Oxidized LDL Regulates Vascular Endothelial Growth Factor Expression in Human Macrophages and Endothelial Cells Through Activation of Peroxisome Proliferator–Activated Receptor-γ

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Abstract—Vascular endothelial growth factor (VEGF) has been recognized as an angiogenic factor that induces endothelial proliferation and vascular permeability. Recent studies have also suggested that VEGF can promote macrophage migration, which is critical for atherosclerosis. We have reported that VEGF is remarkably expressed in activated macrophages, endothelial cells, and smooth muscle cells within human coronary atherosclerotic lesions, and we have proposed the significance of VEGF in the progression of atherosclerosis. To clarify the mechanism of VEGF expression in atherosclerotic lesions, we examined the regulation of VEGF expression by oxidized low density lipoprotein (Ox-LDL), which is abundant in atherosclerotic arterial walls. A recent report has revealed that peroxisome proliferator–activated receptor-γ (PPARγ) is expressed not only in adipocytes but also in monocytes/macrophages and has suggested that PPARγ may have a role in the differentiation of monocytes/macrophages. Furthermore, 9- and 13-hydroxy-(S)-10,12-octadecadienoic acid (9- and 13-HODE, respectively), the components of Ox-LDL, may be PPARγ ligands. Therefore, we investigated the involvement of PPARγ in the regulation of VEGF by Ox-LDL. PPARγ expression was detected in human monocyte/macrophage cell lines, human acute monocytic leukemia (THP-1) cells, and human coronary artery endothelial cells (HCAECs). Ox-LDL (10 to 50 μg/mL) upregulated VEGF secretion from THP-1 dose-dependently. VEGF mRNA expression in HCAECs was also upregulated by Ox-LDL. The mRNA expression of VEGF in THP-1 cells and HCAECs was also augmented by PPARγ activators, troglitazone (TRO), and 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2). In contrast, VEGF expression in another monocyte/macrophage cell line, human histiocytic lymphoma cells (U937), which lacks PPARγ expression, was not augmented by TRO or PGJ2. We established the U937 cell line, which permanently expresses PPARγ (U937T). TRO and Ox-LDL augmented VEGF expression in U937T. In addition, VEGF production by THP-1 cells was significantly increased by exposure to 9-HODE and 13-HODE. In conclusion, Ox-LDL upregulates VEGF expression in macrophages and endothelial cells, at least in part, through the activation of PPARγ. (Arterioscler Thromb Vasc Biol. 2001;21:560-566.)

Key Words: vascular endothelial growth factors ■ atherosclerosis ■ macrophages ■ endothelial cells ■ peroxisome proliferator–activated receptor-γ

Vascular endothelial growth factor (VEGF) has been recognized as an angiogenic factor that induces endothelial proliferation and vascular permeability.1 It may play a role in tumor growth, wound healing, age-related macular degeneration, rheumatoid arthritis, diabetic retinopathy, and collateral formation in ischemic tissue. However, recent studies have demonstrated that the gene for the VEGF receptor flt-1 is also expressed in human monocytes and that the VEGF-induced chemotactic response of human monocytes is mediated via this receptor.2 Clauss et al3 have also documented that VEGF induces monocyte activation and migration. These data strongly suggest that VEGF plays a role in the chemotaxis of monocytes/macrophages, which is crucial in inflammatory reactions and in wound-repair processes. We and others have reported that VEGF is remarkably expressed in activated macrophages, endothelial cells, and smooth muscle cells within human coronary atherosclerotic lesions, and we have proposed the significance of VEGF in the progression of atherosclerosis.4–7

Oxidized LDL (Ox-LDL) has been demonstrated to be a key molecule in the atherosclerotic process.8,9 It has been reported that Ox-LDL, which abundantly exists in atherosclerotic arterial walls, plays an important role in endothelial functional alterations, the recruitment and retention of mono-
cyte/macrophages, and foam cell formation. It has been also revealed that Ox-LDL modulates the secretion of cytokines or growth factors from endothelial cells and macrophages.

In this context, we hypothesized that Ox-LDL might play a role in the expression of VEGF in injured endothelial cells or accumulated macrophages in atherosclerotic lesions. In the present study, to clarify the mechanism of VEGF expression in atherosclerotic lesions, we examined the regulation of VEGF expression in endothelial cells and macrophages by Ox-LDL.

