Regulated Vectorial Secretion of Cholesteryl Ester Transfer Protein (LTP-I) by the CaCo-2 Model of Human Enterocyte Epithelium*

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We have investigated the human CaCo-2 enterocyte model for secretion of the plasma cholesteryl ester transfer protein, LTP-I. CaCo-2 cells secrete a cholesteryl ester transfer protein which possesses molecular identity with plasma LTP-I, demonstrated by anti-LTP-I immunoblot analysis and immunoinhibition of all cell-secreted cholesteryl ester transfer activity. When CaCo-2 are cultured on permeable membranes, cholesteryl ester transfer activity is detected only in the lower culture compartment. Thus, CaCo-2 vectorially sort and secrete LTP-I, as well as the intestinal apolipoproteins, from the basolateral cellular domain. Over a 24-h period, CaCo-2 secrete cholesteryl ester transfer activity in a time-dependent manner, at approximately twice the rate of HepG2. Furthermore, CaCo-2 enterocytes, but not HepG2 hepatocytes, regulate LTP-I secretion in response to fatty acid concentrations in the culture medium. Based on these observations, we speculate that the intestine may be the principal regulated source of human plasma LTP-I.

We have reported that the human plasma cholesteryl ester (CE)1 transfer protein, LTP-I (1), mediates the transfer of neutral lipids, such as triglycerides, and negative lipids, such as phosphatidylcholine, in addition to CE, between the plasma lipoproteins. In situ evidence is rapidly accumulating which implicates LTP-I in a fundamental role in plasma sterol metabolism: LTP-I activity has been shown to modulate the lecithin:cholesterol acyltransferase reaction (1), presumably by relieving CE end product inhibition of the acyltransferase by transfer of CE from the acyltransferase substrate, HDL, to other lipoproteins (2). LTP-I is also suggested to play a role in reverse cholesterol transport (3), a postulated pathway whereby cholesterol from extrahepatic tissues is transported to the liver (4). Cholesterol taken up by the liver which is not used in resynthesis of hepatic lipoproteins is ultimately excreted into bile (5), virtually the only means of excreting cholesterol.

Consistent with its proposed role in reverse cholesterol transport, LTP-I is capable of mediating transfer of CE from peripheral cells and to hepatocytes. For example, recent in vitro models demonstrate LTP-I-mediated CE efflux from both cellular and extracellular components of aorta (6), and preliminary data reveal LTP-I-mediated efflux of CE from macrophages (7) and to HepG2 hepatocytes (8). It is intriguing that both the human monocyte-derived macrophage (9) and the HepG2 hepatocyte (10) synthesize and secrete LTP-I. However, the principal cell and tissue sources of LTP-I in human plasma remain a matter of speculation.

It has recently been reported that human intestine contains the mRNA for LTP-I (11). However, neither the specific cell source of this mRNA, nor the nature of the secreted protein or its regulation are known. We have therefore adopted the CaCo-2 membrane model of enterocyte epithelium (12) as a paradigm for studying intestinal synthesis and secretion of LTP-I.

The following experiments demonstrate for the first time: 1) CaCo-2 enterocytes secrete functional LTP-I; 2) as with the intestinal apolipoproteins, postconfluent CaCo-2 cells cultured on permeable membranes secrete LTP-I in a polar fashion, from the basolateral (serosal) cellular domain; 3) LTP-I secretion by the CaCo-2 epithelium cultured on membranes is regulated by "luminal" fatty acids.

EXPERIMENTAL PROCEDURES

Materials

The human CaCo-2 enterocyte and HepG2 hepatocyte lines were purchased from ATCC (American Type Culture Collection, Rockville, MD). All culture media, supplements, and reagents were from Gibco Laboratories and Sigma Chemical Co. T-75 culture flasks were from Corning (Ithaca, NY); "Transwell" plates were from Costar (Van Nuys, CA). Histochemical stains and reagents were purchased from Sigma. All radioisotopes were from Du Pont-New England Nuclear. Supplies and reagents for electron microscopy were from Ted Pella, Inc. (Tustin, CA). All centrifugation equipment and supplies were from Beckman Instruments.

