CD36 LIMPII Analogous-1, a Human Homolog of the Rodent Scavenger Receptor B1, Provides the Cholesterol Ester for Steroidogenesis in Adrenocortical Cells

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CD36 and LIMPII analogous-1 (CLA-1), a human homolog of the rodent scavenger receptor B1 (SR-B1), binds high-density lipoprotein (HDL) and mediates the selective uptake of HDL cholesterol ester (CE) by cultured transfected cells. CLA-1 is strongly expressed in steroidogenic tissues, including the adrenal gland, suggesting that CLA-1 plays a role in providing substrates for steroidogenesis. To address this, we established an adrenocortical cell line that highly expresses CLA-1. These cells increased CE uptake from HDL to 140.5% of the level in mock-transfected cells. After incubation of the transfected cells with HDL, corticosterone secretion from CLA-1–transfected cells increased to about two times the level in mock-transfected cells. These results indicate the possibility that CLA-1 (a close structural homolog of SR-B1)-mediated uptake of HDL CE may be a significant source of precursor cholesterol for steroidogenesis in humans as it is in mice.

IN HUMANS, the primary pathway for cellular cholesterol uptake involves a low-density lipoprotein (LDL) receptor[1,2] and other members of the LDL receptor family.[3] These receptors function via endocytic uptake and lysosomal degradation of lipoprotein particles to release cholesterol and other lipids into the cell.[1,2] An alternate process, occurring primarily with high-density lipoprotein (HDL), is the selective uptake pathway in which HDL cholesterol ester (CE) is taken into the cell without the uptake and lysosomal degradation of the HDL particle.[4,5]

Despite the widespread nature of the pathway for HDL CE selective uptake, little is known about the biochemical mechanism by which CE is transferred into the cell without the uptake and degradation of the HDL particle. Recent studies have shown that mouse scavenger receptor B1 (SR-B1) mediates the selective uptake of HDL CE into transfected Chinese hamster ovary cells, providing an important link between a defined-cell surface receptor and the selective uptake pathway.[6] The quantitative significance of selective HDL cholesterol uptake may depend on the species examined. In mice, it accounts for at least 90% of the cholesterol destined for steroid production in steroidogenic cells.[7] Recently, Temel et al[8] demonstrated that mouse SR-B1 supplies substrate cholesterol for steroid hormone synthesis in mouse adrenocortical cells. Many studies have suggested that the major source of cholesterol for steroidogenesis may be LDL in humans.[9] Therefore, the role of HDL receptor–mediated selective cholesterol uptake in human adrenals remains to be established.

Human CD36 LIMPII analogous-1 (CLA-1) was cloned from a cDNA library prepared from differentiated HL-60 cells based on the existence of regions with an amino acid sequence highly conserved between CD36 and LIMPII.[10] A previous report provides evidence that CLA-1 can function as a receptor for HDL, can mediate the selective uptake of CE, and is expressed in liver and steroidogenic tissues, like SR-B1, suggesting that CLA-1 is indeed functionally related to the rodent SR-B1.[11]

In this study, the function of CLA-1 in steroidogenic cells was tested using adrenocortical cells that express CLA-1. The results establish that CLA-1 serves as the route for the selective uptake of HDL CE and the delivery of HDL cholesterol to the steroidogenic pathway in cultured adrenal cells.

MATERIALS AND METHODS

Cell Culture

Y-1, a mouse adrenocortical tumor cell line, was provided by Human Science Research Resources Bank (Osaka, Japan). Y-1 cells were cultured in Ham's F-10 medium supplemented with 15% horse serum, 2.5% fetal calf serum, 100 μg/mL streptomycin, and 100 U/mL penicillin in a humidified atmosphere containing 5% CO_2.