 Peroxisome proliferator–activated receptor-γ (PPARγ) is a member of the nuclear receptor superfamily of lipid-activated transcription factors. PPARγ and retinoid X receptor contain the heterodimer to bind regulatory elements in the promoter region of a number of adipocyte-specific genes and to stimulate transcription in response to PPARγ-specific and retinoid X receptor–specific ligands. PPARγ mRNA is most highly expressed in adipose tissue and plays an important role in regulating adipocyte differentiation and glucose metabolism. A new class of antiadipic agents, thiazolidinediones, have been developed and have been demonstrated to improve insulin resistance by binding and activating PPARγ.

Recently, Tontonoz et al. have reported that PPARγ is expressed not only in adipocytes but also in monocytes/macrophages, and they have suggested that PPARγ may have a role in the differentiation of monocytes/macrophages. Moreover, they have identified 2 of the major oxidized lipid components of Ox-LDL, 9-hydroxy-(S)-10,12-octadecadienoic acid (9-HODE) and 13-hydroxy-(S)-10,12-octadecadienoic acid (13-HODE), as endogenous activators and ligands of PPARγ. PPARγ expression in human endothelial cells has also been revealed.

Thus, we sought to determine whether PPARγ might work through the pathway of regulation of VEGF expression in endothelial cells and macrophages stimulated by Ox-LDL. In the present study, we further investigate the regulation of VEGF expression by Ox-LDL in macrophages and endothelial cells via the possible activation of PPARγ activators.

**Methods**

**Agents**

Troglitazone (TRO) was obtained from Sankyo; 15-deoxy-Δ12,14-prostaglandin J1 (PGJ1), prostaglandin F6, (PGF6), 9-HODE, and 13-HODE were purchased from Cayman. LDL (Sigma Chemical Co) was dialyzed against 3 changes of PBS to remove EDTA. Then LDL was oxidized at a concentration of 500 μg/mL by exposure to 10 μmol/L CuSO4 for 24 hours at room temperature, followed by dialysis at 4°C for 24 hours against 3 changes of PBS. LDL was then oxidized for 5 hours as minimum modified LDL (mm-LDL) and LDL incubated for 24 hours as Ox-LDL. The extent of lipid peroxidation was estimated by electrophoresis in agarose gel.

**Cell Culture**

Human monocytic leukemia (THP-1) cells and human monoblastic leukemia (U937) cells were obtained from the American type Culture Collection and cultured in RPMI 1640 medium (GIBCO-BRL) with 10% FBS at 37°C in 5% CO2. Cells were cultured in serum-free medium just before the experiments. The U937 cell line permanently expressing PPARγ was established as follows: PPARγ expression vector, pCMX-mPPARγ, possessing cyto-megalovirus enhancer and mouse full-length PPARγ1 cDNA (1.4 kb, GenBank No. U10374), which was kindly donated by Prof. K. Umesono (Kyoto University Graduate School of Medicine), and 10 mg neomycin resistance gene plasmid expression vector (pCNXNeo) were cotransfected to U937. Electroporation was performed by using a Bio-Rad Gene Pulser set at 220 V and 960 μF. Neomycin (GIBCO-BRL) was added to a final concentration of 1 g/mL. Individual clones were isolated by seeding transfected cells at a limiting dilution in 96-well plates in the medium containing 20% FBS. We designated this U937 cell line expressing PPARγ as U937T cells.

Human coronary artery endothelial cells (HCAECs) were purchased from Clonetics, and they were cultured in EGM-2MV (endothelial cell growth medium; Clonetics) with 5% FCS and changed to serum-free medium just before the experiments.