Methods

Cell Culture—HepG2 hepatocarcinoma cells were cultured and maintained as described (10). Cell stocks of CaCo-2 colonic adenocarcinoma cells were cultured in Dulbecco's modified minimal essential medium (DMEM) with 15% fetal calf serum and maintained as described (13). For experiments described here, cells were seeded on permeable culture membrane (PCM) cups essentially as described by Traber et al. (12). Fetal calf serum DMEM growth medium in both upper and lower chambers was replaced daily from the time of plating to initiation of experiments at approximately 14 days postconfluence. Confluent cell monolayers at approximately 14 days postconfluence which had developed functional tight junctions (see below) were washed twice with Earle's saline buffer supplemented with Ca2+ and Mg2+ to maintain tight junction integrity (14), followed by a single DMEM wash, and finally replaced in serum-free (SF) DMEM to condition medium for analysis of secreted lipid transfer activity (SF-DMEM consisted of DMEM supplemented with 4 mg/ml lactalbumin hydrolysate). This final SF-DMEM wash was frozen for later use as a negative control in the radioassay of CE transfer activity. Following experiments, HepG2 and CaCo-2 cell viability was estimated by trypan blue exclusion, and cell monolayers were extracted with 0.1 N NaOH for determination of monolayer protein. All CaCo-2 cells used

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1 The abbreviations used are: CE, cholesteryl ester; HDL, high density lipoprotein; DMEM, Dulbecco's modified minimal essential medium; PCM, permeable culture membrane; SF, serum-free.
in these experiments were from passages 20–25; HepG2 were from passages 75–80.

Electron Microscopy—Transmission electron microscopy of CaCo-2 cultured on membranes, to confirm polar cell morphology, was performed by standard methods as described (13).

Assessment of CaCo-2 Monolayer Integrity—Functional integrity of CaCo-2 epithelial monolayers was assessed essentially as described (15). Briefly, permeability of intercellular tight junctions was tested by monitoring bidirectional diffusion of horseradish peroxidase across the cellular monolayer, at both 4 and 37 °C. Horseradish peroxidase diffusion was tested across every CaCo-2 monolayer, and only those manifesting a permeability barrier to macromolecular diffusion were used for further experiments.

Fatty Acid Challenge—CaCo-2 cells were grown on PCM to approximately 14 days postconfluency. CaCo-2 monolayers which were cultured on membranes, to confirm polar cell morphology, was used for further experiments.

Thus, the only source of CE transfer activity detected in these samples was LTP-I. LTP-I immunoreactive protein was not detected in medium of the basolateral compartment of the PCM apparatus. Thus, CaCo-2 transfer CE transfer activity was vectorially sorted to, and secreted from, the basolateral cellular domain, as are intestinal apolipoproteins secreted by this cell (12).

Molecular Identity of CaCo-2 CE Transfer Protein—That LTP-I protein was responsible for CE transfer activity detected in cell-conditioned medium was suggested by immunoinhibition of 100% of CaCo-2- and HepG2-secreted CE transfer activity by incubation with specific anti-LTP-I (Fig. 2); immunoinhibition followed a dose-dependent pattern. Thus, only secreted CE transfer activity detected in these samples was LTP-I. LTP-I immunoreactive protein was not present.

RESULTS
CaCo-2 and HepG2 Secretion of CE Transfer Activity—CaCo-2 cells cultured on PCM to approximately 14 days postconfluency secreted cholesteryl ester transfer activity, with 8.7 ± 2.2 total units/μg of cell protein CE transfer activity secreted over a 24-h period during serum-free incubation (mean ± S.D., N = 10, range = 5.8–12.5 units/μg). This is approximately twice the rate we observed for secretion of CE transfer activity by HepG2 hepatocytes (10). As with HepG2 secretion of CE transfer activity, CaCo-2 cells secreted CE transfer activity in a linear, time-dependent manner for at least 24 h (Fig. 1). In cell-conditioned medium from CaCo-2 cultured on membranes, all CE transfer activity was detected in medium of the basolateral compartment of the PCM apparatus. Thus, CaCo-2 transfer CE transfer activity was vectorially sorted to, and secreted from, the basolateral cellular domain, as are intestinal apolipoproteins secreted by this cell (12).

