Transforming Growth Factor-β1 Inhibits Macrophage Cholesteryl Ester Accumulation Induced by Native and Oxidized VLDL Remnants

Carmen A. Argmann, Caroline H. Van Den Diepstraten, Cynthia G. Sawyecz, Jane Y. Edwards, Robert A. Hegele, Bernard M. Wolfe, Murray W. Huff

Abstract—Transforming growth factor β1 (TGF-β1) is secreted by various cells, including macrophages, smooth muscle cells, and endothelial cells. TGF-β1 is present in atherosclerotic lesions, but its role in regulating macrophage foam cell formation is not understood. Hypertriglyceridemic very low density lipoprotein (VLDL) remnants (VLDL-REMs) in their native or oxidized form will induce cholesteryl ester (CE) and triglyceride (TG) accumulation in macrophages. Therefore, we examined whether TGF-β1 can modulate the macrophage uptake of native or oxidized VLDL-REMs (oxVLDL-REMs). Incubation of J774A.1 macrophages with VLDL-REMs and oxVLDL-REMs compared with control cells increased cellular CE (13- and 21-fold, respectively) and TG mass (21-and 18-fold, respectively). Preincubation with TGF-β1 before incubation with VLDL-REMs or oxVLDL-REMs significantly decreased CE (73% and 54%, respectively) and TG mass (42% and 41%, respectively). TGF-β1 inhibited the activity and expression of 2 key components needed for VLDL-REM uptake: lipoprotein lipase and low density lipoprotein receptor. TGF-β1 inhibited CE mass induced by oxVLDL-REMs in part by decreasing the expression of scavenger receptor type AI/II and CD36. Furthermore, TGF-β1 enhanced cholesterol efflux through upregulation of the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1. Thus, TGF-β1 inhibits macrophage foam cell formation induced by VLDL-REMs or oxVLDL-REMs, which suggests an antiatherogenic role for this cytokine. (Arterioscler Thromb Vasc Biol. 2001;21:2011-2018.)

Key Words: atherosclerosis ■ macrophages ■ transforming growth factor-β1 ■ lipoproteins

Macrophage foam cells play an important role in the development of atherosclerosis.1 The lipoproteins that accumulate in the arterial intima include LDL and VLDL remnants (VLDL-REMs), which exist in either native or oxidized form.2,3 After the recruitment of circulating monocytes into the intima and their transformation into macrophages, these cells take up native lipoproteins via the LDL receptor (LDL-R)4 and oxidized lipoproteins via scavenger receptors (SRs), including SRAI/II, CD36, and macroscialin.5-7 The marked accumulation of cellular cholesteryl ester (CE), results in foam cell formation. Alteration of receptor expression, in atherosclerosis-susceptible mice, has a significant impact on atherogenesis. For example, transplantation of irradiated C57Bl/6 mice with LDL-R knockout (−/−) bone marrow significantly decreased diet-induced atherosclerosis.8,9 Furthermore, disruption of the SRAI/II10,11 and CD3612 genes resulted in a significant reduction of hypercholesterolemia-induced atherosclerosis in mice.

The development of atherosclerosis may be modulated by the interactions between arterial cells, which are mediated by a variety of adhesion molecules and cytokines, including transforming growth factor-β1 (TGF-β1).1 For example, in apoE(−/−) mice treated with anti-CD40 ligand antibodies, the lesion profile was converted from a lipid-rich to a lipid-poor collagen-rich lesion that was characterized by increased concentrations of TGF-β1.13 This response may be related to known effects of TGF-β1 on macrophages, including inhibition of NO,14 enhanced expression of the interleukin-1 receptor antagonist,15 inhibition of metalloelastase secretion,16,17 and induction of inhibitors of metalloproteinases.16,18 Furthermore, the presence of TGF-β1 within the arterial intima of mice fed a cholesterol-enriched diet was shown to prevent intimal lipid deposition,19 suggesting that TGF-β1 may modulate macrophage foam cell formation.

Foam cell formation can be modulated through multiple pathways, including regulation of lipoprotein uptake and cholesterol efflux.20,21 TGF-β1 has been shown to regulate proteins involved in lipoprotein uptake, including inhibition of SRAI/II and CD36 expression.22,23 Although TGF-β1 increases LDL-R expression in cultured smooth muscle cells,24 its effect in macrophages is unknown. Recently, TGF-β1 was demonstrated to enhance macrophage chol-
terol efflux, through upregulation of the ATP-binding cassette (ABC) transporter A1 (ABCA1). However, although TGF-β1 has the potential to modulate macrophage lipoprotein uptake and cholesterol efflux, its overall effect on foam cell formation induced by either native or oxidized lipoproteins has not been fully elucidated.