**RNA Extraction, Northern Blot Analysis, and RT-PCR**

Total cellular RNA was isolated from HCAECs and THP-1 cells by using TRIzol reagents (GIBCO-BRL). RNA (20 μg per lane) was electrophoresed on 1% agarose/6% formaldehyde gels and transferred to nylon membranes (Biodyne, Pall BioSupport). Hybridization was performed at 42°C for 20 hours in 50% formamide, 5X SSC, 0.05 mol/L phosphate buffer, 5X Denhardt’s solution, 0.1% SDS, 0.1 mg/mL denatured salmon sperm DNA, and 1X×10^6 cpm/mL 32P-labeled cDNA probes. A 320-bp Pst-EcoRI fragment (nucleotides 484 to 501) of rat VEGF189 cDNA was used as a probe. After hybridization, the filters were washed in 1X SSC/0.1% SDS for 10 minutes at room temperature, for 30 minutes at 55°C, and then in 0.1X SSC/0.1% SDS for 30 minutes at 55°C. Filters were autoradiographed, and the resulting bands were quantified by the BAS 2000 system (Fuji Photo Film Co, Ltd). Reverse transcriptase (RT)–polymerase chain reaction (PCR) for VEGF mRNA was performed as follows: cDNA was synthesized from 5 μg of total RNA primed with oligo-dT by use of RT (Super Script II) at 42°C for 1 hour. cDNA (0.5 μg) was subjected to PCR with 50 fmol of primers. Primer sequences were 5'-GGAATCTTCCAGGAGTA-3' (human VEGF 185 cDNA nucleotides 232 to 250) and 5'-TGGACGCGAGTCTGTG-3' (nucleotides 555 to 573) for detecting human VEGF mRNA. Amplification was carried out for 30 cycles with 30 seconds of incubation at 94°C, 30 seconds at 55°C, and 1 minute at 72°C. A 10-μL aliquot of each RT-PCR reaction mixture was electrophoresed on a 1.5% agarose gel.

**VEGF Protein Quantification**

After incubation of the THP-1, U293, and U937T under the indicated conditions, the conditioned medium was collected and centrifuged at 100 g for 5 minutes, and the supernatant was the material for measurement of VEGF protein with a Quantikine Human VEGF ELISA kit (R&D Systems, Inc). The sensitivity of the assay was 1 pg/mL. All samples were measured in duplicate.

**Immunohistochemical Staining**

HCAECs were cultured by use of flexiPERM (Heraeus Instruments), and before staining, they were permeabilized by Triton-X (Nakarai Tesque). For the identification of VEGF, a rabbit polyclonal antibody that was directed against the 20 amino terminal residues of human VEGF (Santa Cruz Biotechnology) was used. Immunohistochemical staining was performed by the LSAB (labelled streptavidin-biotin) method as described.

**Western Blotting**

Nuclear extracts of U937, U937, and U937T cells were obtained as described. Samples were applied to 10% SDS gels and transferred to nitrocellulose membranes (Bio-Rad) by use of semidry blotting. Western blotting was performed with the antibody at a dilution of 1:1,000 for 2 hours. After they were washed, the membranes were stained with horseradish peroxidase-conjugated rabbit anti-goat antibody. Antigen detection was performed with a CSL kit (Amersham-Pharmacia).
Figure 1. VEGF mRNA expression in HCAECs by Ox-LDL. A, Time course of Ox-LDL effect on VEGF mRNA expression assayed by Northern blot analysis. HCAECs were incubated with or without Ox-LDL (50 μg/mL) or native LDL (N-LDL, 50 μg/mL) for various times. B, Effect of Ox-LDL, mm-LDL, and N-LDL on VEGF mRNA expression in HCAECs. HCAECs were incubated with or without LDLs for 6 hours. C, Immunostaining of VEGF in cultured HCAECs. HCAECs were cultured with Ox-LDL (100 μg/mL) or FCS (20%) for 24 hours. Immunoreactivity of VEGF was increased in HCAECs stimulated by Ox-LDL and FCS. Original magnification ×100 (a through c) and ×400 (d through f).

Statistical Analysis
All results were expressed as mean±SD. Statistical analyses of the data were performed by ANOVA. A value of P<0.05 was considered significant. The experiments presented were the representatives of at least 2 separate experiments.

Results

Ox-LDL Increases VEGF Expression in Endothelial Cells and Monocytes/Macrophages
We have previously reported that human endothelial cells express VEGF in atherosclerotic lesions.5 We examined whether Ox-LDL stimulates VEGF expression in HCAECs. As shown in Figure 1A, Ox-LDL (10 to 50 μg/mL) increased VEGF mRNA expression in HCAECs in a time-dependent manner. When HCAECs were exposed to 10 to 50 μg/mL Ox-LDL for 6 hours, the VEGF mRNA level increased in a dose-dependent manner (Figure 1B). In contrast, native LDL at the same concentration had no effect on VEGF mRNA levels. We also used immunohistochemical techniques to examine whether VEGF was present in cultured HCAECs. As shown in Figure 1C, strong positive staining for VEGF was observed in HCAECs cultured with 100 μg/mL Ox-LDL for 12 hours. Native LDL had no effect on VEGF staining. No evidence of a toxic effect of Ox-LDL was observed during the incubation with HCAECs, as assessed by morphology and trypan blue exclusion.