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detected in CaCo-2 apical conditioned medium, providing further evidence that CaCo-2 cells vectorially sort and secrete LTP-I from the basolateral cellular domain. Furthermore, immunoblot analysis of secreted proteins revealed a single band of LTP-I immunoreactive material in both CaCo-2- and HepG2-conditioned media, with an apparent Mᵣ = 63,000 (Fig. 2, inset). This is consistent with the size of LTP-I purified to homogeneity from human plasma (1), and with that reported for LTP-I visualized by monoclonal antibody immunoblot (20). The apparent molecular weight and broad nature of the CaCo-2 and HepG2 LTP-I bands suggests that LTP-I is secreted from these cells in its glycosylated form (21), as LTP-I cDNA encodes a Mᵣ = 54,000 protein which contains four potential sites for N-linked glycosylation (11).

Preliminary data from solution-hybridization of CaCo-2 RNA using a portion of the LTP-I cDNA (11) indicates that CaCo-2 do indeed transcribe mRNA for LTP-I, corroborating immunoinhibition and Western blot data that the molecular identity of CaCo-2 lipid transfer protein is LTP-I (not shown).

Regulation of CaCo-2 LTP-I Secretion—CaCo-2 cells cultured on permeable membranes upregulated secretion of CE transfer activity in response to increasing concentrations of oleate in the apical PCM culture compartment (Fig. 3); LTP-I activity was detected only in the basolateral medium compartment. The effect appeared to saturate at approximately 250 µM sodium oleate under our culture conditions. Secretion of cholesteryl ester transfer activity by HepG2 hepatocytes was unaltered by similar concentrations of fatty acid (Fig. 3). Thus, although HepG2 hepatocytes were confirmed to constitutively synthesize and secrete LTP-I in vitro, HepG2 cells did not regulate LTP-I secretion in response to this lipid challenge.

Spontaneous (background) transfer of labeled lipid substrates under our assay conditions was <5% of the experimental activity in all cases. In addition to the usual controls, aliquots of the above concentrations of sodium oleate in SF-MEM were incubated with donor-acceptor lipoprotein substrate in the presence or absence of a known source of LTP-I (chromatographed from human plasma, eluted from phenyl-Sepharose with water as described (1), purified approximately 250-fold). This was necessary to determine the effect of these concentrations of sodium oleate on apparent transfer activity of a constant source of LTP-I, since it has been suggested that increasing fatty acid concentration may increase the apparent CE transfer activity of LTP-I (22). However, we observed no effect of sodium oleate on CE transfer activity manifest by a constant source of LTP-I under our experimental conditions.

**DISCUSSION**

The principal rationale for investigating the CaCo-2 enterocyte model for synthesis and secretion of the plasma cholesteryl ester transfer protein was the Northern blot demonstration of LTP-I mRNA in polyadenylated RNA extracted from human intestine (11). The present data suggest that the enterocyte is responsible for at least some of this intestine LTP-I mRNA. We have demonstrated CaCo-2 secretion, and confirmed HepG2 secretion, of the LTP-I protein by: 1) secreted cholesteryl ester transfer activity, 2) anti-LTP-I immunoinhibition of 100% of secreted CE transfer activity, and 3) immunoblot technique revealing a single reactive protein, confirming the molecular identity of the protein responsible for all CE transfer activity secreted by CaCo-2 and HepG2 cells. We have previously demonstrated immunoinhibition of 100% of HepG2-secreted Ce transfer activity (10). Curiously, this is in contrast to a subsequent report of 20% residual CE transfer activity following immunoprecipitation of HepG2-secreted LTP-I, which suggests secretion of more than one cholesteryl transfer protein by HepG2 (21).

Our present findings are consistent with our earlier demonstration that a single protein is responsible for the CE transfer activity detected in human plasma (1) and for CE transfer activity secreted by HepG2 cells (10).

