Novel 5′ Exon of Scavenger Receptor CD36 Is Expressed in Cultured Human Vascular Smooth Muscle Cells and Atherosclerotic Plaques

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Abstract—CD36, a member of the scavenger receptor family, is centrally involved in the uptake of oxidized low density lipoproteins (oxLDLs) from the bloodstream. During the atherosclerotic process, the lipid cargo of oxLDL accumulates in macrophages and smooth muscle cells (SMCs), inducing their pathological conversion to foam cells. Increased expression of CD36 occurs in human atherosclerotic lesions, and CD36 knockout mice show reduced uptake of modified LDLs and reduced atherosclerosis. Here, we describe a novel exon 1b and extended CD36 promoter in human SMCs. Exon 1b is specifically transcribed in activated aortic SMCs and mainly expressed in atherosclerotic plaques. Thus, switching to exon 1b transcription may be an important step for the activation of SMCs and their conversion to foam cells. Using an antisense oligonucleotide to exon 1b, we inhibit CD36 translation and highly reduce oxLDL uptake. The antisense to exon 1b does not affect CD36 in cell lines not expressing the new exon. The possibility of a novel antiatherosclerotic therapy and the use of exon 1b as a marker of atherosclerosis are discussed. (Arterioscler Thromb Vasc Biol. 2002;22:412-417.)

Key Words: scavenger receptors • CD36 • atherosclerosis • oxidized LDL • gene structure

Atherosclerosis and coronary heart disease are the leading causes of death in the developed world. During the atherosclerotic process, lipoproteins, such as LDL, are converted to oxidized LDL (oxLDL), containing modified proteins and lipids.1 A family of genes, the scavenger receptors, recognizes and internalizes modified lipoproteins, making them susceptible to degradation. However, uncontrolled expression of scavenger receptors can lead to foam cell formation.2 Although each scavenger receptor shows a broad specificity, clear preferences for certain ligands have been described.3 The CD36 scavenger receptor is specific for nitrated LDL and oxLDL, the most atherogenic forms of modified LDL.4 CD36 knockout mice exhibit a reduced uptake of modified LDL and reduced atherosclerosis.5 Moreover, increased expression of CD36 occurs in human atherosclerotic lesions.2,6 CD36 is implicated in cytoadherence to Plasmodium falciparum–infected erythrocytes,7 and a CD36 gene mutation has been associated with malaria protection, suggesting a CD36 role in host-parasite interactions.8 CD36 is expressed in numerous tissues and cell lines, such as heart and muscle, capillary endothelial cells, megakaryocytes/platelets, monocytes/macrophages, epithelial cells, and adipocytes. This tissue and cell type specificity suggests that CD36 expression must be highly regulated at transcriptional and posttranscriptional levels. To date, the regulation of CD36 expression was studied mainly in human monocytes, macrophages, platelets, and B lymphocytes. In macrophages, CD36 expression is upregulated by the content of oxLDL, such as the peroxidation products 9-HODE and 13-HODE.6,9,10 These agents stimulate transcription of the CD36 gene by activating the peroxisome proliferator–activated receptor-γ (PPARγ), which binds a regulatory element in the CD36 promoter. Moreover, phosphorylation of PPARγ, mediated by transforming growth factor (TGF)-β1/2–activated mitogen-activated protein kinase, inhibits CD36 expression in THP-1 macrophages.11 At the atherosclerotic plaque, active TGF-β1 is decreased, and the type II TGF-β1 receptor is mutated, suggesting that overexpression of CD36 in plaques may be a result of impaired TGF-β signaling.12,13 Similarly, interleukin-4 stimulates CD36 expression by coordinate induction of PPARγ and 12/15-lipoxygenase expression, which increases the level of the endogenous ligands for PPARγ.14

The human CD36 gene structure has previously been determined in melanoma cells.15 Later, two 5′-untranslated exons were identified in monocytic cell lines.16 Furthermore, 2 alternatively spliced 5′-untranslated exons (exons 2a and 2b) were identified in erythroid leukemia cells,17 and several further splicing variants have been described.18 Previously, we have described the expression of the CD36 scavenger receptor in human vascular smooth muscle cells.