VLDL and VLDL-REM levels are elevated in patients with moderate hypertriglyceridemia, thereby increasing their risk for developing atherosclerosis, because these lipoproteins induce macrophage foam cell formation in their native or oxidized form. VLDL and VLDL-REM cause lipid accumulation via a 2-step process. Initially, they interact with cell surface lipoprotein lipase (LPL), which hydrolyzes core triglyceride (TG) to free fatty acids, which are then taken up by the cell and reesterified into TG. The CE-rich remnants, therefore supporting the hypothesis that TGF-β1 reduces macrophage foam cell formation induced by native and oxidized human VLDLs, VLDL-REMs, and LDLs in part by inhibiting their uptake and enhancing cholesterol efflux. Our results demonstrate that TGF-β1 significantly decreases cellular CE and TG by multiple mechanisms. The decrease in VLDL and VLDL-REM uptake was associated with decreased expression and cell surface activity of (1) LPL and (2) the LDL-R. The decrease in oxVLDL, oxVLDL-REM, and oxidized LDL (oxLDL) uptake was due in part to the reduced expression of SRAI/II and CD36. TGF-β1 also reduced foam cell formation by enhancing cholesterol efflux through increasing mRNA expression of ABCA1 and ABCG1. These findings suggest that TGF-β1 can inhibit foam cell formation, thereby supporting the hypothesis that TGF-β1 is atheroprotective.

**Methods**

**Subjects**

Subjects were recruited from the Outpatient Endocrinology Lipid Clinics at the London Health Sciences Center University Hospital campus (London, Ontario, Canada). The studies were approved by the University of Western Ontario Health Science Standing Committee on Human Research. The criteria of Schaefer and Levy were used to classify the lipoprotein phenotypes of the type IV subjects. No subject carried the apoE2 isoform.

**Lipoprotein Preparation**

Large VLDL (Svedberg flotation constant [Sf] 60 to 400) was isolated from the plasma of type IV hypertriglyceridemic subjects (HTG-VLDL), whereas LDL (Sf 0 to 12) and HDL, were isolated from normal subjects by sequential ultracentrifugation. Remnant-like particles of HTG-VLDL (Sf 60 to 400), designated VLDL-REMs, were formed in vitro (20% to 40% TG hydrolysis) as described previously.

Lipoproteins were oxidized in vitro in the presence of copper sulfate, and LDL was acetylated (AcLDL) by the addition of acetic anhydride. The extent of modification was confirmed by changes in relative electrophoretic mobility.

**Cell Culture, Cellular Lipid Analysis, and LPL Activity**

The murine macrophage–like cell line J774A.1 (American Type Culture Collection) was cultured and set up for experiments as outlined previously. The human macrophage–like cell line THP-1 (American Type Culture Collection) was grown in suspension in bicarbonate-buffered RPMI 1640 (Life Technologies) containing 10% FBS (vol/vol, Sigma) and 50 μM/L β-mercaptoethanol.

Mouse peritoneal macrophages (MPMs) were isolated, as previously described, from either LDL-R(–/–) mice (Jackson Laboratories, Bar Harbor, Me) and their controls (C57Bl/6 mice, Charles River, St. Constant, Quebec, Canada) or SRAI/II(–/–) mice (a kind gift from Dr U. Steinbrecher, The University of British Columbia, British Columbia, Canada) and their controls (CD-1 mice, Charles River); all mice were aged 6 to 8 weeks. MPMs were cultured (cells from 1 mouse per well) as described for J774A.1 cells and used for experiments within 72 hours of isolation.

Cells treated with TGF-β1 (R&D Technologies) received fresh TGF-β1 in the medium to a final concentration of 5 ng/mL unless stated otherwise, and were incubated for a total of 40 hours. For the first 24 hours, duplicate wells were pretreated with the cytokine only, followed by 16 hours of treatment with fresh TGF-β1 in DMEM supplemented with 5% human lipoprotein–deficient serum (LPDS) in the absence or presence of lipoproteins. Native VLDLs and VLDL-REMs or oxVLDLs and oxVLDL-REMs were used at a concentration of 50 μg of lipoprotein total cholesterol (TC) per milliliter medium, whereas native or modified LDL was used at 150 μg TC/mL medium. In some experiments, the cells were incubated with bovine milk LPL, which was added along with lipoproteins at a concentration of 0.25 IU/mL medium. Cellular CE, TC, free cholesterol (FC), TGs, and protein mass were determined as previously described. LPL activity was measured as described in detail previously.

