Cholesteryl ester hydrolase in human monocyte/macrophage: cloning, sequencing, and expression of full-length cDNA

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**Ghosh, Shobha.** Cholesteryl ester hydrolase in human monocyte/macrophage: cloning, sequencing, and expression of full-length cDNA. *Physiol. Genomics* 2: 1–8, 2000.—The sensitive technique of RT-PCR was used to identify cholesteryl ester hydrolase (CEH) expressed in human macrophages. This enzyme is thought to regulate the availability of intracellular free cholesterol for efflux. The expected 667-bp product was obtained starting with RNA from human peripheral blood and THP-1 monocytes and macrophages. The cDNA for human macrophage CEH was then cloned by PCR-based screening of a λgt11 cDNA library. The full-length cDNA was sequenced and found to exhibit 76% homology (at the nucleotide and conceptually translated protein level) to hepatic CEH, an enzyme shown to be involved in hepatic cholesterol homeostasis and regulated by cholesterol at the transcription level via sterol response elements in the proximal promoter. Identification of the conserved catalytic triad (Ser221, His468, and Glu554) and the SEDCLY motif places human macrophage CEH in the family of carboxylesterases. A greater than 20-fold increase in CEH activity was observed when COS-1 and COS-7 cells were transiently transfected with an eukaryotic expression vector, pcDNA3.1/V5/HisTOPO, containing the cDNA for human macrophage CEH. Using this full-length cDNA as a probe, a 2.2-kb transcript was identified by Northern blot analysis of total RNA from human peripheral blood and THP-1 macrophages. Overexpression of human macrophage CEH resulted in an impairment of upregulation of low-density lipoprotein (LDL) receptor mRNA in Chinese hamster ovary (CHO-K1) cells grown in cholesterol-deficient environment. These data identify the human macrophage CEH, demonstrate its expression in human peripheral blood macrophage and human macrophage cell line, THP-1, and suggest its role in the intracellular cholesteryl ester metabolism.

The formation of macrophage-derived foam cells is a central event in the development of fatty streaks within the arterial wall and progression of atherosclerosis. The unregulated uptake of modified lipoproteins by macrophages via scavenger-receptors leads to the deposition of cholesterol esters and the formation of foam cells. Stored cholesterol esters, present as cytoplasmic droplets, exist in dynamic equilibrium with unesterified cholesterol undergoing continuous hydrolysis and reesterification (2) in a process known as the "cholesterol ester cycle." Neutral cholesteryl ester hydrolase (CEH) catalyzes the hydrolytic reaction, whereas reesterification is catalyzed by acyl-CoA cholesterol acyltransferase (ACAT). Free cholesterol released by CEH moves to the plasma membrane and is subsequently transferred to a cholesterol acceptor (e.g., high-density lipoprotein (HDL)), resulting in net cellular cholesterol efflux. Macrophages with high neutral CEH activity accumulate less cholesterol esters in the presence of other atherogenic β-migrating very low-density lipoproteins (β-VLDL) in comparison to macrophages with low CEH activity (13). Animal models of atherosclerosis, such as the hypercholesterolemic rabbit and the White Carneau pigeon, appear to possess macrophages in which stored cholesterol esters are resistant to hydrolysis and subsequent mobilization (20, 26). Hence, CEH activity may be a limiting factor in the mobilization of cholesterol esters from foam cells and therefore may play a role in determining the susceptibility to atherosclerosis.

Despite the obvious significance of cholesterol ester hydrolytic enzymes in atherogenesis, the identity of CEH in macrophages remains obscure. Several lines of evidence suggest that the enzyme responsible for cholesteryl ester hydrolysis in murine macrophages is similar to hormone-sensitive lipase (HSL) present in adipose and steroidogenic tissues (9, 14, 25). Although Reue et al. (23) detected HSL mRNA in the human monocyte cell line THP-1, Contreras et al. (4) failed to detect HSL mRNA in human macrophages. Li and Hui (19) recently reported the absence of HSL in human macrophages and demonstrated the expression of bile salt-stimulated CEH, similar to secretory pancreatic CEH. Since this enzyme was secreted from the cells, Li and Hui (19) proposed that it was unlikely to play a role in the intracellular cholesterol metabolism and suggested that another CEH may be responsible for cholesteryl ester metabolism in human macrophages.

This study was undertaken to identify the CEH in human monocyte/macrophages. An enzyme homologous to hepatic CEH (7) was identified in the human monocyte/macrophage cell line THP-1 and human peripheral blood monocyte/macrophages by RT-PCR. Furthermore, the present study describes, for the first time, cloning, sequencing, and expression of a human macrophage CEH cDNA. Implications of the identification and availability of human macrophage CEH cDNA in relation to stimulating cholesterol efflux from macrophage foam cells present in arterial fatty streaks and/or atherosclerotic plaques are discussed.

