., 1992; Yesner et al., 1996; Figure 6A). As shown above for HL60 cells, TPA also induces expression of PPARγ in THP-1 cells. Treatment of THP-1 cells with either troglitazone alone or LG268 alone resulted in a small increase in *CD36* mRNA expression; however, the combination of both ligands was almost as effective as TPA. Moreover, the combination of troglitazone and TPA led to an additive induction of *CD36* mRNA expression. Interestingly, none of the nuclear receptor ligands analyzed were able to induce significant expression of mRNA encoding SR-A type I (Figure 6A) or SR-A type II (data not shown). In fact, troglitazone antagonized the induction of SR-A type I by TPA. Similar results were obtained when 15d-PGJ2 was used as the PPARγ ligand (data not shown).

We further examined the effect of PPARγ:RXR ligands on surface expression of CD36 by flow cytometry. As shown in Figure 6B, treatment of THP-1 cells and primary human monocytes with either troglitazone or oxLDL alone induced a significant change in CD36 expression. Consistent with the Northern analysis (Figure 6A), simultaneous treatment with LG268 had a synergistic effect. We also directly compared the ability of PPARγ:RXR ligands to modulate surface expression of CD36 and SR-A. Figure 6C demonstrates that while TPA was an effective inducer of both CD36 and SR-A expression on THP-1 cells, the combination of 15d-PGJ2 and LG268 selectively induced expression of CD36. Similar results were obtained when troglitazone was used as the PPARγ ligand (data not shown). Thus, the differential effects of TPA and PPARγ:RXR activators on scavenger receptor mRNA expression are reflected in surface expression of these proteins. We also analyzed expression of two additional candidate oxLDL receptors, CD68 and CD32. No significant effect of PPARγ and RXR ligands on expression of either protein was observed (data not shown). Taken together, these results suggested that the ability of PPARγ and RXR ligands to stimulate binding and uptake of oxLDL in THP-1 cells might be mediated at least in part by the scavenger receptor CD36.

PPARγ:RXR Ligand-Induced Binding of OxLDL to THP-1 Cells Is Mediated Primarily by CD36

To investigate whether the induction of CD36 and the stimulation of oxLDL binding by PPARγ:RXR ligands were linked, we utilized a monoclonal antibody to CD36 (OKM5) that has been demonstrated previously to interfere with the binding of oxLDL (Endemann et al., 1993). Following 5 days of culture in the presence of 15d-PGJ2 and LG268, THP-1 cells were incubated for 3 hr at 4°C with 10 μg/ml DiI-oxLDL in the presence of OKM5 or control IgG monoclonal antibody and cell association quantitated by flow cytometry. An excess of unlabeled oxLDL (200 μg/ml) served as a control for the specificity of oxLDL binding. As shown in Figure 6C, the anti-CD36 antibody was an effective inhibitor of DiI-oxLDL binding, resulting in a 65% reduction in total cell association and a 75% reduction in specific association (as defined by competition with 20-fold excess of oxLDL). While it is possible that other scavenger receptors may make minor contributions to PPARγ:RXR-induced oxLDL association, these data indicate that the effect is mediated primarily by the scavenger receptor CD36.

The *CD36* Promoter Is a Direct Target of the PPARγ:RXRα Heterodimer

The observation that *CD36* mRNA was increased upon treatment with PPARγ and RXR activators raised the possibility that CD36 might be a direct target of the PPARγ:RXR heterodimer. To determine whether PPARγ

