TGF-β1 downregulates CD36 and scavenger receptor A but upregulates LOX-1 in human macrophages

GEORG DRAUDE AND REINHARD L. LORENZ
Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Universität München, 81245 Munich, Germany

Draude, Georg, and Reinhard L. Lorenz. TGF-β1 downregulates CD36 and scavenger receptor A but upregulates LOX-1 in human macrophages. Am J Physiol Heart Circ Physiol 278: H1042–H1048, 2000.—Transforming growth factor-β1 (TGF-β1), a key cytokine for control of cell growth, extracellular matrix formation, and inflammation control, is secreted by many cells present in the atherosclerotic plaque. Lipid accumulation in the vessel wall is regarded as an early step in atherogenesis and depends on uptake of modified low-density lipoprotein (LDL) by macrophages through scavenger receptors and their transformation into foam cells. Prominent members of the scavenger receptor family are the class A type I and II receptors (ScR-A), the class B receptor CD36, and the recently detected lectin-like oxidized LDL receptor-1 (LOX-1), which, unlike the native LDL receptor (LDL-R), are not feedback controlled. CD36 is responsible for >50% of modified LDL uptake into human monocyte-derived macrophages. We therefore studied whether TGF-β1 influences expression and function of ScR-A, CD36, and LOX-1 in monocytes using RT-PCR and flow cytometry. Total uptake of oxidized LDL by monocytic cells, reflecting the combined function of all scavenger receptors, was significantly reduced by TGF-β1. At initially low picomolar concentrations, TGF-β1 decreased CD36 mRNA and protein surface expression and ScR-A mRNA levels in the human monocytic cell line THP-1 and in freshly isolated and cultivated human monocytes, whereas LOX-1 mRNA was increased. Expression of LDL-R and β-actin was not affected by TGF-β1. In conclusion, depression of scavenger receptor function in monocytes by TGF-β1 in low concentrations reduces foam cell formation. Together with matrix control by TGF-β1, this may be important for atherogenesis and plaque stabilization.

transforming growth factor-β1; scavenger receptor class A

TRANSFORMING GROWTH FACTOR-β1 (TGF-β1) is a key cytokine that is physiologically involved in morphogenesis, growth control, extracellular matrix formation, and inflammatory and immunologic responses (9, 19, 26, 27). The TGF-β1 precursor protein can be secreted by many cells, including smooth muscle cells, endothelial cells, and monocytes, and has to be activated by plasmin cleavage (15, 18). TGF-β1 was found to be overexpressed in atherosclerotic lesions, and exogenous TGF-β1 enhanced neointima formation in some animal models of atherosclerosis (14). The contribution of the multiple actions of TGF-β1 to atherogenesis is, however, complex. In fact, a strong negative correlation of active TGF-β1 levels to the severity of coronary atherosclerosis has been reported (3, 10). TGF-β1 is a weak mitogen in some cases and inhibits cell growth in most cell types. TGF-β1 limits, for example, platelet-derived growth factor-induced cell proliferation in man. TGF-β1 increases the deposition of extracellular matrix, reduces its proteolytic degradation (4), and attenuates inflammatory monocyte infiltration. Therefore, rather than acting as a growth factor driving plaque progression, TGF-β1 could promote vascular repair and support stabilization of plaques prone to rupture. A large lipid core is regarded as a hallmark of unstable plaques, and regression or prevention of new lesions also requires negative effects on cell immigration and lipid accumulation in the vessel wall. Lipid accumulation in the vessel wall depends on the intracellular uptake by macrophages transforming into foam cells. Overloaded foam cells finally decay, leaving extracellular lipid deposits. Lipid overload of macrophages is brought about by several classes of scavenger receptors that, unlike the native low-density lipoprotein (LDL) receptor (LDL-R), take up modified LDL and are not feedback controlled. High concentrations of TGF-β1 have been shown to downregulate ScR-A expression in a monocytic cell line (5), and autocrine TGF-β1 mediated the lack of ScR-A expression in a subclone of this cell line (23). However, this effect has never been demonstrated in human monocyte-derived macrophages. Over 50% of the uptake of modified lipoproteins into human monocytes occurs via CD36 (24), whereas ScR-A (28) has to share the rest with several other scavenger receptors, including the lectin-like oxidized LDL receptor-1 (LOX-1) (8, 25). Furthermore, CD36 functions as an adhesion receptor for collagen and thrombospondin, which are involved in macrophage anchoring in the plaque (2, 29). We therefore studied whether low concentrations of TGF-β1 influence ScR-A, LOX-1, and CD36 expression in monocytic cells and human macrophages.