**MATERIALS AND METHODS**

The human monocyte cell line THP-1, CHO-K1, COS-1, and COS-7 were purchased from American Type Culture
Collection (Rockville, MD). The human macrophage cDNA library (in λ-gt11) was obtained from Clontech Laboratories; ThermoScript RT-PCR system, cell culture media, FBS, and cell culture reagents were obtained from Life Technologies (Gaithersburg, MD). Phorbol 12-myristate 13-acetate (PMA), Histopaque 1077 and Kodak LS film were obtained from Sigma (St. Louis, MO). Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Bedford, MA). Dextran T-500 was purchased from Pharmacia. GeneScreen membrane and α-[32P]dCTP were obtained from NEN Research Products (Boston, MA). All other reagents and chemicals were purchased from Fisher Chemical (Cincinnati, OH).

**Cell culture.** THP cells were maintained in RPMI-1640 medium containing 10% FBS according to the instructions supplied. Monocytes were obtained by centrifugation of an aliquot of the suspension culture. For induction of macrophages, PMA (250 nM) was added to the medium and the cells were seeded into 6-well tissue culture dishes at 1 × 10^6 cells/well and maintained in a humidified atmosphere of 95% air and 5% CO₂. Media containing PMA was replaced every 2 days, and experiments started after 7 days in culture, when the cells were phenotypically macrophage (10).

**Isolation of human peripheral blood monocytes.** Human monocytes were isolated from the peripheral blood of healthy donors using the Ficoll-Histopaque density gradient centrifugation (1). Separated mononuclear cells were washed three times with PBS and plated in 75-cm² flasks at a density of 1 × 10⁶ cells/flask. Nonadherent cells were removed after an overnight incubation, and either the monocytes were harvested for total RNA preparation or fresh RPMI-1640 medium with 10% FBS was added to the adherent cells. For cells remaining in culture, the medium was changed every 3 days, and the cells were used as monocyte-derived macrophages after 9 days in culture as described by Li and Hui (19). Greater than 90% cells showed a phenotype characteristic of macrophages.

**RT-PCR.** Total RNA from THP-1 and human peripheral blood monocytes and macrophages was prepared by CsCl gradient centrifugation according to the procedure of Chirgwin et al. (3). Total RNA (10 μg) was reverse transcribed using ThermoScript RT-PCR system (GIBCO-BRL) according to the manufacturer’s instructions. No reverse transcriptase was added to negative controls. Oligo dT(20) was used as a primer for first-strand cDNA synthesis. PCR primers used for CEH amplification were primers 1 and 2 (Table 1). Amplification reactions were carried out for 30 cycles with denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 70°C for 2 min. The extension was increased to 10 min for the final cycle. PCR products were analyzed on 1% agarose gel. The 667-bp PCR product from THP-1 and blood macrophages was cloned into TA cloning vector pCR2.1-TOPO (Invitrogen). EcoR I digested plasmid DNA was separated on 1% agarose gel and processed for Southern blot as described previously (7). The recombinant plasmid containing the 667-bp PCR product was sequenced in both directions by ABI automated DNA sequencing system.

**Isolation of full-length cDNA.** The λ-gt11 cDNA library was screened using one-sided Touchdown PCR. Reverse complements of the primers used above were synthesized (primers 3 and 5) and used in conjunction with λ-forward (primer 4) and λ-forward (primer 6) primers to amplify the 5’ and the 3’ end of the cDNA, respectively. The thermocycling conditions were as follows: denaturation at 94°C for 30 s, annealing and elongation for the first 10 cycles at 65°C, followed by annealing at 58°C for 30 s and elongation at 72°C for 2 min for additional 20 cycles. The elongation time was extended to 10 min for the last cycle. The PCR products were cloned into pCR2.1-TOPO and sequenced in both directions using plasmid-specific primers. Two additional primers were synthesized based on the 5’ and 3’ regions (primers 7 and 8) of these first-round PCR products. These two primers were used to amplify the full-length cDNA from the λ-gt11 cDNA library. To overcome the inherent problems with the strong secondary structure of primer 7, the thermocycling conditions were as follows: denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 2 min. The elongation time was increased to 10 min for the last (25th) cycle. The PCR product (~2 kb) was cloned into pcDNA3.1/V5/HisTOPO. Plasmid DNA from six individual colonies was sequenced in both directions to confirm the sequence. The location of the primers and the cloning strategy is summarized in Fig. 1.