THP-1 cells were exposed to Ox-LDL at concentrations between 10 and 50 μg/mL for 24 hours. VEGF levels were determined in culture medium by ELISA. VEGF concentration was increased significantly by exposure to Ox-LDL (for VEGF concentration, vehicle was 548.7±28.4 pg/mL, 10 μg/mL Ox-LDL was 742.0±26.7 pg/mL [P<0.05 versus vehicle], and 50 μg/mL Ox-LDL was 1078.9±100.6 pg/mL [P<0.05 versus vehicle]). Native LDL had no effect on VEGF protein levels (599.1±3.3 pg/mL). No evidence of a toxic effect of Ox-LDL was observed during the incubation with THP-1 cells.

PPARγ Activators Increase VEGF Expression in Endothelial Cells and Monocytes/Macrophages
HCAECs were exposed to TRO (10⁻⁷ to 10⁻⁵ mol/L). As shown in Figure 2A, VEGF mRNA expression was upregulated by TRO in a dose-dependent manner (percent over the control, 10⁻⁷ mol/L TRO 159%, 10⁻⁶ mol/L TRO 193%, and 10⁻⁵ mol/L TRO 250%) The mRNA expression of VEGF in THP-1 was also dose-dependently augmented by TRO and PGJ2 at 10⁻⁵ to 10⁻⁷ mol/L (percent over the control, 10⁻⁷ mol/L TRO 134%, 10⁻⁶ mol/L TRO 146%, 10⁻⁵ mol/L TRO 170%, 10⁻⁶ mol/L PGJ2 122%, 10⁻⁵ mol/L PGJ2 143%, 10⁻⁵ mol/L PGJ2 168%, and 12-0-tetradecanoylphorbol-13-acetate [TPA] 203%; Figure 2B). In contrast, VEGF expression in U937 was not augmented by TRO or PGJ2 (data not shown). As shown in Figure 2C, RT-PCR analysis also confirmed that TRO (10⁻⁵ mol/L) as well as lysophosphatidylcholine (LPC, 10⁻⁵ mol/L), which is one of the major components of Ox-LDL, increased VEGF mRNA expression. The augmentation of VEGF mRNA expression by TRO or LPC in THP-1 cells was not observed in the presence of actinomycin D, the inhibitor of transcription.

PPARγ Activators Increase VEGF Protein Levels in Culture Medium
To examine whether PPARγ activators stimulate VEGF production by THP-1 cells, VEGF levels were determined by
ELISA in culture medium from THP-1 cells incubated with or without PPARγ activators for 24 hours. Consistent with the results of alterations of mRNA expression, VEGF production by THP-1 cells was increased by exposure to TRO (5 and 10 μmol/L) in a time-dependent manner (Figure 3A). TRO and PGJ2 stimulated VEGF production by THP-1 cells in a concentration-dependent manner (Figure 3B and 3C). In the culture medium collected after treatment with 10 μmol/L TRO, the VEGF concentration was 3.9-fold higher than that of control (Figure 3B). We further examined the effect of PGF2α, an agent known to inhibit PPARγ activation, on TRO-induced secretion of VEGF from THP-1. The addition of PGF2α (200 nmol/L) significantly decreased the effect of TRO on VEGF secretion (Figure 3D).

**Ox-LDL Upregulates VEGF Expression Partly Through PPARγ Activation**

To further characterize the role of PPARγ in Ox-LDL-regulated VEGF expression in monocytes/macrophages, we established the permanent cell line of U937 expressing PPARγ (U937T cells). As shown in Figure 4A, in U937T cells, TRO treatment resulted in a marked upregulation of VEGF secretion similar to that in THP-1 cells, whereas it had no effect on VEGF secretion from U937 cells. As shown in Figure 4B, PPARγ expression in THP-1 cells was barely detected by Western blot, but after TRO or TPA stimulation, we could detect the expression of PPARγ in THP-1 cells. U937T cells showed significant PPARγ expression, whereas U937 cells did not. Figure 5 shows the responsiveness of THP-1, U937, and U937T cells to Ox-LDL. VEGF secretion from these cell lines was significantly upregulated by Ox-LDL stimulation with different magnitudes. VEGF secretion from U937T cells was increased to 190% and 212% by 10 and 50 μg/mL Ox-LDL stimulation, respectively, whereas the secretion from THP-1 cells was increased to 135% and 197% by Ox-LDL at 10 and 50 μg/mL, respectively. The increase of VEGF secretion from U937 cells by Ox-LDL (50 μg/mL) was...
only 125% of the control value. These differences suggest that PPARγ plays some role in the VEGF expression in THP-1 cells induced by Ox-LDL.