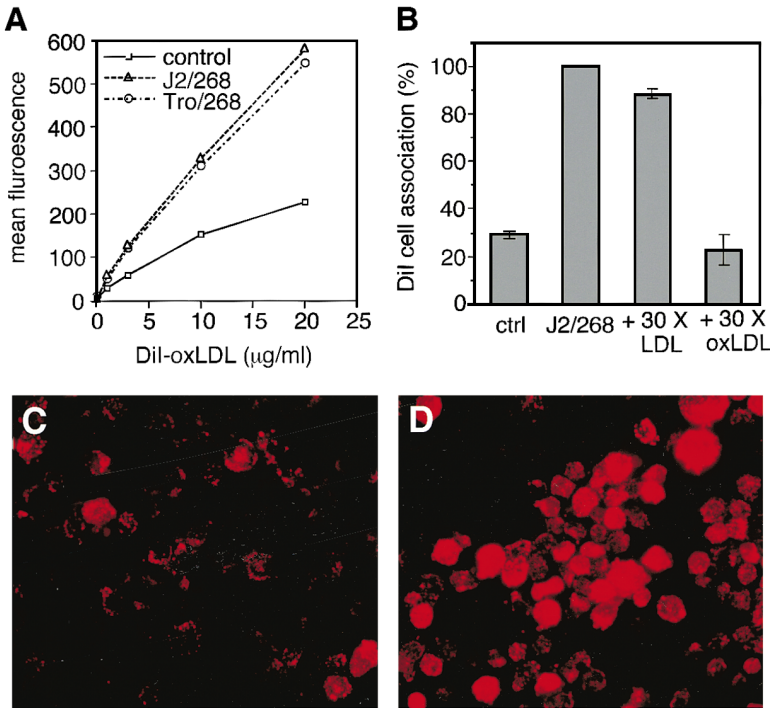

Figure 5. PPAR and RXR Activators Stimulate Binding and Uptake of OxLDL

(A) PPARγ and RXR activators stimulate cell association of DiI-oxLDL in THP-1 cells. Following 5 days of culture in the presence of vehicle, 3 μM 15d-PGJ2 and 100 nM LG268, or 15 μM troglitazone and 100 nM LG268, THP-1 cells were replated and incubated for 4 hr at 37°C with increasing concentrations of DiI-oxLDL as described in the Experimental Procedures. Cell-associated fluorescence was quantitated by flow cytometry. Each point represents the mean fluorescence of a 10,000 cell population. The experiment was repeated three times with similar results.

(B) Oxidized but not native LDL competes for PPARγ:RXR-inducible cell association of DiI-oxLDL to THP-1 cells. Following 5 days of culture in the presence of vehicle or 3 μM 15d-PGJ2 and 100 nM LG268, cells were replated and incubated for 4 hr at 37°C in the presence of 10 μg/ml DiI-oxLDL. A 30-fold excess of unlabeled human oxLDL or native LDL was added as a competitor as indicated. The mean fluorescence ± SE of the counted populations from three independent experiments is presented. The mean fluorescence of the 15d-PGJ2/LG268-treated population was assigned a value of 100%.

(C and D) PPARγ and RXR ligands stimulate uptake of DiI-labeled oxLDL by THP-1 cells. Following 5 days of culture in the presence of vehicle (C) or 3 μM 15d-PGJ2 and 100 nM LG268 (D), THP-1 cells were replated and incubated for 4 hr with 20 μg protein/ml DiI-oxLDL. The cells were washed, cytospun onto glass slides, and analyzed by fluorescence microscopy. Objective magnification 40×.

could function to regulate transcription of the *CD36* promoter, the region corresponding to −273 to +47 of the *CD36* promoter (Armesilla and Vega, 1994) was cloned into the luciferase reporter vector pGL-BASIC (−273/luc). This construct was cotransfected with *CMX-mPPARγ* and *CMX-hRXRα* expression vectors into CV-1 cells. Following transfection, the cells were treated with vehicle or the PPARγ-specific ligand BRL49653. Figure 7A demonstrates that the PPARγ:RXRα heterodimer activated transcription of the −273/luc construct in a ligand-dependent manner.

The PPARγ:RXR complex activates transcription of target genes by binding to DR-1 (direct repeat with one nucleotide spacer) type hormone response elements (Kliewer et al., 1992b). We analyzed the promoter region of the *CD36* gene (Armesilla and Vega, 1994) and identified a sequence (−273 to −260) with homology to a DR-1 motif (Figure 8B). The ability of a synthetic oligonucleotide spanning this region to bind PPARγ was investigated by gel mobility shift assay using nuclear extracts prepared from 3T3-F442A adipocytes and THP-1 cells. As shown in Figure 7B, a single nucleoprotein complex was bound by the *CD36* DR-1 oligonucleotide using either nuclear extract. This complex was competed by a 50-fold excess of unlabeled *CD36* DR-1 oligonucleotide (spf), but not by a similar excess of an oligonucleotide with a mutation in the DR-1 site (mut). Moreover, the complex was abolished and a supershift formed when incubated with antiserum to PPARγ (Figure 7B) and RXRα (data not shown), but not antiserum to PPARα. Thus, the *CD36* promoter contains a bona fide binding site for the PPARγ:RXRα complex.

To confirm that this site was mediating the observed induction of the *CD36* promoter by PPARγ:RXRα, we constructed a second *CD36* promoter-luciferase reporter containing the sequence from −263 to +47, which lacks the PPARγ binding site (−263/luc). The −263/luc construct showed minimal responsiveness to PPARγ (Figure 7A). Collectively, these results suggest that the PPARγ:RXRα heterodimer modulates *CD36* gene expression through direct interaction with the proximal promoter.