**Transient transfection of COS-1 and COS-7 cells.** COS-1 and COS-7 cells were maintained in DMEM supplemented with 10% FBS. Cells were transfected with pcDNA3.1/V5/HisTOPO containing CEH cDNA in the correct orientation using Lipofectamine (GIBCO-BRL) according to the manufacturer’s instructions. Mock-transfected controls were simultaneously treated with Lipofectamine alone. After 48 h of incubation, the cells were harvested in homogenizing buffer containing protease inhibitors (6). The cell suspension was sonicated using Heat Systems Ultrasonic Processor, and the cell lysates were assayed for CEH activity as described earlier (7). The substrate, cholesteryl [1-14C]oleate was dissolved in acetone.

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**Table 1. Sequences and locations of the primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence (5’-3’)</th>
<th>Location</th>
<th>Size of the PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GGACAACATTGGCAACACTTGG</td>
<td>606–626</td>
<td>1 + 2 = 667 bp</td>
</tr>
<tr>
<td>2</td>
<td>CACATCCTCACAAGGATCCTTG</td>
<td>1251–1272 (RC)</td>
<td>3 + 4 = 728 bp</td>
</tr>
<tr>
<td>3</td>
<td>GACACCAGACCAACTGGTAGT</td>
<td>gt11 1096–1117 (RC)</td>
<td>2 + 4 = 65 bp</td>
</tr>
<tr>
<td>4</td>
<td>CCAAATGGCAGATGTGCCG</td>
<td>606–626 (RC)</td>
<td>3 + 4 = 728 bp</td>
</tr>
<tr>
<td>5</td>
<td>CCAGGACTGCTGTAGAGATGT</td>
<td>1251–1272</td>
<td>3 + 4 = 728 bp</td>
</tr>
<tr>
<td>6</td>
<td>GCTGGCCAGGACTGCTGGCC</td>
<td>gt11 1083–1054</td>
<td>3 + 4 = 728 bp</td>
</tr>
<tr>
<td>7</td>
<td>CGAGAACCTGGGCTTTCAGAAGTG</td>
<td>−21 to 27</td>
<td>7 + 8 = 1,881 bp</td>
</tr>
<tr>
<td>8</td>
<td>GCTCCGGCTTTATTCCGCTTG</td>
<td>1830–1850</td>
<td>7 + 8 = 1,881 bp</td>
</tr>
</tbody>
</table>

**Primer 1 and 2 were synthesized based on the hepatic cholesteryl ester hydrolyase (CEH) cDNA sequence (7). Sequences of primers 3 and 5 were obtained from the Lambda Library Protocol handbook supplied by Clontech along with the cDNA library. Primer 7 was designed on the basis of the sequence of the 728-bp PCR product obtained in the first round of PCR screening. Similarly, the sequence of primer 8 was obtained from the 745-bp PCR product.**

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and presented as droplets representing the physiological state of the cytosolic stores of cholesteryl esters in macrophage foam cells.

**Northern blot analysis.** Total RNA from THP-1 and human peripheral blood monocytes and macrophages was electrophoresed on a 1% agarose gel in the presence of formaldehyde. The RNA was stained with ethidium bromide, and the integrity of RNA was verified by the presence of 28S and 18S rRNA bands. The RNA was transferred to GeneScreen membrane and hybridized with 32P-labeled full-length macrophage CEH cDNA according to manufacturer's instructions. The blots were washed under high stringency (0.2× SSC + 0.1% SDS at 42°C), and positive hybridization was detected by exposure to Kodak LS film for 18 h at −70°C.

**Low-density lipoprotein receptor expression.** CHO-K1 cells were transfected with pcDNA3.1/V5/His-TOPO containing CEH cDNA in the correct orientation as described above. After 24 h, the growth medium was replaced with either medium containing 10% FBS or 10% lipoprotein-deficient serum (LPDS). Cells were harvested 48 h posttransfection for CEH assay and total RNA extraction. Twenty micrograms of total RNA was separated on formaldehyde-agarose gel, transferred to GeneScreen membrane, and probed with either 32P-labeled hamster LDL-receptor cDNA or human macrophage CEH cDNA. For normalization, the blot was reprobed with 32P-labeled β-actin probe. The intensity of the hybridized bands was quantified by scanning the autoradiograms using personal densitometer from Molecular Dynamics. Data is presented as intensities normalized to β-actin.

**RESULTS**

**Molecular identity of CEH in human macrophages.** Total RNA from THP-1 and human peripheral blood, monocyte, and macrophages was used to determine the molecular identity of the CEH expressed in these cells. An RT-PCR product of 667 bp was consistently obtained (Fig. 2) when hepatic CEH-specific primers, *primers 1* and *2*, were used. No amplified product was seen in the absence of reverse transcriptase (data not shown).

