Vitamin E Reduces the Uptake of Oxidized LDL by Inhibiting CD36 Scavenger Receptor Expression in Cultured Aortic Smooth Muscle Cells

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Background—Vitamin E is well known as an antioxidant, and numerous studies suggest that it has a preventive role in atherosclerosis, although the mechanism of action still remains unclear.

Methods and Results—The original aim of this study was to establish whether α-tocopherol (the most active form of vitamin E) acts at the earliest events on the cascade of atherosclerosis progression, that of oxidized LDL (oxLDL) uptake and foam-cell formation. We show here that the CD36 scavenger receptor (a specific receptor for oxLDL) is expressed in cultured human aortic smooth muscle cells (SMCs). Treatment of SMCs and HL-60 macrophages with α-tocopherol (50 μmol/L, a physiological concentration) downregulates CD36 expression by reducing its promoter activity. Furthermore, we find that α-tocopherol treatment of SMCs leads to a reduction of oxLDL uptake.

Conclusions—This study indicates that CD36 is expressed in cultured human SMCs. In these cells, CD36 transports oxLDL into the cytosol. α-Tocopherol inhibits oxLDL uptake by a mechanism involving downregulation of CD36 mRNA and protein expression. Therefore, the beneficial effect of α-tocopherol against atherosclerosis can be explained, at least in part, by its effect of lowering the uptake of oxidized lipoproteins, with consequent reduction of foam cell formation. (Circulation. 2000;102:82-87.)

Key Words: tocopherol ■ receptors ■ lipoproteins ■ atherosclerosis

A dominant feature of atherogenesis is the migration of smooth muscle cells (SMCs) into the intima of the arterial wall. They proliferate, synthesize extracellular matrix, and together with macrophages accumulate cholesterol, leading to the progression of the atherosclerotic plaque. These changes are thought to be a genetic response to a number of stimuli, such as increased plasma cholesterol levels, hypertension, and oxidative stress. Together with proliferation and migration, the most characteristic events at the atherosclerotic lesion are cholesterol accumulation and conversion of macrophages and SMCs to foam cells. In SMCs, α-tocopherol specifically inhibits protein kinase C-α (PKC-α), which is strongly involved in the proliferative signal transduction pathway. Activation of PKC is known to lead to an increased activity of the transcription factor AP-1; thus, inhibition of PKC by α-tocopherol could lead to changes of the gene expression patterns altered in atherosclerosis. Candidate genes for such alteration are the scavenger receptors, which take up modified LDL, leading to foam cell formation at the atherogenic lesion. These receptors include SR-A, SR-B1, CD36, CD68, and LOX-1. Knockout mice for the macrophage scavenger receptor have shown reduced incidence of atherosclerosis, although smooth muscle–derived foam cells can still form in the remaining lesions. Expression of some of the scavenger receptors is increased at the atherosclerotic lesion, but to date the regulatory mechanisms for most of these genes are not yet known and could lead to a better understanding of the process, prevention, and therapy of this disease.

The CD36 scavenger receptor has been reported to be expressed in megakaryocytes/platelets, monocytes/macrophages, mammary epithelial cells, and adipocytes. It is also expressed in capillary endothelial cells of adipose, cardiac, and muscle tissue and at low levels in the vascular endothelium of the brain, lung, and kidneys. CD36 binds to a large variety of ligands: thrombospondin, collagens type I and IV, fatty acids, anionic phospholipids, Plasmodium falciparum–infected erythrocytes, HDL, and oxLDL. Monocytes/macrophages from CD36-deficient patients show a reduced capacity to bind and internalize oxLDL. In contrast to the LDL

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receptor, which is downregulated by a negative feedback loop mediated by cholesterol, CD36 appears to be upregulated by the cargo of oxLDL.11,12

We show here that the CD36 scavenger receptor is also expressed in cultured human aortic SMCs. This finding is important in the light of the still unknown mechanism of foam-cell formation from human aortic SMCs.13 Furthermore, we find that both the CD36 mRNA and the corresponding protein are downregulated by α-tocopherol. Moreover, the reduction of CD36 expression by α-tocopherol leads to a reduction of oxLDL uptake, which explains the beneficial effect of α-tocopherol on atherogenesis.