Plasmid Construction and Stable Transfection

The full-length cDNA of the 509–amino acid residue form of CLA-1 cloned into the eukaryocytic expression vector pcDNA 3 (Invitrogen, CA) was linearized with *PvuI*. Y-1 was transfected with 10 μg of the linearized plasmid DNA as described previously.[12] Stable transfectants were selected by resistance to G418 sulfate (400 μg active drug/mL), and a clone showing high CLA-1 expression was identified by Western blot analysis.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was isolated from the transfected adrenocortical cell lines, mock-transfected Y-1 and CLA-1–transfected Y-1, by single-step acid guanidinium thiocyanate-phenol-chloroform extraction.[13] CLA-1 mRNA expression was detected by polymerase chain reaction (PCR) analysis of the reverse-transcribed RNA as described previously.[14] A primer pair matched the published sequence[10] of CLA-1 (sense 5′-ATG-ATC-GTG-ATG-GTG-CCG-TC-3′ and antisense 5′-ACT-GAA-CCT-GCA-GGT-GCT-GA-3′). These primers amplify a 930–base pair (bp) region of CLA-1 cDNA and a 930-bp region of mouse SR-B1 cDNA[6] because of their high homology. The PCR products amplified from CLA-1 have a restriction enzyme site for *XhoI*, but do not possess the sites for amplified mouse SR-B1. To distinguish CLA-1 from mouse SR-B1, we digested PCR products with *XhoI* in accordance with the manufacturer's recommendations. The digests were separated by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide.

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Submitted July 14, 1998; accepted October 29, 1998.
Supported in part by Uehara Memorial Foundation (Japan).
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0026-0495/99/4805-0015$10.00/0

Western Blot Analysis

Transfected Y-1 cells were washed and scraped with phosphate-buffered saline (PBS) and lysed in a lysis buffer as described previously.[15] The proteins were resuspended under reducing conditions, and 15 μg was fractionated by size on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane for immunoblotting. The membrane was blocked by 0.2% Tween 20 in PBS and then incubated with anti–CLA-1 antibody (diluted 1:2,000 from whole antiserum) as described previously.[11] The membrane was rinsed and then incubated with horseradish peroxidase–linked anti–guinea pig immunoglobulin G ([IgG] diluted 1:1.500), and antibody binding was visualized by chemiluminescence detection (ECL; Amersham, Arlington Heights, IL).

[^{3}H]HDL Uptake

Human HDL (d = 1.063 to 1.21 g/mL) was isolated by preparative ultracentrifugation and labeled with [^{3}H]cholesteryl oleolyl ether as described previously.[16,17] The specific activity of [^{3}H]HDL was 3.9 dpm/ng protein. Transfected Y-1 cells were maintained and experiments performed in a 37°C incubator humidified with 95% air/5% CO_2. For all experiments, six-well plates (Falcon, NJ) were seeded with transfected Y-1 cells. The transfected cells were grown in the complete medium for 12 hours, and then the medium was removed and replaced with Ham's F-10 medium. After 1 hour of incubation, [^{3}H]HDL was added at 10 μg/mL and the incubation was continued for 0.5 to 5.5 hours. The cells were washed twice with 0.1% bovine serum albumin in PBS and twice with PBS, and lysed overnight with 250 μL 0.2N NaOH. Radioactivity was quantified as described previously.[17]

Corticosterone Production

CLA-1– or mock-transfected Y-1 cells were plated in six-well plates in complete medium. After overnight incubation, the medium was replaced with Ham's F-10 medium with or without 100 to 500 μg/mL HDL. After a 24-hour incubation at 37°C, the medium was collected and the corticosterone concentration in the medium was measured with a corticosterone radioimmunoassay (RIA) kit (Amersham) in accordance with the manufacturer's recommendation. The protein content was measured by the method of Lowry et al.[18]

Statistical Analysis

Statistical comparisons were made by one-way ANOVA and Student's *t* test, with a *P* level less than .05 considered significant.

RESULTS

Overexpression of CLA-1 in CLA-1–Transfected Y-1 Cells

Western blot analysis of proteins extracted from cells stably expressing CLA-1, using the antibody directed against an extracellular portion of CLA-1, revealed a single band with an estimated molecular mass of 83 kilodaltons (kDa) (Fig 1A). Mock-transfected cells also showed a band of the same size but at a much lower level, because the antibody for CLA-1 that we used recognized not only CLA-1 but also mouse SR-B1. To confirm this, we used both methods, reverse transcriptase–PCR (RT-PCR) and restriction enzyme digestion. Although the oligonucleotide primer pair used in this investigation amplifies