**ACAT and NCEH Activity**

The effect of TGF-β1 on cholesterol esterification and acyl coenzyme A:cholesterol acyltransferase (ACAT) activity was directly determined in J774A.1 macrophages. Neutral CE hydrolase (NCEH) activity was determined by using a previously described method with the following modification: cells were preincubated with [14C]oleic acid and type IV VLDL (50 μg TC/mL medium).

**Measurement of mRNA Abundance**

THP-1 macrophages were seeded in 100-mm plates (Falcon Scientific) in RPMI 1640 supplemented with 10% FBS and phorbol dibutyrate (0.16 μmol/L for 6 days, Sigma). J774A.1 macrophages were plated in 100-mm plates in DMEM containing 10% FBS and grown to 70% to 80% confluence. All cells were treated for 24 hours in media supplemented with 5% LPDS and either 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2, 3.0 μmol/L, Cayman Chemical), oxVLDL (50 μg TC/mL medium), or oxLDL (150 μg TC/mL medium) in the absence or presence of TGF-β1 (5 ng/mL). Total RNA was isolated by using Trizol reagent (Life Technologies), and the mRNA abundances were measured. For further details, please see http://atvb.ahajournals.org.

**Cholesterol Efflux Assay**

J774A.1 macrophages were converted to foam cells by incubation with 100 μg/mL AcLDL in medium containing 0.2% fatty acid–free BSA (Sigma) and 1% (25 mM)-l-[3H]cholesterol (1 μCi/mL, Amershams) for 24 hours. Cells were then washed with PBS and incubated for 16 hours in the presence or absence of TGF-β1 (5 ng/mL medium, DMEM, and 0.2% BSA). Subsequently, cells were incubated (12 hours) in medium containing either 0.2% BSA only or supplemented with human apoA-I (10 μg/mL, Sigma), LPDS (5%), 4,4’-disothioyanothibene-2,2’-disulfonic acid (DIDS, 400 μmol/L, Sigma), or HDL, (100 μg/mL) in the absence or presence of TGF-β1. After the collection and centrifugation of the medium (10 minutes, 10 000 rpm), the monolayers were extensively washed and solubilized in 0.1N NaOH. Cholesterol efflux was expressed as

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Results

TGF-β1 Inhibits HTG-VLDL− and VLDL-REM−Induced Macrophage Lipid Accumulation

Incubation of J774A.1 macrophages with type IV HTG-VLDL or VLDL-REM compared with control cells resulted in a 19- and 13-fold increase, respectively, in CE and a 19- and 21-fold increase, respectively, in TG content (P<0.05; Figure 1A). The cellular FC content was not significantly changed from control (data not shown).

When cells were pretreated with TGF-β1, followed by incubation with either HTG-VLDL or VLDL-REM, CE accumulation was significantly reduced by 74% and 73%, respectively (P<0.05 for both, Figure 1A), and TG accumulation was decreased by 41% and 42%, respectively (P<0.05 for both, Figure 1A). LDL increased cellular CE 9-fold, which was reduced by 60% (P<0.05) in the presence of TGF-β1. Macrophage FC content was unaffected by TGF-β1 (data not shown).

The inhibitory effect of TGF-β1 on lipid accumulation induced by HTG-VLDL was concentration dependent (data not shown). The minimal concentration of TGF-β1 required to significantly decrease CE (51%) and TG (26%) accumulation was 2.5 ng/mL medium. Normal human plasma concentration of TGF-β1 has been reported to be 5 ng/mL.37

TGF-β1 Decreases OxVLDL-Induced and OxVLDL-REM−Induced Macrophage Lipid Accumulation

Although TGF-β1 is known to inhibit expression of receptors for modified lipoproteins,22,23 it has not been demonstrated that this translates into a reduction in foam cell formation, ie, reduced CE mass accumulation. Figure 1B shows that incubation of macrophages with oxVLDL or oxVLDL-REM increased CE mass by 25- and 21-fold, respectively, and TG mass by 16- and 18-fold, respectively (P<0.05), and that preincubation of cells with TGF-β1 significantly decreased the accumulation of CE (−42% to −54%, respectively; P<0.05) and TG (−55% to −41%, respectively; P<0.05). Macrophages incubated with oxLDL or AcLDL (data not shown) increased CE mass 14- and 50-fold, respectively (P<0.05). Preincubation of cells with TGF-β1 resulted in a 28% (Figure 1B) and 32% decrease in cellular CE mass, respectively (both P<0.05). OxLDL and AcLDL had no effect on cellular TG because of the very low TG content of these lipoproteins.