PPARγ Is Expressed in the Foam Cells of Atherosclerotic Lesions

The above findings raise the intriguing possibility that PPARγ may be involved in the regulation of foam cell lipid metabolism in vivo. A pivotal question, then, is whether PPARγ is actually expressed in the foam cells of atherosclerotic lesions. In fact, very few transcription factors have ever been shown to be present at detectable levels in foam cells. Expression of PPARγ protein in atherosclerotic lesions was examined by immunohistochemistry using sections of aorta from a mouse that carries human *apoB* and *apo(a)* transgenes and lacks expression the LDL receptor (Sanan et al., 1998). These mice develop atherosclerosis spontaneously at a young age when fed a normal diet. Subscapular brown adipose tissue was used a control for PPARγ expression. As seen in Figure 8, strong nuclear staining (brown) of the adipocytes is observed with PPARγ antisera (Figure 8B) but not with preimmune sera (Figure 8A). The aortic sections shown in Figures 8C, 8D, and 8E show atherosclerotic lesions characterized by the accumulation of

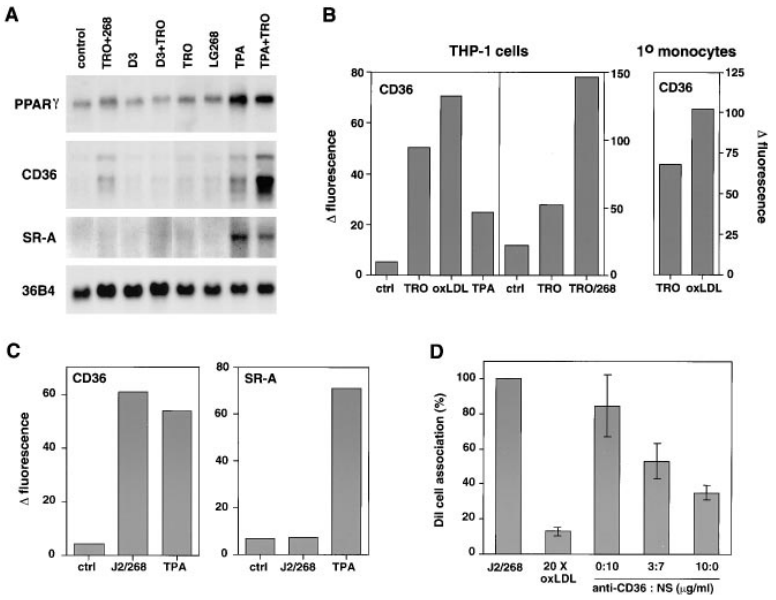

Figure 6. Regulation of Scavenger Receptor Expression by PPARγ and RXR Ligands

(A) Differential regulation of macrophage scavenger receptor mRNA expression by nuclear receptor ligands. THP-1 cells were cultured for 6 days in the presence of 100 nM LG268, 15 μM troglitazone, 30 nM 1,25-dihydroxyvitamin D3, and/or for 24 hr in the presence of 40 ng/ml TPA. Total RNA (15 μg per lane) was analyzed by Northern blotting using ^{32}P-labeled cDNA probes.

(B) Regulation of surface CD36 expression on THP-1 cells and primary human monocytes by PPARγ and RXR ligands. THP-1 cells were treated for 5 days with 5 mM BRL49653, 3 μM 15d-PGJ2, and 100 nM LG268 as indicated, or for 48 hr with 40 ng/ml TPA. Cells were analyzed by flow cytometry using unconjugated anti-CD36 and FITC-conjugated anti-IgG. A population of 5000 viable cells was analyzed for each treatment. Data are presented as the difference in mean fluorescence between anti-CD36 antibody and control IgG.

(C) Regulation of surface CD36 and SR-A type I/II expression on THP-1 cells by PPARγ and RXR ligands. THP-1 cells were cultured for 5 days in the presence of 100 nM LG268 and 3 μM 15d-PGJ2, or for 48 hr with 40 ng/ml TPA and analyzed by flow cytometry using unconjugated anti-CD36 and anti-SR-A type I/II antibodies as in (B).

(D) Inhibition of PPARγ:RXR-stimulated oxLDL association by a monoclonal antibody to CD36. THP-1 cells were treated for 5 days with vehicle or 3 μM 15d-PGJ2 and 100 nM LG268, washed, and incubated for 3 hr at 4°C in the presence of 10 μg/ml DiI-oxLDL. Unlabeled oxLDL (200 μg/ml), anti-CD36 monoclonal antibody (OKM5) or control IgG monoclonal antibody was added as indicated. The cell-associated fluorescence ± SE of the counted populations from two independent experiments is presented. The fluorescence of the 15d-PGJ2/LG268 treated population in the absence of competitor was assigned a value of 100%.

macrophage-derived foam cells and lipid within the subendothelial space. In contrast to the normal cellular components of the vessel wall, the foam cells within these lesions show remarkably strong nuclear staining for PPARγ (Figures 8C, 8D, and 8E).