**9-HODE and 13-HODE Regulate VEGF Secretion From Human Macrophage Cell Lines**

Two of the major oxidized lipid components of Ox-LDL, 9-HODE and 13-HODE, were reported to be endogenous activators and ligands of PPARγ. Thus, we investigated whether 9-HODE and 13-HODE could stimulate VEGF production by human macrophage cell lines. As shown in Figure 6, VEGF production by THP-1 cells was increased by exposure to 9-HODE and 13-HODE (0.1 to 5 µg/mL) for 24 hours, but VEGF production by U937 cells, in which we could not detect PPARγ mRNA, was not increased by 9-HODE or 13-HODE. The result further supports the possibility that Ox-LDL induces VEGF expression through PPARγ activation. In addition, LPC also stimulated VEGF production by U937 cells.

**Discussion**

We and others have previously demonstrated the expression of VEGF protein and mRNA in the progression of human coronary atherosclerosis.4-7 We have reported that VEGF is remarkably expressed in activated macrophages, endothelial cells, and smooth muscle cells within human coronary atherosclerotic lesions and proposed the significance of VEGF in the progression of atherosclerosis. Because Ox-LDL is abundant in atherosclerotic arterial walls and is critically involved in atherogenesis, we hypothesized that Ox-LDL might have a role in the expression of VEGF in atherosclerotic lesions. Ramos et al.46 have previously reported the induction of VEGF expression in a mouse macrophage-like cell line (RAW 264 cells) in response to Ox-LDL. The present study was compatible with their result and further revealed that Ox-LDL-induced upregulation of VEGF occurred not only in macrophages but also in endothelial cells.

The significance of PPARγ in atherosclerosis is beginning to be elucidated. It is reported that PPARγ activators inhibit neointimal formation after balloon injury of the rat carotid artery.31 Recently, many lines of evidence have elucidated that PPARγ regulates the expression of several genes import
also induces VEGF expression in HCAECs. As shown in Figure 5, Ox-LDL stimulated VEGF secretion in U937 cells with less magnitude. Inasmuch as we observed significant stimulation by LPC of VEGF expression in U937 cells (Figure 2C and 6), LPC is also involved in Ox-LDL–regulated VEGF expression.

The exact role of VEGF in atherosclerotic lesions remains to be determined. It has been reported that the in vivo introduction of human VEGF165 cDNA into rabbit carotid arteries, by use of the hemagglutinating virus of Japan–liposome method, induces prominent angiomatoid proliferation of endothelial cells and thickening of the intima due to fibromuscular hyperplasia. Angiogenesis in atheromatous plaques is suggested to be an important process of atherogenesis and plaque rupture. Thus, VEGF produced in accumulating macrophages in atheromatous plaque could have a proatherogenic effect. On the other hand, VEGF can also play a role in protecting endothelial cells from injury in the early phase of atherosclerosis. Upregulation of VEGF expression by Ox-LDL, the key molecule of atherosclerosis, through the PPARγ activation observed in the present study can be relevant in the progression of atherosclerosis. In conclusion, we have shown that Ox-LDL regulates VEGF expression in human atherosclerotic lesions through, at least in part, ligand activation of PPARγ. These findings suggest that the PPARγ signaling pathway may play a role in atherogenesis induced by Ox-LDL.

Acknowledgments

This work was supported in part by research grants from the Japanese Ministry of Education, Science, and Culture; the Japanese Society for the promotion of Science “Research for the Future” program (JSPS-RFTF 96100204 and JSPS-RFTF 98L00801); and the smoking research foundation. We appreciate technical advice concerning the preparation of Ox-LDL from Dr Kiyotaka Kugiyama (Kumamoto University, Japan). We thank Prof Alan R. Saltiel (University of Michigan, Pfizer Inc), Dr Heidi Camp (Pfizer Inc), and Liyun Ding (Pfizer Inc) for their technical advice and gift of PPARγ antibody. We thank Ayumi Sone, Akane Nonoguchi, and Yukiko Takada for their excellent secretarial work.

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doi: 10.1161/01.ATV.21.4.560
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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