The principal cellular and tissue sources of human plasma LTP-I remain a matter of speculation. Cholesteryl ester transfer activity has been detected in concentrated rabbit liver perfusate, suggesting that liver is a source of plasma LTP-I (23). Furthermore, synthesis and secretion of LTP-I by the human HepG2 hepatocyte model (10) suggests that the liver parenchymal cell may be responsible for cholesteryl ester transfer activity detected in liver perfusate, although the hepatic Kupffer macrophage may contribute as well (9). Enterocyte synthesis of LTP-I is suggested by the presence of LTP-I mRNA in intestinal extract and confirmed by the present data. However, the relative hepatic and intestinal contributions to the total plasma LTP-I pool is unknown.

Data on the physiological correlates of CE transfer activity in vivo suggest that LTP-I protein mass is upregulated in response to both acute (24) and chronic (25) lipemia. As the immediate site of lipid absorption, the intestinal enterocyte is suspect as a source and regulatory site for proteins involved in lipid metabolism (26), including LTP-I. In vivo, dietary triglycerides are lipolyzed to free fatty acids and monoglycerides in the intestinal lumen. These readily enter the enterocyte epithelium, where they are used to resynthesize triglycerides to be packaged and secreted with intestinal apolipoproteins. The present data are consistent with tissue-specific upregulation of LTP-I secretion by intestinal epithelium in response to fatty acid flux. Thus, dietary fat intake may acutely upregulate LTP-I levels in order to meet increased demands for lipid transport in postprandial plasma. In this context, it is noteworthy that LTP-I, the cholesteryl ester

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**Fig. 3. Fatty acid regulation of LTP-I secretion by CaCo-2 cells.** Conditioned media, from CaCo-2 and HepG2 cells following 24-h exposure to the concentrations of sodium oleate indicated, were assayed for CE transfer activity. Relative amounts of LTP-I activity secreted are expressed as percent of control (serum-free conditioned media = 100%). Data points represent the mean of six determinations from three dishes each; S.D. were <10%.
transfer protein, also mediates the net mass transfer of triglycerides and phospholipids between the plasma lipoproteins. Thus, plasma and tissue lipid homeostasis during alimentary lipemia may be maintained by upregulating LTP-I, as demonstrated here, and perhaps coordinately by upregulating lecithin:cholesterol acyltransferase activity, as suggested elsewhere (27).

It has been difficult to assess in vivo the question of whether the correlation of LTP-I-mediated CE transfer activity with plasma lipemia is causal or consequential. The data presented here suggest that acute lipid challenge causes upregulation of LTP-I secretion by enterocytes but not hepatocytes. Based on these observations, we anticipate in vivo data to corroborate the intestine as a principal regulated source of plasma LTP-I in humans.

The specific mechanism by which free fatty acids may upregulate protein secretion is unknown. However, sterols have been shown to control levels of protein mass and mRNA for other proteins which regulate cell cholesterol levels (28-30). A similar mechanism may be operating in the present case, perhaps indirectly through an intracellular sterol carrier or fatty acid binding protein (31). The present data do not speak to the question of whether the observed regulation occurs at transcriptional, translational, or secretory levels. We will need to collate solution-hybridization quantitation of specific LTP-I mRNA with quantitation of secreted LTP-I lipid transfer activity and mass in order to address this question.

The precise physiological roles and clinical significance of LTP-I remain uncertain. The hypothesized role of LTP-I in reverse cholesterol transport (3), and reports of LTP-I-mediated CE transfer from peripheral cells (6, 7) and to liver cells (8), support a preventive role of LTP-I in arterial lipid deposition. Alternatively, transfer of additional CE to the atherogenic apo-B-containing lipoproteins could potentially exacerbate an atherogenic condition. Moreover, growing evidence of synthesis and secretion by many cell types suggests that LTP-I may play a more pervasive role in lipid metabolism than previously suspected. The advent of our present, and previous (9, 10), models of cellular LTP-I production should help us understand the regulation of LTP-I synthesis and secretion, as well as its role in lipid metabolism. Finally, the present data lend further validity to CaCo-2 cells cultured on membranes as a model of enterocyte epithelium.

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