Received September 4, 2001; revision accepted November 6, 2001.
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(human vascular SMCs [T/G cells]). In transfection experiments, we showed that the previously described core promoter was active when transfected into T/G cells and inhibited by treatment with α-tocopherol. In the present study, we identify an extended CD36 promoter and novel exon (exon 1b) in T/G cells. (GenBank accession number of the CD36 promoter and exon 1b is AF266759.) Exon 1b is mainly expressed in atherosclerotic plaques, indicating that CD36 promoter and exon switching may be involved in the cell activation during the formation of the atherosclerotic lesion. Antisense oligonucleotides to exon 1b were evaluated as inhibitors of CD36 expression and oxLDL uptake. The possibility of a novel antiatherosclerotic therapy is discussed.

**Methods**

**Cell Lines and Culture**

Human aortic SMCs (T/G cells, American Type Culture Collection No. CRL-1999) were cultured in DMEM/10% FCS and used between passages 4 and 10. Treatments were performed with subconfluent cells, as indicated in the text. ISS10, simian virus 40 (SV40)-immortalized human aortic SMCs, were kindly provided by Dr Y. Sasaguri (Department of Pathology, University of Occupational and Environmental Health, Kitakyushu, Japan) and grown in DMEM/10% FCS. Primary coronary SMCs (Cascade Biologies, Inc) were grown in medium 231 containing smooth muscle growth supplement (Cascade Biologies, Inc). HL-60 monocytes were grown in RPMI/10% FCS and differentiated to macrophages by treatment with 9-cis-retinoic acid (100 nmol/L) for 4 days. U937 and HEL cells were cultured in RPMI/10% FCS/0.2% glucose and 1 mmol/L sodium pyruvate. Skin fibroblasts and MCF-7 breast cancer cells were grown in medium 231 containing smooth muscle growth supplement (Cascade Biologies, Inc). The HepG2 hepatoblastoma cell line was grown in MEM/10% FCS and 1 mmol/L sodium pyruvate. The CaCo-2 colon adenocarcinoma cell line was grown in MEM/20% FCS.

**Aortic Tissues**

Aortic samples, with and without plaques, were obtained from transplant-organ donors and had the quality of transplantable material. They were immediately frozen in liquid nitrogen and kept at −80°C if not immediately processed.

**5′-RACE Protocol**

Total RNA was isolated from T/G cells at 80% confluence by using the Trizol reagent (GIBCO-BRL). The mRNA was isolated by using twice the Oligotex Midi mRNA isolation kit (Qiagen). The mRNA was isolated by using the anchor primer provided with the kit as an upstream oligonucleotide and primer Cdxex5 (5′-CTCTGGATAAGCAGGTCCTC-3′), which is specific to exon 3 of the human CD36 mRNA. A poly(A) tail was added to the 5′ end of the cDNA and then amplified by using the anchor primer provided with the kit as an upstream oligonucleotide and primer Cdxex3 (5′-CTCTGGATAAGCAGGTCCTC-3′), which is specific to exon 3 of the human CD36 mRNA, as a downstream oligonucleotide. The polymerase chain reaction (PCR) fragments were then cloned into the pGEM-T easy vector (Promega), and several clones containing an insert were sequenced.

**Reverse Transcription–PCR**

Semi-quantitative reverse transcription (RT)-PCR was performed basically according to the RT-PCR kit from Perkin-Elmer. A PCR product specific for the new exon 1b was obtained by using primer CDexnew (5′-GAAGTTATATAGGGACAGG-3′), which is specific for the new exon 1b, and primer CDex3a (5′-GTTCAGAGGTGAATTAGTG-3′), which anneals to exon 3, with the same conditions as described above for 30 cycles, leading to a 205-bp PCR fragment. RT-PCR for CD36 expression was performed with primer CD36PCRF (5′-ATCCCCATCTCT-AAAATC-3′), which anneals to exon 6 and primer CD36PCR (5′-TCGATTTAGGGCAACTTAC-3′), which anneals to exon 7.