The induction of PPARγ expression by oxLDL exposure, the ability of PPARγ:RXR ligands to promote monocyte differentiation and uptake of oxLDL, and the high level of expression of PPARγ in foam cells in vivo collectively suggest that PPARγ may play a role in the pathogenesis of atherosclerosis.

Discussion

Coronary artery disease is the leading cause of death in industrialized societies. A detailed understanding of the molecular and cellular events that underlie formation

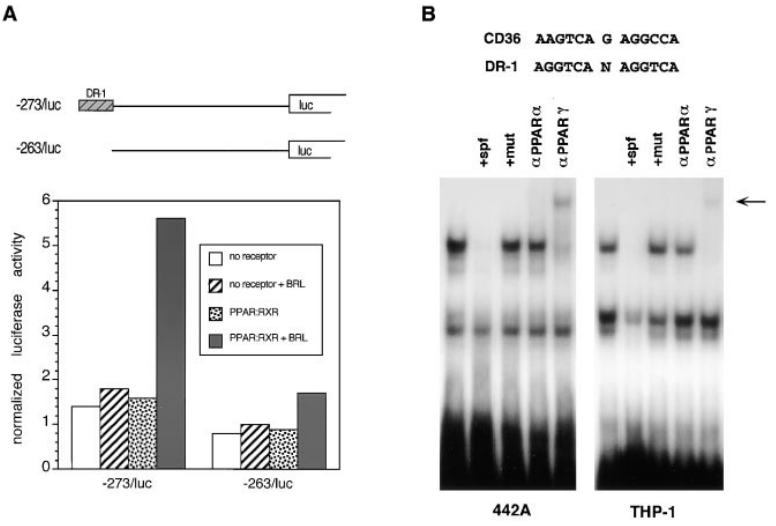

Figure 7. The *CD36* Promoter Contains a Functional Binding Site for the PPARγ:RXRα Heterodimer

(A) The PPARγ:RXRα heterodimer activates the *CD36* promoter. CV-1 cells were transfected with −273/luc or −263/luc reporter; CMX-β-gal, CMX-mPPARγ1, and CMX-hRXRα; and cultured in the presence or absence of 5 μM BRL49653 as indicated. Average normalized luciferase activity (arbitrary units) of triplicate points is presented. The experiment was repeated three times with similar results.

(B) A DR-1 motif in the *CD36* promoter binds PPARγ:RXRα from 3T3-F442A adipocyte and THP-1 nuclear extracts. An alignment of the CD36 DR-1 motif with the consensus DR-1 element is shown at top. Double-stranded ^{32}P-labeled *CD36 DR-1* oligonucleotide was used as a probe in a DNA mobility shift assay with 3T3-F442A adipocyte and THP-1 monocyte nuclear extract. Antisera to PPARα or PPARγ was included in the binding reaction (1:200 dilution) as indicated. Competitor oligonucleotides were added in 30-fold molar excess.