Effect of TGF-β1 on Oleate Incorporation Into CE and TG and NCEH Activity in J774A.1 Cells

The TGF-β1−induced reductions in CE and TG mass in cells incubated in the presence of HTG-VLDL were paralleled by reductions in radiolabeled oleate incorporation into CE (−48%, P<0.05) and TG (−23%, P<0.05; data not shown). TGF-β1 had no direct affect on ACAT activity, as determined in microsomes isolated from J774A.1 cells (data not shown).

Incubation of macrophages containing prelabeled cholesteryl oleate with TGF-β1 plus an ACAT inhibitor had no significant effect on the rate of CE hydrolysis compared with the rate in cells without TGF-β1 (0.489±0.057 versus 0.474±0.034 nmol CE remaining per milligram cell protein at 24 hours, respectively).

TGF-β1 Inhibits LPL Activity and Expression

Preincubation of cells with TGF-β1 (5 ng/mL) significantly reduced cell surface LPL by 88% (P<0.05; please see Figure I, which can be accessed online at http://atvb.ahajournals.org). This inhibition occurred in a dose-responsive manner with an IC50 dose of 2.5 ng/mL for TGF-β1 (data not shown). Furthermore, TGF-β1 significantly reduced cell surface LPL activity when cells were incubated with either HTG-VLDL (−71%, P<0.05) or VLDL-REM (−86%, P<0.05) compared with cells incubated with lipoproteins alone (online Figure I). The inhibition of LPL activity by TGF-β1 was also observed in cells incubated with oxVLDL-REM (−84%, P<0.05; data not shown). The effect of TGF-β1 on secreted LPL activity paralleled that seen for cell surface LPL (online Figure I). In addition, TGF-β1 did not have an effect on LPL activity when it was added directly to the LPL activity assay (data not shown);
mRNA Abundances of Various Receptors and Enzymes Involved in Lipoprotein Uptake in Macrophages

<table>
<thead>
<tr>
<th>mRNA Expression in J774A.1 Macrophages</th>
<th>LDL-R*</th>
<th>LPL†</th>
<th>CD36*</th>
<th>SRAI/II†</th>
<th>ABCA1*</th>
<th>ABCG1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.68±0.07</td>
<td>0.18±0.02</td>
<td>0.95±0.20</td>
<td>0.13±0.01</td>
<td>0.35±0.07</td>
<td>1.76±0.30</td>
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<tr>
<td>+TGF-β1</td>
<td>0.40±0.06‡</td>
<td>0.10±0.01‡</td>
<td>0.42±0.10‡</td>
<td>0.076±0.01‡</td>
<td>0.48±0.10‡</td>
<td>2.4±0.40‡</td>
</tr>
</tbody>
</table>

CD36 mRNA Expression in THP-1 Macrophages

<table>
<thead>
<tr>
<th>mRNA Expression in THP-1 Macrophages§</th>
<th>Alone</th>
<th>+PGJ2</th>
<th>+OxVLDL</th>
<th>+OxLDL</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0±1.80</td>
<td>339.3±46.54‡</td>
<td>198.5±4.50‡</td>
<td>138.0±3.00‡</td>
</tr>
<tr>
<td>+TGF-β1</td>
<td>61.7±1.50‡</td>
<td>108.1±2.89‡</td>
<td>125.2±1.76¶</td>
<td>82.0±2.00¶</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*Results expressed as ratio of band intensity relative to GAPDH (n=3).
†Results expressed as ratio of picograms of RNA relative to picograms of GAPDH RNA (n=4).
‡Different from control (P<0.05).
§Results expressed as percentage of control (ratio of band intensity relative to GAPDH, n=3).
¶Different from PGJ2 alone (P<0.05).

In contrast, exogenous LPL had no effect on the TGF-β1–induced decrease of CE or TG during incubation with oxVLDL or oxVLDL-REM (data not shown). This result confirms our previous observations that these lipoproteins are no longer optimal substrates for LPL activity.30 Thus, the ability of TGF-β1 to decrease macrophage uptake of oxVLDL and oxVLDL REM is independent of LPL activity.