METHODS

Materials. Human TGF-β1 was purchased from Promocell (Heidelberg, Germany), phenol from Amresco (Solon, OH), Moloney murine leukemia virus (M-MLV) reverse transcriptase was from Life Technologies (Eggenstein, Germany), and random hexamers were from Boehringer Mannheim (Mannheim, Germany). The DEAE column was from Applied Biosystems (Weterstadt, Germany), Ficol was from Biochrom

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
(Berlin, Germany), Falkon Primaria cell culture dishes were from Becton-Dickinson (Lincoln Park, N.J.), and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was from Molecular Probes (Leiden, The Netherlands). Mouse IgM negative control and FITC-labeled IgM were from Dako (Glostrup, Denmark), mouse anti-human CD36 was from Serotec (Oxford, UK), and Taq (Thermus aquaticus) polymerase and cell culture media and ingredients as well as all other chemicals were purchased from Sigma (Deisenhofen, Germany).

Subjects and cell culture. Human monocytoid THP-1 cells were obtained from American Type Culture Collection and cultivated in RPMI 1640 in the presence of 10% FCS and 200 mM glutamine. Differentiation of THP-1 cells was stimulated with 160 nM phorbol 12-myristate 13-acetate (PMA). Four to five million cells were seeded in 100 mm dishes with 10 ml of medium. Human TGF-β1 was dissolved in 5 mM citrate buffer (pH 3.5), and aliquots were diluted and frozen in PBS with 2 mg/ml BSA. Circulating monocytes from peripheral blood obtained from healthy, nonsmoking, informed male volunteers were isolated and cultivated as previously described (8).

Isolation, oxidation, and labeling of LDL. LDL (density 1.019–1.063 g/ml) was prepared from plasma of healthy, normolipidemic fasting volunteers by sequential ultracentrifugation. Oxidized LDL (oxLDL) was prepared by incubation of LDL (200 µg/ml) in EDTA-free, O2-saturated PBS containing 5 µM CuSO4 at 37°C for 6 or 20 h. Oxidation was stopped by the addition of EDTA (final concentration 0.24 mM). OxLDL was washed five times with PBS containing 0.24 mM EDTA using Centriflo ultrafiltration cones (CF25 Amicon) (11). OxLDL was labeled with DiI according to the manufacturer’s instructions at 37°C in the presence of lipoprotein-deficient serum (30 mg protein/ml) and ascorbic acid (100 µM) as previously described in detail (31). Labeled oxLDL was reisolated by ultracentrifugation, and all reagents were removed by repeated dialysis (four times) against Tris buffer (pH 7.4).

Quantitative RT-PCR. For detection of mRNA levels of LDL-R, ScR-A, CD36, and LOX-1, a quantitative RT-PCR was performed. After reverse transcription of total RNA into cDNA, PCR resulted in specific amplificates according to the mRNA levels of the several receptors. To quantify these products, they were analyzed by HPLC and the results normalized to levels of a housekeeping gene. The amplificates were always produced only one specific peak in the HPLC chromatograms at specific time points according to the length of the amplificates; this was confirmed in a control experiment performed with an ethidium bromide-stained agarose gel.