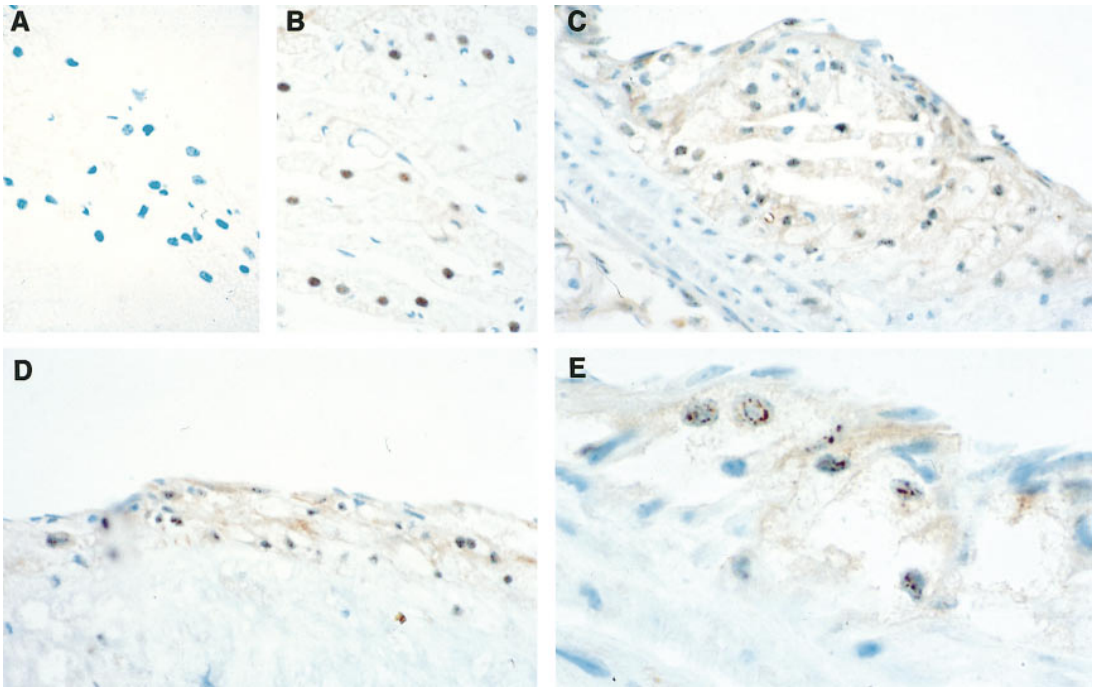

Figure 8. PPARγ Is Expressed in the Foam Cells of Atherosclerotic Lesions

Expression of PPARγ protein was localized by immunohistochemistry using affinity-purified anti-PPARγ antisera. Peroxidase activity was visualized by DAB and sections were counterstained with 0.4% methylgreen.

(A and B) Subscapular brown adipose tissue stained with preimmune serum (A) or PPARγ antibody (B). Objective magnification 40×.

(C–E) Expression of PPARγ in atherosclerotic lesions from the aorta of an 8-month-old LDLR$^{-/-}$; Tg(apoB);Tg(apoa) mouse. Objective magnification 40× for (C) and (D) and 100× for (E).

of the atherosclerotic plaque is a prerequisite to the rational design of therapeutics for this disease.

In this work, we have presented evidence implicating the nuclear receptor PPARγ in a novel signaling pathway that regulates differentiation and lipid metabolism in monocytic cells (Figure 9). PPARγ is induced in monocytes by exposure to oxLDL and is expressed at high levels in the foam cells of atherosclerotic lesions. Moreover, ligand activation of this receptor leads to a transcriptional induction of the scavenger receptor CD36 and endows cultured monocytes with the ability to bind and internalize oxLDL. Together, these results suggest that PPARγ and its congeners may play an unexpected role in the development of the atherosclerotic lesion.

Macrophages participate in diverse biologic processes, including phagocytosis of pathogens and debris, antigen presentation, and regulation of the immune response through cytokine production. Subspecialization of macrophages likely involves distinct alterations in patterns of gene expression. For example, the osteoclast is a specialized macrophage-like cell that plays a central role in bone remodeling. In this cell, the vitamin D receptor, another member of the nuclear hormone receptor superfamily, functions to regulate a specific set of genes involved in bone resorption (Ross et al., 1994). During atherogenesis, the deposition of large amounts of oxLDL within the arterial wall leads to the development of another functionally specialized type of macrophage, the foam cell (Steinberg et al., 1989, and references therein). The subset of genes important for macrophage accumulation and metabolism of cholesterol and lipid may be at least partially distinct from those important for inflammatory and immune responses. Interestingly, expression of a number of growth factors and cytokines has been reported to be influenced by oxLDL, including TNFα, IL-8, IL1-β, and iNOS (Hamilton et al., 1995; Wang et al., 1996). Our observation that oxLDL exposure induces PPARγ expression suggests that PPARγ may be important for the modulation of gene expression in this context. In fact, we have recently demonstrated that certain oxidized lipid components of the oxLDL particle can function as endogenous activators of PPARγ (Nagy et al., 1998). This observation reveals a novel mechanism whereby oxLDL may modulate macrophage gene expression.

While the association of a PPAR signaling pathway with macrophage lipid metabolism is unexpected, it fits well with the previously defined role for PPARγ in adipogenesis. Although prior work has focused primarily on its role in adipocytes, it is likely that this nuclear receptor also functions as a regulator of lipid homeostasis in certain other specialized cell types. Changes in lipid metabolism are a prominent feature of macrophage differentiation into foam cells. Such changes include increased free and esterified cholesterol content, increased content of lipoprotein oxidation products, expression of scavenger receptors, 15-lipoxygenase and lipoprotein lipase, and increases in acyl CoA: cholesterol acyltransferase (ACAT)