Methods

Cell Culture

RRR-α-tocopherol and β-tocopherol (Henkel) and probucol (Sigma) were dissolved in ethanol, and the concentrations of the stock solutions were confirmed spectrophotometrically. Human aortic SMCs (T/G) (ATCC CRL-1999) were cultured in DMEM/10% FCS and used between passages 4 and 10. Treatments were done with confluence as indicated in the text. HL-60 monocytes (kindly obtained from Dr M. Thelen, University of Bern, Theodor Kocher Institute, Switzerland) were grown in RPMI/10% FCS.

Immunofluorescence

Cells were plated on glass coverslips, allowed to attach overnight, and then treated with tocopherol as indicated in the figure legends. After the treatment, the cells were washed with PBS and fixed with 4% formaldehyde, 0.4% methanol, and 5% sucrose for 5 minutes. Nonspecific binding sites were blocked with 3% BSA in PBS for 30 minutes. The cells were incubated with CD36 antibody (monoclonal anti-human CD36-FTIC [Ancell] diluted 1/50 in PBS/1% BSA) for ≥30 minutes at room temperature. Then the cells were washed twice with PBS, incubated with PBS containing 1 μg/mL bisbenzimide (Hoechst 33258, Fluka), and washed 3 times with PBS. The slides were covered with 20 μL of 1× PBS/9% glycerin/1% p-phenylenediamine (Fluka) and then analyzed.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated with an RNA extraction kit from Qiagen. Semiquantitative assays for CD36 mRNA expression were performed with a reverse transcription–polymerase chain reaction (RT-PCR) kit (Perkin Elmer) with AmpliTaq polymerase and primer CD36PCR: 5’-ATCCCATATCTATCAAAATC-3’, which anneals to exon 6, and primer CD36PCR: 5’-TCGATTATGGCAACTTTAC-3’, which anneals to exon 7, for 22 cycles at 95°C, 30 seconds; 50°C, 30 seconds; and 72°C, 30 seconds. [α-32P]ATP (2 μCi) was included per reaction. Control reactions were performed with primers specific for human GAPDH: GAP1, 5’-AGGCCATCTGCTAAGACACC-3’ and GAP2, 5’-TGGGCTTGGTCTAGTCTTCTC-3’ for 18 cycles at 95°C, 30 seconds; 68°C, 30 seconds; and 72°C, 30 seconds. These conditions were found to be in the linear range of amplification. The identity of the amplified fragments was confirmed by sequencing. PCR without the reverse transcriptase step and PCR with genomic DNA did not result in amplification. The PCR products were loaded on a 6% polyacrylamide gel, and the radioactive signals were quantified with a phosphorimager (Biorad).

Western Blots

Western blot was done according to standard methods with monoclonal mouse anti-human CD36 primary antibody (Ancell) and sheep anti-mouse IgM secondary antibody coupled to horseradish peroxidase (Pierce). An anti-α-actin antibody (Santa Cruz) was used as internal control. Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer’s instructions (Amersham). Chemiluminescence was monitored by exposure to film (Hyperfilm ECL), and the signals were analyzed under nonsaturating conditions with an image densitometer (Biorad).

Labeling and Uptake of oxLDL

OxLDLs (90% to 100% oxidation) were purchased from Intracell Corp. Small amounts of LDL were oxidized with CuSO4 (20 μmol/L) at 37°C for 18 to 22 hours. LDL oxidation was confirmed by the formation of a characteristic smear band on an agarose gel. Labeling of oxLDL was done basically as previously described.14 OxLDLs were incubated at 37°C with Dio (Molecular Probes) in lipoprotein-deficient serum (Sigma) for 15 hours. The labeled oxLDLs (oxLDL-Dio) were purified by ultracentrifugation over a KBr gradient and dialyzed against several changes of saline-EDTA (1.5 mol/L NaCl–0.01% EDTA) for 6 hours.