Specific ampliacates were quantified by HPLC separation on a nonporous DEAE column with a 0.3–0.6 M NaCl gradient buffered at pH 9.0, detected on an ultraviolet detector (Gilson-Abaem) at 260 nm, and with integration of corresponding absorption peak areas. Specific mRNA levels of LDL-R, ScR-A, CD36, and LOX-1 were normalized to levels of β-actin mRNA, which served as an endogenous control. The amplificates always produced only one specific peak in the HPLC chromatograms at specific time points according to the length of the amplificates; this was confirmed in a control experiment performed with an ethidium bromide-stained agarose gel.

Fig. 1. Time course of transforming growth factor-β1 (TGF-β1) effects on mRNA expression of low-density lipoprotein (LDL) receptor (LDL-R) (A), scavenger receptor A (ScR-A) (B), and CD36 (C). THP-1 cells were incubated for 12, 24, 48, or 72 h with 20 pM TGF-β1 added at start (solid lines). Expression of a receptor at each time point is expressed as a percentage of expression in control cells exposed to carrier only for same time (t). To compensate for potential TGF-β1 consumption or decay, we changed medium in some experiments and added fresh TGF-β1 at 48 h (dotted lines, arrows). Data are means ± SE; n = 5 experiments.
CD36 surface expression and oxLDL uptake. Surface expression of CD36 protein was measured by flow cytometry with a specific CD36 antibody, a secondary FITC-labeled mouse anti-human IgG, and a mouse IgM isotype control as described previously (11). A Becton-Dickinson flow cytometer (FACScan) was used.

The specific uptake of Dil-labeled oxLDL into cells after incubation for 3 h at 37°C was measured by FACScan and corrected for unspecific binding of Dil-labeled oxLDL in the presence of a 25-fold excess of unlabeled oxLDL.

Statistical analysis. The overall response to increasing concentrations of TGF-β1 in monocytic cells was tested by regression analysis. Comparisons of cells obtained from the same donor and incubated either under control conditions or in the presence of TGF-β1 were made by the matched pairs signed rank Wilcoxon test.

RESULTS

Freshly passaged THP-1 cells were cultured in the presence of 20 pM TGF-β1 for various time intervals to explore the time course of a potential TGF-β1 effect on lipoprotein receptor expression (Fig. 1). The level of expression of LDL (apolipoprotein B) receptor-specific mRNA, measured as a control marker, was not affected by incubation of the cells with TGF-β1 for ≥72 h. In contrast, ScR-A- and CD36-specific mRNA expression were suppressed below 50% by TGF-β1, reaching a minimum after ~48 h. This incubation time was therefore used in all subsequent experiments. Recovery of ScR-A and CD36 expression from suppression by TGF-β1 was assessed by flow cytometry using specific antibodies.

Statistical analysis. The overall response to increasing concentrations of TGF-β1 in monocytic cells was tested by regression analysis. Comparisons of cells obtained from the same donor and incubated either under control conditions or in the presence of TGF-β1 were made by the matched pairs signed rank Wilcoxon test.
TGF-β1 at 72 h was most likely caused by breakdown of TGF-β1 in the medium or consumption of the very low concentration of TGF-β1 used by cells. Repeated addition of fresh TGF-β1 (20 pM) after 48 h prevented the recovery of ScR-A expression, and partially prevented that of CD36 expression.

To establish the minimal concentrations of TGF-β1 influencing scavenger receptor expression and the maximal effect of TGF-β1, we then incubated THP-1 cells with increasing concentrations of TGF-β1 for 48 h. An approximately log-linear suppression effect on ScR-A- and CD36-specific mRNA was observed over the range of TGF-β1 concentrations tested (Fig. 2). For CD36, the effect was clearly detectable already at 2.5 pM TGF-β1. The ED50 was −10 pM, and at 120 pM TGF-β1, CD36 mRNA levels were suppressed ~90%. ScR-A expression was also clearly suppressed already by 5 pM TGF-β1, and the dose-response curve closely resembled that of CD36. β-Actin- and LDL-R-specific mRNA expression were not influenced by TGF-β1 over the whole range of concentrations tested. LOX-1 mRNA expression in THP-1 cells is very low (8) and was not tested in these cells. Cell count, viability, and morphological differentiation were not detectably affected by TGF-β1 exposure. TGF-β1 was added simultaneously with PMA to the cells, no preincubation with PMA was necessary, and no signs of toxicity were noted.