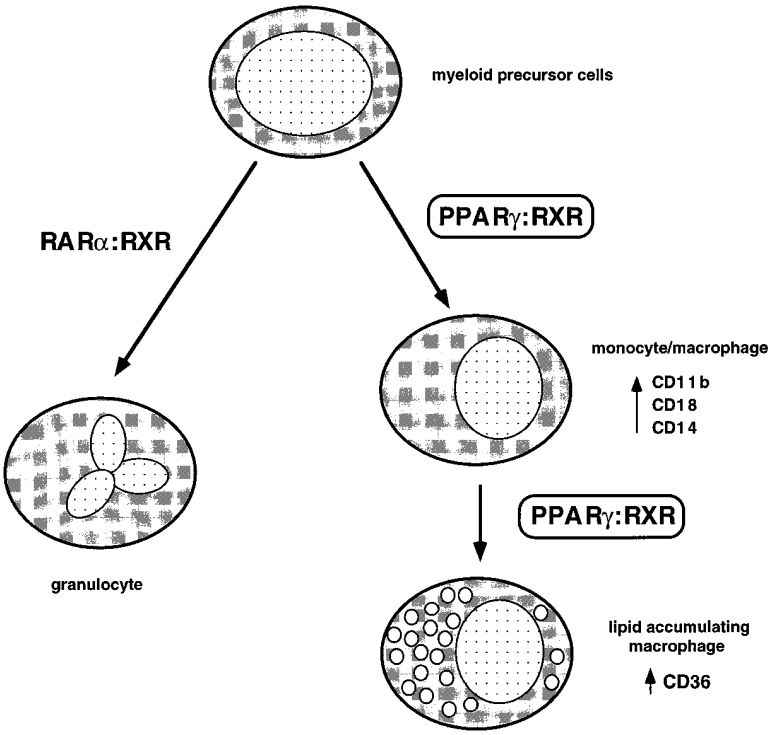

Figure 9. A Role for PPARγ in Monocyte/Macrophage Differentiation

and acid cholesterol-ester hydrolase activities (Stary et al., 1994, and references therein). In addition to the scavenger receptor CD36 (this work), the lipoprotein lipase gene has also been shown to be a target for PPARγ (Schoonjans et al., 1996).

There is strong evidence that both SR-A and CD36 participate in the uptake of oxLDL in vivo (Nozaki et al., 1995; Suzuki et al., 1997). While expression of SR-A is restricted to macrophages, CD36 is expressed in a number of other cell types, including mammary epithelium and adipose tissue. This pattern of expression overlaps significantly with that of PPARγ. Interestingly, CD36 is induced during the differentiation of preadipocytes and has been suggested to function as a fatty acid transporter in adipose tissue (Abumrad et al., 1993). CD36 expression is up-regulated by long chain fatty acids in preadipocyte cell lines (Sfeir et al., 1997), and has also been shown to be induced in murine models of diabetes and in mice maintained on a high-fat diet (Greenwalt et al., 1995). Recently, it was reported that both native and modified LDL can induce CD36 expression in J774 macrophages by a transcriptional mechanism (Han et al., 1997). All of these observations are consistent with our identification of *CD36* as a PPARγ target gene.

Previous work has demonstrated that expression of both the *SR-A* and *CD36* genes is induced by M-CSF and TPA in THP-1 cells (Moulton et al., 1992; Yesner et al., 1996). Surprisingly, however, we find that expression of these two proteins is differentially regulated by nuclear receptor pathways. *CD36*, but not *SR-A*, is transcriptionally induced by PPARγ agonists in THP-1 cells. The combined stimulus of a PPARγ agonist and TPA has an additive effect on *CD36* expression. In the case of *SR-A*, however, PPARγ ligands antagonize induction by TPA. A similar antagonism of PPARγ ligands and TPA has recently been shown to occur on the *SR-A* promoter in U937 cells (Ricote et al., 1998). The uptake of oxLDL in vivo likely requires integration of multiple signaling pathways, and therefore, the net effect of PPARγ and RXR agonists on macrophage uptake of oxLDL may vary between different cellular and hormonal contexts. Our findings that *CD36* is a direct target of PPARγ, that exposure to oxLDL induces PPARγ expression, and that the oxLDL particle itself is a rich source of PPARγ activators (Nagy et al., 1998), collectively suggest that PPARγ may play a role in foam cell formation. Clearly, in vivo studies will be required to further address this possibility.

Two groups have reported recently that PPARγ activators such as 15d-J2 and thiazolidinediones can inhibit production of certain inflammatory cytokines by TPA- and IFNγ-activated macrophages in culture (Jiang et al., 1998; Ricote et al., 1998). However, the molecular mechanism involved in this inhibition and the implications of these observations for the physiologic role of PPARγ in monocytic cells are not clear. We have shown here that in undifferentiated myeloid precursors and resting monocytes PPARγ functions as an inducer of monocytic gene expression and differentiation. Thus, control of gene expression by PPARγ in this cell type is likely to involve both positive and negative regulation of target genes and to depend on the activation state of the cell.