**Promoter Cloning**

For cloning the new promoter sequence, PCR was performed with human genomic DNA (Roche) and primer CDPromf (5′-CTGGGCCTCGACTTACTTGCTAGG-3′), which anneals to the CD36 promoter, and primer CDnewrev (5′-CCTCTATATAACCTTGAGGACAGG-3′), which anneals to the new exon 1b. Conditions for PCR were as follows: 95°C, 30 seconds; 55°C, 30 seconds; and 72°C, 1 minute, for 35 cycles. To avoid amplification of mutated products, Expand High Fidelity Polymerases (Roche) were used. The PCR fragments were cloned into pGEM-T easy vector (Promega), and several clones containing an insert were sequenced.

**Transfection and Promoter Assays**

The extended promoter fragment was inserted into pGL3-basic (Promega), leading to pCDb-basic and pCDb-basic/as, and into pGL3-enhancer (Promega), leading to pCDb-enh and pCDb-enh/as. Plasmids pCD-basic and pCD-enh have been previously described. The plasmids were transfected into T/G cells with Superfect (Qiagen), as described by the manufacturer, and pRL-TK (Promega) was used as an internal control vector. Promoter activity was measured by using the dual luciferase kit (Promega) with a TD-20/20 luminometer (Turner Designs). Duplicate transfections were always made with standard deviations indicated in the figures.

**Antisense Experiments**

Phosphorothioate oligonucleotides specific for exon 1b (CD1bantiA [5′-TATATACCTCTTAGAGATA-3′]), exon 1b (CD1bantiB [5′-CTTCCCTCTATTATAACCTTG-3′]), exon 1a (CD1aantiA [5′-TC-AAAATGCTCCAAACATTGTG-3′]), exon 3 (CD3ex3antiA [5′-GCCCATTTCCTTTCTTGTCAGG-3′]), and a scrambled control (CD1bscrambm [5′-AACCTAATTTCTTCCTAGT-3′]) were purchased from Microsynth. The oligonucleotides (final concentration 0.2 or 0.6 μmol/L) were transfected with Superfect for 4 hours, the medium was changed, and the cells were grown for 48 hours. Higher concentrations were found to be toxic. Total protein isolation, Western blots, labeling, and uptake of fluorescently labeled oxLDL (oxLDL-DIO) were performed as previously described.

**Results**

**Identification of New 5′ Exon of the Human CD36 Gene**

The 5′ end of CD36 was previously determined in melanoma cells. We speculated that the CD36 gene expression in smooth muscle could be due to an alternative promoter, which is specifically used in vascular SMCs. 5′-RACE was performed with mRNA isolated from T/G cells, and the PCR products were cloned and sequenced. Interestingly, in addition to the previously characterized 5′ end, a new exon was found spliced to exon 2a in T/G cells, in 3 of 4 CD36-positive clones.

**Identification of Extended CD36 Promoter Specific for SMCs**

To locate the new CD36 exon and extended promoter, PCR with human genomic DNA was performed. Amplification...
occurred with primer CDprof, which is specific for the previously described CD36 promoter, and primer CDnewrev, which is specific for the new exon. The amplified fragment was 500 bp long, indicating that the new exon (now exon 1b) is located downstream from the previously described exon 1 (now exon 1a, Figure 1). The sequence of the amplified fragment reveals the presence of the consensus promoter and potential regulatory elements, such as TATA boxes, nuclear factor (NF-κB), peroxisome proliferator-activated receptor γ (PPARγ), myocyte-specific enhancer factor (MEF)-2, interferon-γ, and E-boxes. NF-κB, PPARγ, and MEF-2 have been shown to be activated in the plaque, and SMC-specific E-box binding proteins have recently been described. Analysis of the sequence of the newly isolated exons indicated that the 5' ends were in good agreement with the consensus CAP sequence. Moreover, in 1 of the 4 CD36-positive clones, a further transcription initiation site was identified, which was located 12 bp upstream from the previously described 5' end.

CD36 Promoter Activity in T/G Cells

The extended CD36 promoter was cloned into the pGL3-basic and pGL3-enhancer promoter test vectors and transfected into T/G cells. As seen in Figure 2, the extended promoter pCDb-basic activity was similar to the previously described (pCD-basic) activity. Only background was measured when the CD36 promoters were in the antisense orientation.