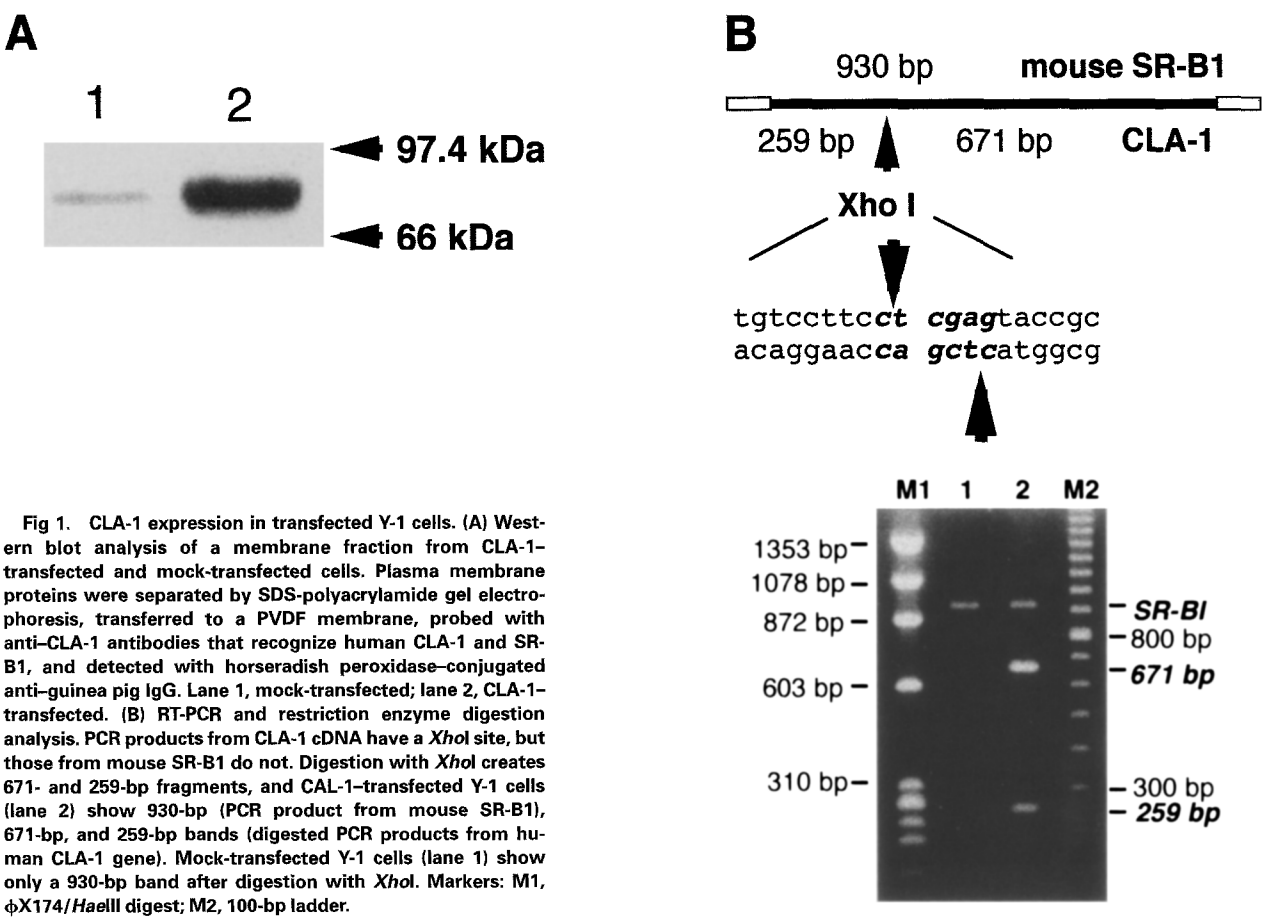

Fig 1. CLA-1 expression in transfected Y-1 cells. (A) Western blot analysis of a membrane fraction from CLA-1–transfected and mock-transfected cells. Plasma membrane proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane, probed with anti–CLA-1 antibodies that recognize human CLA-1 and SR-B1, and detected with horseradish peroxidase–conjugated anti–guinea pig IgG. Lane 1, mock-transfected; lane 2, CLA-1–transfected. (B) RT-PCR and restriction enzyme digestion analysis. PCR products from CLA-1 cDNA have a *Xho*I site, but those from mouse SR-B1 do not. Digestion with *Xho*I creates 671- and 259-bp fragments, and CAL-1–transfected Y-1 cells (lane 2) show 930-bp (PCR product from mouse SR-B1), 671-bp, and 259-bp bands (digested PCR products from human CLA-1 gene). Mock-transfected Y-1 cells (lane 1) show only a 930-bp band after digestion with *Xho*I. Markers: M1, ϕX174/*Hae*III digest; M2, 100-bp ladder.