As an obligate heterodimeric DNA binding partner for a number of nuclear receptors, RXR stands at the crossroads of multiple cellular signaling pathways (Yu et al., 1991; Kliewer et al., 1992a; Leid et al., 1992). Interpreting

the effects of RXR ligands in biological systems can therefore be difficult. It is now clear that certain heterodimers, such as PPAR:RXR, can be activated by an RXR ligand in the absence of ligand for the partner receptor (a permissive heterodimer), while others, such as the RAR:RXR heterodimer, can be activated by RXR ligand only in the presence of ligand for the partner (a nonpermissive heterodimer; Kurokawa et al., 1994; Forman et al., 1995b; Chen et al., 1996; Minucci et al., 1997). In the case of PPAR:RXR, a number of studies have provided compelling evidence that the transcriptional activity of the heterodimer is maximal in the presence of ligands for both receptors (Kliewer et al., 1992b). In fact, recent work has demonstrated that RXR-specific ligands have similar activity to PPARγ ligands in biologic systems, including the ability to stimulate adipogenesis in cultured cell lines and the ability to increase insulin sensitivity in animal models of diabetes (Mukherjee et al., 1997; Tontonoz et al., 1997). The work presented here has in many instances utilized the combination of a PPARγ ligand and an RXR ligand in an effort to activate the PPARγ pathway maximally. The possibility that a portion of the effect of the RXR ligand on myelomonocytic cells is mediated by a permissive heterodimeric partner other than PPARγ cannot be excluded.

It is tempting to speculate that the elucidation of a nuclear receptor signaling pathway involved in macrophage lipid metabolism may open the door for therapeutic intervention in this process. A receptor agonist or antagonist that could specifically inhibit cholesterol uptake by macrophages in the arterial wall would obviously have tremendous pharmacologic potential. It is important to keep in mind, however, both the differential effects of PPAR and RXR ligands on the expression of individual scavenger receptors and the complexity of systemic oxidized cholesterol metabolism. While cholesterol accumulation by subendothelial macrophages is pathologic, the major site of systemic oxidized cholesterol clearance is the liver (Brown and Goldstein, 1983; Nagelkerke et al., 1984). The efficiency of hepatic clearance of oxLDL probably explains why oxLDL does not circulate at significant concentrations and is able to accumulate only in the microenvironment of the atherosclerotic artery wall. Since the pathways that regulate oxLDL uptake in arterial wall macrophages and Kupffer cells are likely to overlap, it is possible that an antagonist of these receptors might actually raise systemic levels of oxLDL. Moreover, since the relationship between lipid homeostasis and atherogenesis is complex, the influence of PPARγ and RXR ligands on systemic fatty acid, triglyceride and cholesterol metabolism, as well as their effects on macrophage cytokine production, must also be taken into account. Clearly, in vivo studies will be required to determine whether agonists or antagonists of the PPARγ:RXR pathway have potential utility in the treatment or prevention of atherosclerosis.

Experimental Procedures

Cell Culture and RNA Analysis

HL60 and THP-1 cells were obtained from ATCC and cultured in RPMI containing 10% fetal calf serum (Summit), penicillin, and streptomycin in an atmosphere of 7% CO_2. Human peripheral blood monocytes from healthy volunteers were isolated using Vacutainer CPT cell separator tubes, purified by adherence to the culture dish, and maintained in RPMI containing 10% autologous human serum. AM580 (Biomol), 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (Cayman), BRL49653 (Biomol), troglitazone (Sankyo), LG268 (Ligand Pharmaceuticals), 1,25-dihydroxyvitamin D3 (Biomol), and TPA (Sigma) were added to the culture media in a minimal volume ($<$0.1%) of ethanol or DMSO. NBT reduction assays were performed as described (Nagy et al., 1995). Total RNA was isolated using TRIzol reagent (GIBCO-BRL) and Northern analysis was carried out as described (Tontonoz et al. 1994a). The cDNA probe used for detection of SR-A was a 215 bp fragment derived from the SR-A collagen-like domain (Matsumoto et al., 1990). A cDNA probe encoding human ribosomal phosphoprotein PO was used as a control for loading and integrity of RNA (Laborda, 1991).