Uptake of oxLDL was studied either with fluorescence/confocal microscopy or fluorescence-activated cell sorting (FACS). For microscopy, the cells were grown overnight on culture slides (Falcon) and then incubated with oxLDL-Dio (5 μg/mL medium) for 6 hours. Thereafter, the cells were washed 5 times with PBS–2 mg/mL BSA and once with PBS. For nucleus staining, the cells were incubated for 10 minutes with 1 μg/mL bisbenzimide (Hoechst 33258, Fluka) and washed 3 times with PBS. The cells were then fixed with 4% paraformaldehyde in PBS for 30 minutes. Finally, the slides were covered with 20 μL of 1× PBS/9% glycerin/1% p-phenylenediamine for 5 min. For FACS, the cells were pretreated for 16 hours with 50 μmol/L tocopherol, probucol, or ethanol solvent (control) and then incubated with oxLDL-Dio (5 μg/mL medium) for 6 hours. For competition experiments, the cells were incubated with monoclonal anti-CD36 antibody (60 μg/5 mL DMEM) (Ancell), with an unspecific isotype-matched antibody (mouse IgM, Ancell), or with unlabeled oxLDL (100 μg/5 mL DMEM) (Intracell Corp). Thereafter, the cells were washed 3 times with PBS and twice with PBS–3 mg/mL BSA and then were detached with trypsin (0.25% trypsin, 0.03% EDTA). The cells were harvested with DMEM/10% FCS, centrifuged, washed twice with PBS, and then fixed with 4% paraformaldehyde in PBS. FACS was performed with a FACScan (Becton-Dickinson). Data were calculated by subtracting the cell autofluorescence from the fluorescence of the treated samples.

Transfection and Luciferase Assay

A CD36 promoter fragment was amplified with the primer pair CDP0R, 5’-CTGGGCTTCTGCTACTTACG-3’ and CDP8R, 5’-CTTTGAAATGCTAGTACAG-3’, and the amplified fragment was cloned into pT7Blue-3 (Novagen). The identity of the amplified fragment was confirmed by sequencing. A KpnI/HindIII CD36 promoter fragment was cloned into pGL3-basic (pCD-basic). CD36 promoter plasmids and a pRL-TK internal control vector were transfected into T/G cells with Lipofectamine (Gibco). Tocopherol and probucol treatments (50 μg/mL) started 12 hours after transfection for 8 hours. Promoter activity was measured with the dual luciferase assay kit (Promega) with a TD-20/20 luminometer (Turner Designs). CD36 promoter–firefly-luciferase activity was normalized to the TK promoter–renilla-luciferase activity, and the activity of the untreated CD36 promoter was set to 100%.

Results

CD36 mRNA and Protein Are Expressed in Human Aortic SMCs and Inhibited by α-Tocopherol

Scavenger receptors are involved in the uptake of modified LDL, allowing the accumulation of lipids and cholesterol in macrophages and aortic SMCs and the consequent foam-cell formation. In macrophages, α-tocopherol reduces the expression of the scavenger receptor SR-A/II, which is mainly responsible for the uptake of acetylated LDL.15,16 However, this receptor is expressed at low levels in human aortic SMCs, the second cell type converting to foam cells at the athero-
sclerotic lesion. One of the aims of this study was to establish whether CD36, a scavenger receptor specific for the uptake of oxLDL, is expressed in aortic SMCs and whether α-tocopherol can inhibit its expression.

Human aortic SMCs (T/G cells) were treated at different time points with α-tocopherol or ethanol, as a solvent control, and total RNA was isolated and quantified by RT-PCR. Interestingly, α-tocopherol decreased CD36 mRNA expression in a time-dependent manner, at a concentration (50 μmol/L) found in moderately supplemented individuals (Figure 1A). When various concentrations of α-tocopherol were used, the reduction was strongest at 50 and 100 μmol/L, whereas β-tocopherol did not lead to any reduction (Figure 1B). We have shown PKC inhibition by α-tocopherol in previous studies. Thus, it was relevant to establish whether the effect seen at the level of CD36 was due to the α-tocopherol–mediated PKC inhibition. The results shown in Figure 1C excluded this hypothesis, because calphostin, a potent PKC inhibitor, did not affect CD36 expression. Western blot with an anti-CD36 monoclonal antibody showed that CD36 protein is expressed in T/G cells (Figure 2). Caco-2 cells and HL-60 monocytes, which do not express CD36 mRNA (data not shown), were used as negative controls (Figure 2A, lanes 1 and 2), whereas differentiated HL-60 cells were positive controls (Figure 2A, lane 3). Treatment of T/G cells with α-tocopherol (50 μmol/L) resulted in a time-dependent decrease of CD36 protein expression (Figure 2B), as was observed at the mRNA level. With immunofluorescence, overall CD36 expression was reduced in cells treated with α-tocopherol, but not with β-tocopherol (Figure 3). Caco-2 cells, which according to RT-PCR results do not express CD36 (data not shown), showed only background staining, confirming the specificity of the antibody. No increase in cytoplasmic staining became evident on α-tocopherol treatment, suggesting that CD36 protein is not translocated to the cytoplasm.