**LDL-R Activity and mRNA Abundance Are Decreased by TGF-β1**

TGF-β1 significantly decreased [125I]-LDL total cell association by 22% (P<0.05), represented as binding plus uptake and [125I]-LDL degradation by 20% (P<0.05) at 37°C (Figure 2). Binding of [125I]-LDL at 4°C was also significantly reduced by 42% (P<0.05). The decreased cell surface LDL-R expression by TGF-β1 was associated with a 41% (P<0.05, Table) reduction in LDL-R mRNA.

**TGF-β1 Does Not Affect HTG-VLDL–Induced CE Accumulation in LDL-R(−/−) MPMs**

The role of the LDL-R in the TGF-β1–induced decrease in macrophage CE accumulation was established in MPMs obtained from LDL-R(−/−) mice. Incubation of wild-type cells with HTG-VLDL increased cellular CE 3-fold (P<0.05, Figure 3). Preincubation with TGF-β1 decreased CE accumulation by 54% (P<0.05). In contrast, the attenuated increase (2-fold) in cellular CE in the LDL-R(−/−) cells was completely unaffected by TGF-β1, providing further evidence that the inhibition of cellular CE involves regulation of the LDL-R. The effect of TGF-β1 on cellular TG was similar for wild-type and LDL-R(−/−) cells (Figure 3), indicating that the effect of TGF-β1 on cellular TG accumulation is independent of the LDL-R.

**TGF-β1 Reduces OxVLDL and OxLDL Uptake in SRAI/II(−/−) MPMs**

Incubation of wild-type cells with oxVLDL and oxLDL increased cellular CE by 64- and 31-fold, respectively (P<0.05, Figure 4). Preincubation of the cells with TGF-β1 significantly decreased CE accumulation induced by oxVLDL (-36%, P<0.05) and oxLDL (-31%, P<0.05). In SRAI/II(−/−) cells

However, as shown in the Table, TGF-β1 significantly reduced LPL mRNA by 55.0% (P<0.05).

The importance of TGF-β1 inhibition of LPL activity on lipid uptake was determined by the addition of exogenous LPL to macrophages preincubated in the presence or absence of TGF-β1. Bovine milk LPL did not further enhance CE or TG accumulation induced by native VLDL, native VLDL-REM, oxVLDL, or oxVLDL-REM (data not shown), indicating that the amount of LPL secreted by these cells was not rate limiting. However, the addition of bovine milk LPL significantly attenuated the TGF-β1–induced decrease in cellular TG accumulation during incubation with native lipoproteins (Figure 1A). These findings further support our previous conclusions that lipoprotein TG hydrolysis is critical for macrophage TG accumulation, thereby facilitating remnant uptake.28,30 However, exogenous LPL only partially rescued the TGF-β1–induced inhibition of CE mass accumulation (Figure 1A), suggesting that TGF-β1 was affecting additional steps in native lipoprotein uptake, beyond LPL hydrolysis.
Effect of TGF-$\beta$1 (5 ng/mL) on lipid mass accumulation (mean±SEM) in MPMs isolated from C57BL/6 and LDL-R(-/-) mice (n=4). Incubation conditions were as described in Figure 1. *P<0.05 vs control; †P<0.05 vs lipoprotein alone.

Figure 3. Effect of TGF-$\beta$1 (5 ng/mL) on CE mass (mean±SEM) in MPMs isolated from CD-1 and SRAI/II(-/-) mice (n=3). Incubation conditions were as described in Figure 1. *P<0.05 vs control; †P<0.05 vs LPDS; ‡P<0.05 vs BSA; ††P<0.05 vs HDL3; †††P<0.05 vs lipoprotein alone.

Figure 4. Effect of TGF-$\beta$1 (5 ng/mL) on CE mass (mean±SEM) in MPMs isolated from CD-1 and SRAI/II(-/-) mice (n=3). Incubation conditions were as described in Figure 1. *P<0.05 vs control; †P<0.05 vs LPDS; ‡P<0.05 vs BSA; ††P<0.05 vs HDL3; †††P<0.05 vs lipoprotein alone.