OxLDL Cell Association

All lipoproteins were obtained from Perimmune Inc. For oxLDL association experiments, cells were pretreated with receptor ligands for 5 days and replated in RPMI containing 2.0% resin-charcoal-stripped FCS and the indicated concentration of DiI-labeled oxidized human LDL. Unlabeled oxidized human LDL and native human LDL were added as competitors as indicated. Cells were incubated at 37°C, washed twice with PBS containing 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$, and analyzed by flow cytometry or fluorescence microscopy. Anti-CD36 antibody inhibition experiments were performed as described (Endemann et al., 1993). Cells were preincubated in the presence or absence of anti-CD36 monoclonal antibody OKM5 (Ortho Diagnostics) or control IgG monoclonal antibody for 30 min at 4°C. DiI-oxLDL was added at a final concentration of 10 mg (protein)/ml, and cells were incubated for an additional 2.5 hr at 4°C, washed, and analyzed by flow cytometry.

Flow Cytometry

Cells were washed with ice-cold PBS containing 0.5% BSA and incubated with fluorochrome-conjugated monoclonal antibody for 30–60 min on ice. For experiments using anti-CD36 and anti-SR-A antibodies, a second incubation with FITC-conjugated anti-immunoglobulin monoclonal antibody was performed. Nonspecific monoclonal antibody (anti-BrdU) or normal mouse IgG was used as a control. Cells were stained with propidium iodide to facilitate selection of the viable cell population and analyzed on a Becton-Dickinson FACScan using CellQuest software. The monoclonal antibodies used were: R-phycoerythrin (RPE)-conjugated anti-human CD14 (clone *TUK4*, DAKO), RPE-conjugated anti-human CD11b (clone *2LPM19c*, DAKO), fluorescein isothiocyanate (FITC)-conjugated anti-human CD18 (clone *MHM23*, DAKO), and FITC-conjugated anti-immunoglobulin (DAKO). The anti-human CD36 monoclonal antibodies (8A6 and OKM5) were generously provided by J. Barnwell (NYU Medical Center) and Ortho Diagnostics, respectively. The anti-SR-A I/II antibody was a gift of C. Glass (UCSD) .

Immunocytochemistry

To prepare polyclonal anti-human PPARγ antisera, an N-terminal fragment of hPPARγ1 (amino acids 1–145, Greene et al., 1995) was expressed in bacteria as a glutathione S-transferase (GST) fusion protein and purified by glutathione affinity chromatography. The PPARγ fragment was purified from the GST carrier by thrombin cleavage followed by FPLC. Rabbits were immunized by standard protocols. Total immunoglobulin was isolated from the crude antisera using the E-Z-Sep kit (Pharmacia) and immunoaffinity purified using a resin prepared by coupling the N-terminal PPARγ protein fragment to Affigel 10/15 agarose beads (Bio-Rad). 3T3-L1 cells and human peripheral blood monocytes were cultured in multichamber wells and processed for indirect immunofluorescence as follows. Cells were fixed in chilled acetone for 20 min. Antibodies were diluted in 20 mM Tris (pH 7.5), 0.5 M NaCl, 2% BSA, and 2 % nonfat dry milk. Affinity-purified rabbit anti-human PPARγ antibody was applied at a dilution of 1:50 at 4°C overnight followed by an FITC-labeled donkey anti-rabbit IgG (Jackson) diluted 1:80 for 45 min. For tissue localization studies, cryostat sections of paraformaldehyde fixed tissue samples of $LDLR^{-/-}$;Tg(apoB);Tg(apoa) mice were used (Sanan et al., 1998). Endogenous peroxidase was blocked with

0.1 M periodic acid for 10 min. Affinity-purified PPARγ antibody was applied at 4°C overnight at 1:100 dilution, followed by a biotinylated goat anti-rabbit IgG (Vector Labs; 1:300 dilution) for 45 min at room temperature. ABC peroxidase (Vectastain Elite, Vector) was used according to the manufacturer's instructions. Peroxidase activity was visualized either by DAB or the Vector VIP (Vector) substrate. Sections were counterstained with 0.4% methylgreen.

Gel Shift and Transfection Assays

Nuclear extract preparation and gel mobility shift assays were performed as described previously (Graves et al., 1992). The sequences of the oligonucleotides used were as follows (only one strand shown): *CD36 DR-1*, 5′-GGGGTCAGTAAGTCAGAGGCCAG GGA-3′; mutant, 5′-GGGGTCAGTAAGTCAGTTTCAGGGA-3′. The human *CD36* promoter region from −273 to +47 and from −263 to +47 (Armesilla and Vega, 1994) was amplified from human placental DNA by PCR and cloned into pGL-Basic (Promega). Transfections were performed in phenol red-free DMEM containing 10% resin-charcoal-stripped fetal calf serum by the lipofection method using DOTAP (Boehringer Mannheim). Luciferase and β-galactosidase assays were carried out as described (Forman et al., 1995a).

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