α-Tocopherol Inhibits oxLDL Uptake

Inhibition of CD36 scavenger receptor expression should lead to reduced oxLDL uptake. The uptake of fluorescent oxLDL-DiO was either visualized by fluorescence/confocal microscopy or quantified by FACS as described in the Methods section. As shown in Figure 4, oxLDL-DiO was taken up by T/G cells and accumulated in endosomes. The uptake of oxLDL-DiO was heterogeneous, because the cells stained with different intensities (data not shown). However, when
oxLDL-Dio uptake was quantified by FACS, the signal was lower in cells treated with α-tocopherol (Figure 5A). The median of the peak was reduced by 33.6 ± 2.3% in cells treated with α-tocopherol. Therefore, the inhibition of CD36 expression by α-tocopherol leads to reduced CD36-mediated oxLDL uptake.

To see whether CD36 is the major scavenger receptor responsible for the uptake of oxLDL in human aortic SMCs, T/G cells were preincubated for 30 minutes with an anti-CD36 monoclonal antibody before oxLDL-Dio was added for 6 hours. As already described with macrophages,19,20 an anti-CD36 antibody reduced the uptake of oxLDL-Dio in T/G cells (Figure 5B). In 2 experiments, the median of the peak was reduced by 60 ± 4.1% in cells treated with anti-CD36 antibody. Similarly, preincubation with unlabeled oxLDL as competitor. D, Cells were treated with probucol (50 μmol/L) or ethanol as control. E, Cells were incubated with unspecific isotype-matched antibody (mouse IgM). All treatments are described in Methods. Black line represents autofluorescence of cells in absence of oxLDL-Dio.

CD36 monoclonal antibody before oxLDL-Dio was added for 6 hours. As already described with macrophages,19,20 an anti-CD36 antibody reduced the uptake of oxLDL-Dio in T/G cells (Figure 5B). In 2 experiments, the median of the peak was reduced by 60 ± 4.1% in cells treated with anti-CD36 antibody. Similarly, preincubation of T/G cells with unlabeled oxLDL reduced the uptake of oxLDL-Dio by 55 ± 4.4% (Figure 5C), and the antioxidant probucol had no effect (Figure 5D). A control experiment with isotype-matched antibody (mouse IgM) did not show any reduction of oxLDL-Dio uptake (Figure 5E). These results show that CD36 is responsible for the α-tocopherol-mediated decrease of oxLDL uptake in aortic SMCs.

**CD36 Regulation by α-Tocopherol Is Promoter-Mediated**

The decrease in CD36 mRNA suggested that there must be a regulatory element in the CD36 promoter that is responsive to α-tocopherol. We isolated a CD36 promoter fragment that harbors the major transcriptional activity in U937 cells21 and cloned it into the luciferase reporter vector pGL3-basic, leading to pCD-basic. The construct pCD-basic was transfected into T/G cells, and luciferase activity was measured as described in the Methods section. The CD36 promoter
by reducing the formation and uptake of cholesteryl ester, by decreasing SMC proliferation via inhibition of the PKC pathway, and by preventing inflammation and monocyte/macrophage adhesion to the endothelium.

We show here that CD36 scavenger receptor is expressed in human aortic SMCs and that α-tocopherol downregulates its expression. The reduction of CD36 expression with α-tocopherol is due to the reduction of CD36 promoter activity, although other mechanisms, such as an increase of CD36 protein trafficking, cannot be excluded at this time. Furthermore, we find that α-tocopherol treatment of aortic SMCs leads to a reduction of oxLDL uptake. A similar action of α-tocopherol has been described in macrophages, in which tocopherol reduced cholesterol esterification and uptake of acetylated LDL. Similarly, α-tocopherol decreased the toxicity of oxLDL in macrophages and reduced tumor necrosis factor-α–mediated LDL/oxLDL accumulation in the artery wall. It appears possible that all these effects of α-tocopherol are due to the downregulation of the CD36 scavenger receptor.