Figure 5. Cholesterol efflux from cholesterol-loaded J774A.1 macrophages preincubated with TGF-$\beta$1 (5 ng/mL, 0.2% BSA) for 24 hours before a 12-hour incubation with BSA (0.2%) and either apoA-I (10 µg/mL), LPDS (5%), or HDL3 (100 µg/mL) in the absence or presence of TGF-$\beta$1. Cholesterol efflux was expressed as the percentage of counts (mean±SEM) in the medium vs total $[^{3}H]$cholesterol counts (media plus cell). *P<0.05 vs BSA; †P<0.05 vs cholesterol acceptor alone (n=3).

TGF-$\beta$1 Enhances Cholesterol Efflux

We determined whether TGF-$\beta$1 affected the release of labeled cholesterol from cholesterol-loaded J774A.1 macrophages by various acceptor molecules. ApoA-I enhanced cholesterol efflux 1.7-fold over BSA alone (P<0.05, Figure 5). Preincubation with TGF-$\beta$1 enhanced apoA-I-mediated cholesterol efflux by a further 37% (P<0.05). Cholesterol efflux to HDL3 was also significantly increased by TGF-$\beta$1 (32%, P<0.05). Because the experiments in which TGF-$\beta$1 inhibited lipoprotein-induced CE accumulation were performed in DMEM containing only LPDS, we determined whether LPDS could act as a cellular cholesterol acceptor. LPDS increased the efflux of cholesterol 1.8-fold (P<0.05) over BSA alone. Importantly, TGF-$\beta$1 further increased cellular cholesterol efflux to LPDS by 30% (P<0.05), a value similar to that observed for apoA-I.

Although the cholesterol acceptors in LPDS have not been completely defined, we determined that LPDS provided a final medium apoA-I concentration of 5 µg/mL, an amount sufficient to effect efflux. Alternate acceptors of cholesterol,
including apoA-II and apoE, were not detected in the LPDS used.

The cholesterol transporters ABCA1 and ABCG1 are known to regulate apoprotein-mediated cholesterol efflux from macrophages. In addition, the compound DIDS has been shown to inhibit ABCA1-mediated cholesterol efflux to LPDS by 37% (data not shown), indicating the involvement of ABCA1. The effect of TGF-β1 on macrophage expression of ABCA1 and ABCG1 mRNA abundance is shown in the Table. TGF-β1 increased ABCA1 mRNA by 37% and ABCG1 by 35% (P<0.05 for both), indicating a possible mechanism by which TGF-β1 stimulated cholesterol efflux.

Discussion

The regulation of macrophage activation and foam cell formation by cytokines has the potential to modulate the progression of atherosclerosis. In the present study, we demonstrate that TGF-β1 inhibits macrophage foam cell formation induced by type IV VLDL and their remnants in their native and oxidized forms. Our results indicate that TGF-β1 mediates its effects through modulation of several processes involved in foam cell formation. These include (1) inhibition of macrophage LPL, (2) inhibition of the expression and activity of the LDL-R, SRAI/II, and CD36, (3) enhancement of macrophage cholesterol efflux.

A novel aspect of the present study is that TGF-β1 decreases macrophage uptake and lipid accumulation induced by native VLDL, VLDL-REM, and, to a lesser extent, LDL. Previously, we have demonstrated that cultured macrophages are capable of taking up native VLDL and VLDL-REM. Furthermore, cell surface expression of LPL is required for initial lipidic modification, and the LDL-R is responsible for the uptake of the TG-depleted remnant. TGF-β1 inhibited gene expression and cell surface activity of LPL; however, the addition of exogenous LPL to cells in the presence of TGF-β1 could only rescue cellular TG accumulation. The failure of LPL to completely restore macrophage CE accumulation indicated that TGF-β1 was also regulating remnant uptake and/or cellular cholesterol efflux. Our results showing decreased macrophage LDL-R activity and expression together with a lack of effect of TGF-β1 in LDL-R(–/−) macrophages support the concept that TGF-β1 contributes to reduced macrophage CE accumulation induced by VLDL and VLDL-REM, through inhibition of LDL-R expression. The importance of macrophage LPL and the LDL-R in atherogenesis is highlighted by results obtained in mice, in which macrophage-specific knockout of LPL or the LDL-R significantly decreased the extent of lesion formation.