The homology of this PCR product to hepatic CEH was confirmed by strong hybridization of the cloned PCR product to full-length rat hepatic CEH cDNA on a Southern blot (data not shown) and by sequencing (see Fig. 7 for the sequence lineup). These results demonstrate that an enzyme similar or identical to hepatic CEH is expressed in human monocytes and human macrophage.

**Cloning of human macrophage CEH.** Screening of the human macrophage cDNA library using *primers 3* and *4* resulted in an amplified product of ~700 bp in length. Sequence analysis of the cloned PCR product identified several amplicons identical in sequence but differing slightly in their lengths (690–728 bp). The longest product was 728 bp long and contained the consensus Kozak box, 42 bp downstream of the 5′ end. Screening of the library with *primers 5* and *6* also resulted in an amplified product of ~700 bp in length. The longest amplicon, identified by sequence analysis of the cloned PCR product, was 745 bp long and contained the consensus polyadenylation signal 14 bp upstream of the start of a 7-bp stretch of adenosines (poly-A tail). The 728 and 745 bp, therefore, correspond to the 5′ and the 3′ end of the CEH cDNA, respectively. On the basis of the sequence at the 5′ and 3′ ends of the products obtained above, *primers 7* and *8* were synthesized and used to amplify a 1,881-bp PCR product from the human macrophage cDNA library. The sequence of this PCR product was identical and overlapped with the 728- and 745-bp products in the 5′ and 3′ ends.
the 3’ regions, respectively. The complete cDNA sequence and the predicted amino acid sequence are shown in Fig. 3.

The first ATG codon, 45 bp downstream from the 5’ terminus was identified by the sequence CCACGAATG, differing only in the fifth nucleotide from the consensus sequence CCACCATG, described by Minchiotti et al. (21). Nucleotide sequence surrounding this ATG codon also corresponds to the optimal consensus sequence AXAXGTGXG, for initiation of translation by eukaryotic ribosomes (15). Beginning with this start codon, a long open reading frame coding for 567 amino acids was observed. A termination codon, TGA, is present at position 1701, leaving a 199-bp 3’-untranslated region. The polyadenylation signal, AATAA, is located 14 bp from the start of the poly(A) tail.

Fig. 3. cDNA nucleotide sequence and deduced amino acid sequence of the human macrophage CEH. cDNA sequence is numbered starting from the first codon ATG. Sequence information from 231 to 1850 is from the 1,881-bp PCR product obtained in the second round of PCR screening. Additional sequence information in the 5’ and 3’ ends of the cDNA is from the other overlapping PCR products (728 and 745 bp) obtained in the first round of PCR screening. Conserved active site motif GXXSG is shown in bold face and underlined. Putative residues of the catalytic triad (Glu354 and His468) are also shown in bold face. The conserved SEDCLY motif is underlined. Polyadenylation signal (AATAA) is shown in bold face.

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Expression of human macrophage CEH cDNA in transiently transfected COS-1 and COS-7 cells. The authenticity of the cloned human macrophage cDNA was verified by expression of the eukaryotic vector pcDNA3.1/V5/His-TOPO containing the full-length CEH cDNA in COS-1 and COS-7 cells. Expression was driven by the CMV promoter located upstream of the 5' terminus of the cDNA. As shown in Fig. 4, 17- to 20-fold higher CEH activity was present in lysates from cells transfected with CEH cDNA compared with mock-transfected controls. A linear relationship was seen with activity and protein concentration in both cell lines.

Detection of CEH mRNA in human monocyte-derived macrophage and PMA-transformed THP-1 macrophages. Total RNA from these macrophages was probed with the full-length human macrophage CEH cDNA on a Northern blot. As shown in Fig. 5, the probe hybridized to a single 2.2-kb band in both the RNA samples, demonstrating the expression of a corresponding mRNA in both these cell types.

Effect of CEH overexpression on low-density lipoprotein receptor mRNA levels. Transfection of CHO-K1 cells with macrophage CEH resulted in overexpression of CEH mRNA as determined by Northern blot analysis (Fig. 6A). Consistent with this increase in CEH mRNA levels, the transfected cells had 7.8 ± 0.04-fold higher CEH activity than mock-transfected control cells. To examine the effects of CEH overexpression on cellular cholesterol homeostasis, low-density lipoprotein receptor (LDLR) mRNA levels were measured in control (mock-transfected) and macrophage CEH-transfected CHO-K1 cells grown in the presence medium containing 10% FBS or LPDS. Compared with cells grown in FBS, the mock-transfected cells grown in LPDS showed a 1.7-fold increase in LDLR mRNA (Fig. 6B). Significantly lower increase (1.2-fold, \( P < 0.05 \)) in LDLR mRNA was seen in cells transfected with human macrophage CEH cDNA (Fig. 6B). Therefore, overexpression of CEH resulted in an impaired ability of cells to increase LDLR expression in response to conditions where exogenous cholesterol is deficient.