The effect of TGF-β1 on CD36 was independently confirmed at the level of CD36 protein surface expression (Fig. 3). Incubation with 20 pM TGF-β1 for 48 h significantly reduced CD36 protein surface expression as measured by flow cytometry to 67% compared with that in control cells incubated with carrier only.

To demonstrate the functional relevance of this reduction in CD36 and ScR-A expression, we determined the specific cell association of oxLDL in THP-1 cells by incubation of cells with 10 µg/ml Dil-labeled oxLDL for 3 h and quantification of cell fluorescence by flow cytometry. Cell association in the presence of a 25-fold excess of unlabeled oxLDL was simultaneously determined to account for unspecific binding. Incubation for 48 h with 20 pM TGF-β1 significantly decreased the specific cell association of oxLDL to 60% of the association seen in control cells incubated in carrier only (Fig. 4).

Finally, human monocyte-derived macrophages freshly isolated from the blood of healthy volunteers were resuspended in autologous serum and cultivated...
for 48 h in the presence of carrier only or 20 and 40 pM TGF-β1 (Fig. 5). Similar to the effects in THP-1 cells, ScR-A and CD36 mRNA levels in macrophages were significantly downregulated by TGF-β1. The human macrophages were only slightly less sensitive to TGF-β1 than were THP-1 cells. CD36 and ScR-A mRNA levels were reduced to ~80% by 20 pM TGF-β1 and to ~50% by 40 pM TGF-β1. The mRNA levels of LOX-1 were significantly increased by 40 pM TGF-β1 to >150% of control. The expression of β-actin- and LDL-R-specific mRNA in macrophages was not influenced by TGF-β1 treatment. Cell count, viability, and morphological differentiation of human macrophages also were not detectably affected by TGF-β1 exposure.

**DISCUSSION**

In the present study, we demonstrated for the first time a marked downregulation of CD36, the dominating scavenger receptor in monocytic cells and human monocyte-derived macrophages, by low concentrations of TGF-β1 both at the mRNA and surface protein level. A comparable downregulation was also demonstrated for the first time for ScR-A in monocytes, whereas the expression of LDL-R was not affected by TGF-β1 treatment. The functional relevance of the TGF-β1-mediated downregulation of CD36 and ScR-A was demonstrated by a marked reduction of oxLDL uptake by these cells. In contrast, the mRNA expression of the oxLDL receptor LOX-1, recently discovered in endothelial cells (25) and also found to be expressed in monocytes and smooth muscle cells (8), increased in human monocytes after TGF-β1 exposure. LOX-1 takes up oxLDL in an amount comparable to that of ScR-A type II in transfected Chinese hamster ovary cells, which may neutralize the chances for oxLDL uptake by these receptors. LOX-1 expression has previously been described to be decreased by lovastatin in monocytes (8) and to be increased by tumor necrosis factor-α and oxLDL exposure in endothelial cells (17), but the promoter region of LOX-1 and the mechanisms involved are unknown. Two studies have previously reported a downregulation of ScR-A in the presence of TGF-β1 in the monocytic cell line THP-1. Binding of acetylated LDL was significantly decreased in THP-1 cells by concentrations of TGF-β1 ranging from 24 to 240 pM, and suppression of ScR-A mRNA expression was demonstrated with 240 pM TGF-β1 (5). In the present study, ScR-A was already decreased in THP-1 cells as well as
in monocytes by 10- to 100-fold lower concentrations of TGF-β1. Although the local concentrations of TGF-β1 in the plaque are unknown, the very low concentration effective in our experiments supports the biological relevance of the TGF-β1 present in the plaque for the attenuation of scavenger receptor expression. Increased autocrine secretion of TGF-β1 has recently been identified as the cause of missing scavenging activity and lack of ScR-A expression in a subclone of cultivated THP-1 cells (23). Signals that increase autocrine TGF-β1 synthesis of monocytes or increase its activation, such as tissue plasminogen activator, might therefore exert a negative feedback control of ScR-A expression.