Our results suggest the existence of signaling pathways that regulate CD36 expression in response to α-tocopherol. Like CD36, the collagenase MMP-1 and the collagen-α1 genes are downregulated by α-tocopherol at the transcriptional level. A number of transcription factors are redox-regulated, and α-tocopherol may influence gene expression by preventing oxidation. The lack of CD36 modulation by β-tocopherol and probucol appears to exclude this hypothesis. Furthermore, because it is known that α-tocopherol inhibits PKC activity, phosphorylation/dephosphorylation of transcription factors may be involved in these responses. However, the CD36 scavenger receptor appears not to be regulated by PKC, because its mRNA expression was not affected by treatment of cells with the PKC inhibitor calphostin. Conversely, expression of the scavenger receptors AI/II is stimulated by PKC-α, and α-tocopherol reduces expression of this receptor and the uptake of acetylated LDL in macrophages, most likely via inhibition of PKC. We found that expression of the class A scavenger receptor is low in T/G cells (data not shown), suggesting that the decreased uptake of oxLDL-DiO is mainly due to the reduction of CD36 expression. Alternatively, α-tocopherol could modulate gene expression directly, by binding to a specific receptor, or indirectly, by inhibiting the action of peroxisome proliferator–activated receptor-γ activators (9-HODE and 13-HODE), which stimulate CD36 expression. Indeed, α-tocopherol reduced the level of 9-HODE and 13-HODE by inhibiting their enzymatic production.

The symptoms of CD36/fatty acid translocase (FAT)–deficient spontaneously hypertensive rats are hypertension and defective fatty acid as well as glucose metabolism. Thus, the complete absence of CD36/FAT expression in these animals leads to deregulation of cardiovascular physiology, insulin resistance, and diabetes. Conversely, CD36 expression is increased in the heart of diabetic rats and mice, suggesting that in diabetes, the cardiac myocytes rely more on fatty acid uptake and oxidation. Because CD36 is expressed to a significant extent at the atherosclerotic lesion, part of the beneficial effect of α-tocopherol may thus be due to the

Figure 6. Inhibition of CD36 promoter activity by α-tocopherol in T/G human aortic SMCs. After transfection with pcDNA-basic, cells were treated for 8 hours with 50 μmol/L α-tocopherol, β-tocopherol, or probucol, and luciferase activity was measured as described in Methods. Experiment was repeated 3 times, and SD is indicated.

Figure 7. Inhibition of CD36 expression by α-tocopherol in HL-60 cells differentiated with 9-cis-retinoic acid. HL-60 cells were differentiated with 9-cis-retinoic acid and treated with α-tocopherol or ethanol (control) as described in text. CD36 expression was assayed by FACS with monoclonal anti–CD36-FITC antibody as described for T/G cells.

Inhibition of CD36 Expression by α-Tocopherol in HL-60 Cells

Because foam cells are formed primarily by monocytes/macrophages, we tested whether HL-60 cells showed a significant effect on differentiation of HL-60 cells, as monitored by the reduction of nitro blue tetrazolium.
reduction of CD36 expression.\textsuperscript{8} Resetting of increased CD36 expression in diabetes and atherosclerosis could explain the beneficial effects of \textalpha-tocopherol during the progression of both of these diseases. Other studies have shown that the predominant cell type in early atherosclerotic lesions is smooth muscle–derived cells.\textsuperscript{13} Moreover, it was shown that scavenger receptors can be detected in the atherosclerotic lesion only in activated SMCs,\textsuperscript{17} and ongoing research in our laboratory confirms these findings (data not shown).

In summary, our findings indicate that, as in macrophages, the uptake of oxLDL in human aortic SMCs is mediated by the CD36 scavenger receptor. Vitamin E interferes with the oxLDL uptake by reducing the expression of CD36. A further characterization of the regulation of this scavenger receptor, in response to a number of proatherogenic and antiatherogenic conditions, could lead to new treatments useful to prevent initiation and reverse progression of atherosclerosis.

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References
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