Cell Type and Tissue Specificity of Exon 1b

The cell specificity of the new exon 1b was verified by its expression in different cell lines and tissues. Whereas CD36 mRNA was expressed in a number of cell lines, exon 1b could be mainly amplified in T/G cells, which represent a model of activated SMCs, and promyelocytic monocytes (U937), and the erythroleukemia cell line (HEL), but not in HepG2 hepatoma cells. CD36 and exon 1b were not expressed in ISS10 (SV40-transformed vascular SMCs), possibly because the CD36 gene is inactivated. However, exon 1b was expressed in proliferating primary SMCs (Figure 4), indicating that our findings are not cell-line specific.

Exon 1b Is Expressed in Atherosclerotic Plaques

To determine the importance of exon 1b for atherosclerosis, the in vivo expression of exon 1b in normal and atherosclerotic parts of human aortas was analyzed. RT-PCR revealed the presence of exon 1b in only the samples isolated from the plaques, whereas in the parts of aorta without lesions, exon 1b expression was almost undetectable (Figure 5). Thus, switching to exon 1b may trigger the pathological activation of the cells at the atherosclerotic lesion. Because the lesion is a composite of activated cells, such as SMCs and macrophages, we cannot completely exclude the possibility that exon switching occurs also in cells other than SMCs. However, in

Figure 1. Sequence of the human CD36 5' exons 1a and 1b and the preceding promoter region. An additional start site of transcription is found 11 bp upstream from the one previously described. The nucleotide positions are indicated relative to the newly characterized start site of exon 1b (+1). Exon 1a (dashed line) and exon 1b (solid line) are underlined. Potential regulatory elements in the new SMC promoter are boxed. Antisense oligonucleotides are indicated below the sequence. IFNγ indicates interferon-γ.

Figure 2. Structure and activity of the CD36 promoter. Top, Gene structure of the CD36 promoter and 5' exons. The previously described exon 1 is indicated as exon 1a, and the newly described exon is indicated as exon 1b. Both exons are spliced to exon 2a, which is separated by a large intron. Two TATA boxes (black boxes) precede start sites of transcription newly mapped in T/G cells. Bottom, Luciferase (LUC) assay with constructs containing the previously described promoter (pCD-basic) and the new promoter (pCDb-basic). A construct with the antisense orientation (pCDb-basic/as) is used as a negative control.

Figure 3. Cell type–specific CD36 exon usage. Lanes are as follows for RT-PCR of total RNA isolated from various cell lines: 1, T/G cells; 2 T/G cells (duplicate); 3, ISS10 (SV40-transformed vascular SMCs); 4, HL-60 monocytes; 5, HL-60 macrophages, differentiated with 9-cis-retinoic acid; 6, U937 monocytes; 7, HepG2 (liver hepatoma); 8, HEL (erythroleukemia); 9, MCF-7 (breast cancer); 10, CaCo2 (colon cancer); 11, skin fibroblasts; and 12, molecular weight marker (top panel, exon 1b–positive control). The additional bands in lanes 3, 7, and 9 (middle panel) were sequenced and characterized as rRNA contamination.
vitro macrophage differentiation led only to weak exon 1b expression (Figure 3), indicating that the major source of exon 1b may come from activated SMCs.

**Inhibition of CD36 Expression by Antisense Oligonucleotides**

CD36-mediated oxLDL uptake in activated SMCs has been described by our group in a previous study showing downregulation of CD36 by physiological concentration of α-tocopherol. However, as recently discussed, all factors involved in CD36 functions, such as scavenger receptors, fatty acid transporters, and mediators of apoptotic cell death, would be affected by putative anti-CD36 drugs.31 A more promising approach to prevent and reduce foam cell formation would be the local delivery of drugs to the vessel wall. We have designed phosphorothioate antisense oligonucleotides against CD36 exons 1b, 1a, and 3. After oligo transfection of cultured SMCs, inhibition of CD36 expression occurred at mRNA (not shown) and protein (Figure 6A) levels, and the degree of inhibition was dependent on the type of oligonucleotide used. The antisense oligonucleotide to exon 1b affected CD36 expression in SMCs but not in U937, MCF-7, or HEL cells, which do not use exon 1b. The Western blot signal was quantified by densitometry and displayed as a bar graph. Each panel is representative of 2 experiments with essentially similar results.

whereas a scrambled control was less efficient (data not shown).