DISCUSSION

This is the first report of identification and molecular cloning of human macrophage CEH. The expression of this CEH mRNA was demonstrated in human monocytes/macrophage cell line THP-1 and in human peripheral blood monocytes/macrophages by RT-PCR (Fig. 2) and by Northern blot analysis (Fig. 5). A greater than 20-fold increase in cholesterol ester hydrolysis was observed in COS-1 and COS-7 cells transiently transfected with eukaryotic expression vector containing macrophage CEH cDNA (Fig. 4). The cloned cDNA exhibits only 39% homology with hormone-sensitive lipase (11), an enzyme thought to be responsible for cholesterol ester hydrolytic activity in murine macrophages. However, high degree of homology (76%, see Fig. 7) exists between human macrophage CEH reported here and rat hepatic CEH, a member of carboxylesterase family shown to be involved in hepatic cholesterol homeostasis (8) and regulated by sterols via sterol response elements at the transcriptional level (22). Direct evidence for the role of human macrophage CEH in intracellular cholesterol homeostasis is provided by the observed impairment (Fig. 6) of upregulation of LDL receptor mRNA in cells overexpressing CEH and grown in a cholesterol-
deficient environment. These results demonstrate the ability of macrophage CEH activity to modulate the intracellular regulatory pools of free cholesterol that influence the transcription of sterol-sensitive genes like LDL receptor.

A search of the GenBank/EMBL database revealed that the isolated cDNA was highly homologous to human liver carboxylesterase (16). Differences were observed in only 16 nucleotides, two of which are located in the 5'-untranslated region and five in the 3'-untranslated region. Within the coding region, absence of three consecutive nucleotides (GCA) at positions 52–54 results in the absence of one amino acid (alanine) in human macrophage CEH. Substitution of a C for G at position 172, a C for a T at position 199, a G for an A at position 271, and an insertion of a G at position 1667 lead to the amino acid changes shown in Fig. 7. Single amino acid substitutions have profound effects on the catalytic properties of enzymes (18), although the mechanism of these effects remains to be elucidated.

On the basis of the observed sequence homologies, the cDNA reported here belongs to the family of carboxylesterases, characterized by the presence of a conserved catalytic triad (serine, histidine, and an acidic residue) and a conserved SEDCLY motif (24). By sequence comparisons, the putative residues in human macrophage CEH cDNA would be Ser(221), His(468), and Glu(354). The SEDCLY motif is located at amino acid residues 113–118 (see Fig. 4). Thus human macrophage CEH becomes the second member of the carboxylesterase family to be involved in intracellular cholesterol ester metabolism and the first human clone to be identified. In a macrophage-derived foam cell, cholesterol esters are stored as cytoplasmic lipid droplets. The ability of CEH protein expressed in transiently transfected COS cells to hydrolyze cholesterol esters presented as lipid droplets in the assay system used further indicates its role in clearance of stored cholesterol esters.

The identification of human macrophage CEH represents the first step toward understanding of (dys)regulation of macrophage-foam cell cholesterol ester metabolism and, therefore, its role in determining the susceptibility to atherosclerosis. Several independent studies provide a correlation between foam cell formation and intracellular CEH activity. Impaired cholesterol efflux from human THP-1 macrophages was found to be due to low levels of neutral CEH activity (10). Kritharides et al. (17) recently showed that the choles-

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terol ester pool of THP-1 cells loaded with acetylated LDL was essentially inactive. It is, therefore, likely that slow hydrolysis of cholesterol esters leads to the impaired cholesterol efflux from these cells (17). Consistent with the susceptibility to atherosclerosis, macrophage CEH activity in C57BL/6J mice was less than one-half of that seen in resistant C3H/HeN mice (12). Furthermore, Escary et al. (5) recently described almost complete hydrolysis of intracellular cholesterol esters in the cholesterol-laden RAW264.7 murine macrophage cell line overexpressing hormone-sensitive lipase and treated with cAMP. Experiments are in progress to demonstrate stimulation of cholesterol ester clearance from cholesterol-laden human THP-1 macrophage by overexpression of human macrophage CEH cDNA, an in vitro mimic of atherosclerotic plaque regression.

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REFERENCES


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