a 930-bp region of both CLA-1 cDNA and mouse SR-B1 cDNA, the former should be digested with *Xho*I and show the presence of 671- and 259-bp CLA-1 fragments. Figure 1B shows that the mock-transfected cells had only a 930-bp band that originated from the mouse SR-B1 gene (lane 1). On the other hand, CLA-1–transfected cells showed 930-, 671-, and 259-bp bands (lane 2). The 930-bp fragment in CLA-1–transfected cells is from the endogenous SR-B1 gene, whereas the 671- and 259-bp fragments are from the transfected CLA-1 gene. These results confirm that the transfected Y-1 cells exhibited high-level expression of CLA-1.

HDL CE Selective Uptake in CLA-1–Transfected Cells

We have previously shown that CLA-1 mediates the selective uptake of HDL CE.[11] To examine HDL CE uptake by CLA-1–transfected Y-1 cells, CLA-1– and mock-transfected cells were incubated with [^{3}H]HDL, which contained [^{3}H]cholesteryl oleolyl ether as a tracer for CE. Although cell-associated [^{3}H]HDL increased continuously in both CLA-1– and mock-transfected cells, the uptake of CE from HDL in CLA-1–transfected cells significantly increased to 140.5% of the level in mock-transfected cells (Fig 2). These results indicate that CLA-1–expressing Y-1 cells increased the transfer of CE into the cells.

Steroidogenesis in CLA-1–Transfected Cells

Since CLA-1–transfected Y-1 cells show increased uptake of CE from HDL, they were used to determine whether CLA-1 is directly involved in providing substrate cholesterol to the steroidogenic pathway. In the presence and absence of HDL in the culture medium, transfected cells were incubated for 24 hours and the amount of secreted corticosterone was measured by a RIA kit. Without adding HDL, corticosterone levels were not significantly different between CLA-1– and the mock-transfected cells. When the HDL concentration was increased

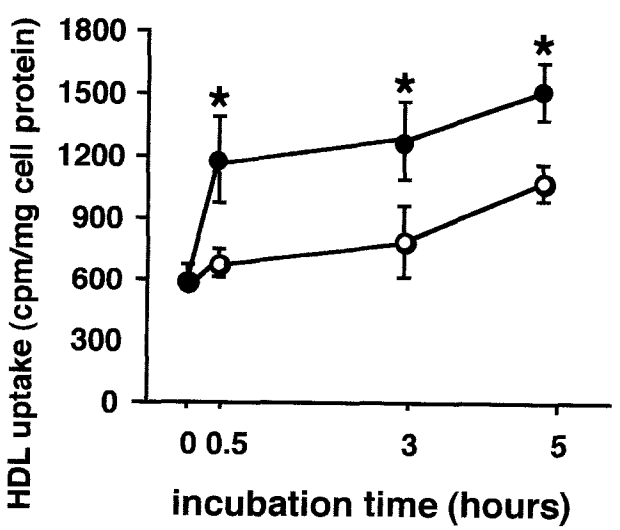

Fig 2. Uptake of CE from HDL by transfected Y-1 cells. Cells were incubated at 37°C with 10 μg/mL [^{3}H]HDL for the indicated periods, harvested, and assayed. Uptake of [^{3}H]cholesteryl oleate was estimated. Results are the mean ± SE of 3 experiments. (●) CLA-1–transfected; (○), mock-transfected. *$P < .05$ *v* mock-transfected.