**Discussion**

Foam cell formation may be a response of the cells at the atherogenic lesion to a number of proatherogenic stimuli, which may result in alterations of gene expression. In this process, alterations of scavenger receptor expression could play a critical role because of their function in the transport of lipids and cholesterol into and out of the cells. Foam cells are formed by macrophages, but a number of studies describe smooth muscle–derived foam cells at the atherosclerotic lesion.32 In fact, the predominant cell type in the atherosclerotic lesions of young adults was of smooth muscle origin.33 A necessary prerequisite for atherosclerosis is a change in the phenotype of SMC from a quiescent contractile state to a proliferative and synthetic form.32 In vitro exposure of SMCs to growth factors leads to scavenger receptor expression and foam cell formation,30 suggesting that activation is required for the atherogenic process. Moreover, primary SMCs isolated from the aorta express PPARγ, CD36, and the macrophage-specific genes SR-A, CD32, and CD68, indicating that SMCs can convert to macrophage-like cells and contribute to the formation of atherosclerotic plaque.29

In the present study, we show that in cultured aortic SMCs, CD36 transcription is initiated at 2 alternative start sites, leading to 2 alternative first exons, 1a and 1b. Both exons are preceded by promoters that are functional in T/G cells. In cultured promyelocytic monocytes (U937), erythroleukemia (HEL), and differentiated macrophages, weak expression of exon 1b was detectable, possibly implying that cell transformation is associated with transcriptional changes leading to usage of exon 1b. In human aorta, the new exon 1b was
detectable only in RNA derived from plaque and not in RNA derived from the normal parts of aortic tissue. This strongly suggests that switching to exon 1b may occur during the process of smooth muscle activation. Although in vitro–differentiated macrophages express only low levels of exon 1b, we cannot exclude the use of exon 1b by activated macrophages.

Our results suggest alternative promoter and exon usage in atherosclerotic plaque and T/G cells. It appears possible that the new promoter carries binding sites for transcription factors that are specific for activated SMCs. Indeed, there are consensus binding sites for NF-κB and MEF-2, which are activated in SMCs of the atherosclerotic plaque.23–25 Moreover, there is 1 binding site for interferon-γ and 4 E-boxes, which may be targeted by c-myc or possibly by smooth muscle–specific basic helix-loop-helix transcription factors, such as the cardiovascular helix-loop-helix factor 1. 27 Recently, an E-box motif was identified in the aortic preferentially expressed gene-1, which mediates specific vascular SMC gene expression.26 PPARγ, which is expressed in CD36-positive activated SMC clones (and also in T/G cells, data not shown), could play an additional role in activating CD36 expression in SMCs and converting them into macrophage-like cells.29 Indeed, a recent report shows that NF-κB and PPARγ cooperate to stimulate transcription in SMCs,34 and binding sites for both factors are located in the presented promoter sequence. Preliminary data suggest that the described consensus sites in the extended CD36 promoter can bind their corresponding transcription factors (data not shown). A more detailed analysis of the bound proteins should clarify the mechanisms for alternative CD36 promoter usage.

Monocytes/macrophages from CD36-deficient patients show a reduced capacity to bind and internalize oxLDLs and long-chain fatty acids.35,36 Conversely, at the atherosclerotic lesion, the expression of the SR-A/II, SR-BI, and CD36 scavenger receptors is increased in macrophages and SMCs (see review37). Moreover, overexpression of CD36 in Chinese hamster ovary cells rendered them susceptible to apoB triggered by oxLDL or 25-hydroxycholesterol. Thus, aberrant regulation of the CD36 scavenger receptor may be involved in the accumulation of oxLDL, cholesterol, and lipids in the aorta, leading to apoptosis and/or foam cell formation at the atherosclerotic plaque.

The evidence reported in the present study of a new CD36 exon, specifically spliced in activated SMCs, hints of a pathological origin. It remains to be shown whether a difference exists in the structure, stability, and rate of translation of the two 5′ untranslated regions carrying exon 1a and 1b.  

Acknowledgments

This study was supported by the Swiss National Science Foundation, by Bayer-Vital, Henkel Corp, and by the Stiftung für die Ernährungsforschung in der Schweiz. Dr R. Ricciarelli is recipient of a Telethon-Italia fellowship.

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doi: 10.1161/hq0302.104517

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