A decreased expression of SRAI/II by TGF-β1 has been demonstrated in THP-1 macrophages, which resulted in reductions in 125I-AcLDL binding and degradation. In the present study, we extend the physiological significance of this observation to include decreased SRAI/II-mediated uptake of oxVLDL. However, there is increasing evidence indicating that the uptake of oxidized lipoproteins involves receptors other than SRAI/II. Lougheed et al reported that uptake of oxLDL was reduced by only 30% in MPMs isolated from SRAI/II(–/−) mice. We have now shown that CE accumulation induced by oxVLDL in SRAI/II(−/−) macrophages is similarly reduced by ~30%, indicating that SRAI/II is only partially responsible for macrophage oxVLDL uptake. Our finding that TGF-β1 could further reduce the CE accumulation in SRAI/II(–/−) macrophages incubated with oxVLDL by ~30% strongly suggests that TGF-β1 regulates additional processes involved in oxidized lipoprotein–induced foam cell formation.

CD36 can be upregulated in THP-1 macrophages by its own ligand, oxLDL, an effect potentiated by PPARγ ligands. In the present study, we demonstrated that oxVLDL also upregulates CD36 mRNA expression but to a greater extent than does oxLDL. Thus, oxVLDL has the potential to amplify its ability to induce foam cell formation. In the present study, we confirm that TGF-β1 inhibits the expression of CD36 in THP-1 cells, an effect mediated through reduced activation of PPARγ-mediated signaling. Furthermore, we now demonstrate that the oxVLDL–induced increase in CD36 expression is reduced substantially by TGF-β1. Thus, oxVLDL may regulate CD36 in a manner similar to that of oxLDL, through a PPARγ-mediated mechanism. These results suggest that the reduction in CD36 expression by TGF-β1 contributes to the decrease in lipid accumulation in macrophages induced by oxVLDL.

Another key step in macrophage processing of lipoprotein–derived cholesterol involves the esterification of FC to CE, catalyzed by the enzyme ACAT, and the subsequent hydrolysis of CE to FC, catalyzed by NCEH. In the present study, NCEH activity was not affected by TGF-β1, a finding similar to that reported in smooth muscle cells. However, we demonstrated that TGF-β1 inhibited VLDL-induced cholesterol esterification. This observation parallels experiments in THP-1 macrophages, in which AcLDL-stimulated ACAT activity was inhibited by TGF-β1. However, TGF-β1 does not regulate ACAT expression in macrophages, and we now report that the addition of TGF-β1 directly to monocytes isolated from macrophages has no effect on ACAT activity. Therefore, inhibition of ACAT activity by TGF-β1 is likely secondary to reduced cholesterol availability in the ACAT substrate pool.

The importance of cholesterol efflux in foam cell formation has been enhanced by elucidation of mutations in ABCA1 as the molecular basis of Tangier disease. ABCA1 is a member of the ABC transporter family, which is involved in the control of apoA-I–mediated cholesterol efflux from macrophages. In the present study, we demonstrate that TGF-β1 enhances ABCA1 mRNA expression and increases cholesterol efflux to apoA-I, HDL3, or LPDS. We reasoned that if increased cholesterol efflux contributed to the TGF-β1–induced decrease in macrophage CE accumulation, then a component of the loading medium must also contain a cholesterol acceptor. Indeed, LPDS alone was found to contain apoA-I at a concentration capable of affecting cholesterol efflux. Recently, Panousis et al reported that TGF-β1 treatment of MPMs isolated from apoE(−/−) mice increased ABCA1 expression and cholesterol efflux to apoA-I and HDL, observations entirely consistent with the present findings. Another member of the ABC family, ABCG1, which is regulated in a manner similar to that of ABCA1, is involved in macrophage cholesterol efflux. A novel observation in the present study is that TGF-β1 induces the expression of ABCG1 to an extent similar to that observed for ABCA1. These data suggest that TGF-β1–
enhanced macrophage cholesterol efflux, mediated by increased expression of ABCA1 and ABCG1, contributes to the reduction in cellular CE in cells incubated with native VLDL, oxVLDL, and their remnants.

The inhibition of macrophage foam cell formation by TGF-β1 involves alteration in gene expression, likely mediated through a signal transduction pathway. After binding of TGF-β1 to its receptor, the primary downstream signaling events are mediated by Smads, a novel family of signaling molecules. Heteromeric complexes of Smads enter the nucleus and, along with other transcription factors, regulate the expression of TGF-β1-responsive genes. Whether Smad complexes mediate the TGF-β1–induced changes in macrophage gene expression observed in the present study remains to be determined. TGF-β1 can also activate the mitogen-activated protein (MAP) kinase (MAPK) cascades, thereby mediating some of the TGF-β1–induced downstream signaling events. Although MAPK–mediated phosphorylation of PPARγ causes the decreased expression of CD36 by TGF-β1, the role of MAPK in the regulation of the LDL-R, LPL, SRAI/II, ABCA1, and ABCG1 by TGF-β1 has not been fully elucidated.