However, CD36, and not ScR-A or LOX-1, functions as the quantitatively dominating route for oxLDL uptake into human monocytes (24) as well as an anchoring receptor of monocytes to the extracellular matrix proteins collagen and thrombospondin. Here we demonstrated for the first time a downregulation of CD36 by TGF-β1. This adds a further mechanism to the complex role of TGF-β1 in atherogenesis. A negative effect of TGF-β1 on CD36 expression was found despite the presence of a nuclear factor (NF)-1 domain in the promoter region of the CD36 gene (1). An NF-1 binding site has previously been identified to mediate the stimulatory effect of TGF-β1 on nitric oxide synthetase in endothelial cells (13). The mechanisms involved in the signaling of TGF-β1 to the NF-1 binding site remain unknown as yet.

The recently described positive signaling pathway of TGF-β1 from the cell membrane to the nucleus implicates a family of serine/threonine kinase effectors, termed Smad proteins (16). Whereas Smad2 and Smad3 are phosphorylated by TGF-β1, Smad6 and Smad7 can suppress this effect (12, 22). Whether Smad7 directly signals inhibitory effects of TGF-β1 is, however, unknown. A new protein called SARA (Smad anchor for receptor activation) was described to be involved in the TGF-β1/Smad signal transduction pathway (30). SARA associates together with Smad2 to TGF-β1 and its receptor. As this complex is phosphorylated by a TGF-β type 1 receptor kinase, Smad2 dissociates and binds Smad4 to a heteromeric complex and regulates gene transcription. Sequences to which the Smad2/4 complex or Smad7 can bind and nuclear binding domains receiving other inhibitory signals from TGF-β1 have not yet been described.

A 50-base sequence termed ARE has been defined in the promoter region of Mix.2, an immediate-early response gene specifically activated by TGF-β superfamily members (6). However, ARE is not present in the promoter regions of ScR-A and CD36. Conversely, a consensus binding domain for C/EBP has recently been reported to mediate the induction of ScR-A by oxidative stress (20), but no negative regulatory elements have so far been described for ScR-A. Therefore, the molecular mechanisms of the TGF-β1 effect on ScR-A remain to be elucidated.

In conclusion, the TGF-β1-induced downregulation of ScR-A and CD36 scavenger receptors in monocyte-

Fig. 5. Effect of 20 and 40 pM TGF-β1 on mRNA expression of LDL-R (A), ScR-A (B), CD36 (C), LOX-1 (D), and β-actin (E) in freshly isolated human monocytes. Blood was obtained from 6 healthy volunteers, and cells were isolated by Ficoll gradient centrifugation and preincubated for 2 h for adhesion. Lymphocytes were withdrawn, and monocytes were incubated for 48 h with 20 or 40 pM TGF-β1 in autologous serum (20%). Data are means ± SE. *P < 0.05.
derived macrophages decreases foam cell formation. Together with the TGF-β1 control of extracellular matrix deposition and smooth muscle cell proliferation, this effect could contribute to the stabilization of atherosclerotic plaques. In fact, in an injury model, direct gene transfer of TGF-β1 has already been shown to alter the vascular response from a proliferative to a more reparative response (21).

We thank Prof. P. C. Weber for helpful discussion in the preparation of the manuscript.

This work is part of the doctoral thesis of G. Draude and was supported by August-Lenz-Stiftung, Munich, Germany. These data were presented at the 5th International Symposium on Multiple Risk Factors in Cardiovascular Disease, Venice, Italy, Oct. 28–31, 1999.

Address for reprint requests and other correspondence: R. Lorenz, Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Universität München, Pettenkoferstr. 9, 81245 Munich, Germany.

Received 3 May 1999; accepted in final form 9 November 1999.

REFERENCES