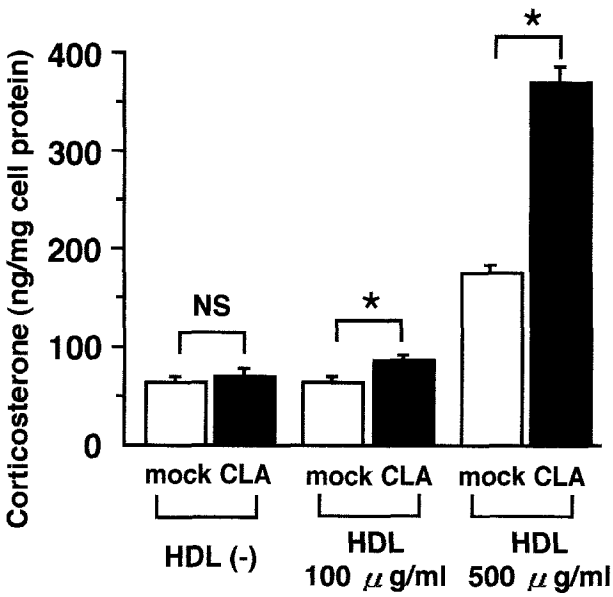

Fig 3. Effects of HDL on corticosterone secretion from transfected cells. Transfected cells were incubated with or without 100 or 500 μg/mL human HDL for 24 hours. The corticosterone concentration in the medium and cell protein contein were measured. Results are the mean ± SE of 3 experiments. CLA, CLA-1–transfected; mock, mock-transfected. *$P < .05$ *v* mock-transfected.

(500 μg/mL), corticosterone secretion in CLA-1–transfected cells increased to about two times the level in mock-transfected cells (Fig 3).

DISCUSSION

This study shows that CLA-1, like its very close homolog, rodent SR-B1, is responsible for the delivery of HDL CE to the steroidogenic pathway in Y-1 adrenocortical cells. Although many studies have suggested that the major source of cholesterol for steroidogenesis in humans is LDL, it is difficult to assess the relative importance of LDL and HDL under in vivo conditions. Certainly, in situations where LDL concentrations are very low (eg, hypobetalipoproteinemia), adrenal functions remain adequate although less able to respond to stress. Under these situations, selective CE transport from HDL via CLA-1 might become physiologically important.

The selective uptake of HDL CE occurs in a variety of human and other mammalian cell types, and appears to be an important pathway for the movement of plasma HDL CE into the liver and steroidogenic cells.[19] In rodents that lack the plasma CE transfer protein, the selective uptake pathway is the predominant means by which plasma HDL CE is delivered to either the liver or steroidogenic cells.[4,5] In mice lacking apolipoprotein A1, CE accumulation in steroidogenic cells is dramatically reduced and corticotropin (ACTH)-stimulated corticosteroid production is blunted, illustrating the dependence of adrenocortical cells on the HDL CE–selective uptake pathway.[7] The SR-B1 is the first HDL receptor to be well defined at a molecular level, and is a mediator of selective cholesterol uptake.[6] Temel et al[8] reported that mouse SR-B1 provides substrate cholesterol for steroid hormone synthesis in adrenocortical cells. These observations are supported by the results that SR-B1–deficient knockout

mice have substantially reduced adrenal cholesterol stores compared with normal controls.[20]

Recently, CLA-1 has been shown to mediate HDL CE selective uptake in transfected cells and to be expressed at high levels in the human adrenal gland.[11] CLA-1 not only displays a high sequence homology but is also functionally related to SR-B1, because both receptors display very similar ligand-binding specificity. Liu et al[21] reported that CLA-1 is abundantly expressed in human adrenocortical tissues at the RNA level. The accumulation of CLA-1 mRNA is upregulated by ACTH, involving the cyclic adenosine monophosphate–dependent protein kinase pathway.[21] These observations raise the possibility that a human gene, CLA-1, is responsible for the delivery of HDL CE to the steroidogenic pathway. However, additional in vivo studies will be required to evaluate the roles of CLA-1 and the HDL CE selective uptake pathway in the steroidogenic cells of humans and other species that transport most of the plasma cholesterol in LDL particles.

In conclusion, this study indicates the possibility that CLA-1–mediated uptake of HDL CE may be a significant source of precursor cholesterol for steroid production in humans as it is in mice.

ACKNOWLEDGMENT

We are grateful to Drs D. Steinberg and O. Quehenberger (University of California, San Diego) for invaluable discussions, and to Masahiko Hori and Kazuko Yamaji for technical assistance.

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