Our discovery that TGF-β1 inhibits macrophage foam cell formation in vitro suggests that it has an antithrombogenic role in vivo. Atherosclerosis studies in mice have demonstrated that higher levels of TGF-β1 are associated with reduced susceptibility to lesion development. In humans, the evidence is less clear-cut. Reduced serum concentrations of TGF-β1 have been correlated with a lower incidence of coronary artery disease. On the other hand, increased colocalization of TGF-β1 with fibrofatty and restenotic lesions has been documented, implicating TGF-β1 as proatherogenic.

In the present study, we clearly show that TGF-β1 can inhibit the macrophage uptake of native and oxidized lipoproteins and enhance cholesterol efflux, thereby reducing foam cell formation. Thus, TGF-β1 has the potential to decrease fatty streak formation and enhance plaque regression. Because macrophage foam cells are also characteristic of unstable plaques, inhibition of foam cell formation would contribute to other known effects of TGF-β1 in macrophages, thereby resulting in enhanced plaque stabilization. Therefore, TGF-β1 has the potential to reduce atherosclerosis through a number of different mechanisms and at several stages of disease development.

Acknowledgments

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References


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Figure I

Cell Surface LPL

- Control
- + TGFβ-1

Secreted LPL

μmoles FFA/mg cell protein/hr at 37°C

Control     HTG-VLDL     VLDL-REM
Control     HTG-VLDL     VLDL-REM
Transforming Growth Factor β-1 Inhibits Macrophage Cholesteryl Ester Accumulation Induced by Native and Oxidized VLDL-Remnants

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Methods:

mRNA Abundance

A Hind III/Xba I fragment from glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ATCC) subcloned into pGEM-7Zf (Promega), an EcoRI/BamHI fragment from murine LPL (ATCC) subcloned into pBluescript IISK (Strategene) and a Sac1/Apa1 fragment from SRAI/II (a kind gift from A. Daugherty, University of Kentucky, Lexington, Kentucky, USA) subcloned into pGem Vector T (Promega), served as templates to synthesize anti-sense RNA probes. These riboprobes were used to measure mRNA concentrations in a modification of the RNase protection/solution hybridization assay of Azrolan and Breslow 1, as described previously 2.

Custom primers were generated corresponding to murine CD36 (accession # L23108, base pairs (bp) 1140-1194), human GAPDH (accession # M32599, bp 718-741), murine LDL-R (accession # X64414, bp 419-477), human ABCA1 (accession# AF165310, bp 198-237) and murine ABCG1 (accession# NM_009593, bp 1499-1545) (Life Technologies). Oligonucleotides (5 pmoles) were 5’-end labeled with [γ32P] ATP.
(7000Ci/mmol, Amersham, 10min, 37°C) using T4 polynucleotide kinase (Life Technologies, 10U/µl) and were re-isolated after filtering through quick spin centrifuge columns (Boehringer Mannheim GmbH Diagnostic, Montreal, Quebec). Each oligonucleotide of interest was simultaneously hybridized with GAPDH to total RNA (20µg) from either control or treated cells and incubated over night at 55°C ³. Samples were then incubated with 300U of S1 nuclease (Boehringer Mannheim GmbH Diagnostic, 30min, 37°C), RNA was precipitated and separated by denaturing polyacrylamide gel electrophoresis (19%) ³. Bands were visualized using a phosphoimager and quantified using Image Quant software.

References


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Figure Legends:

**Figure I**: Effect of TGFβ-1 on lipoprotein lipase (LPL) activity secreted into the medium and bound to the cell surface (heparin releasable) by J774A.1 macrophages incubated with HTG-VLDL or VLDL-REM. LPL activity was assayed from media collected from macrophages after incubation for 24h in the absence or presence of heparin (10U/mL medium), TGFβ-1 (5ng/ml media) and either HTG-VLDL or VLDL-REM (50µg lipoprotein total cholesterol/ml of media). Heparin releasable LPL activity represents LPL activity at the cell surface. Values are expressed as mean +/- SEM. *P<0.05 vs incubation in the absence of TGFβ-1. Data are representative of 2 experiments done in triplicate.