Synthesis and Secretion of Plasma Cholesteryl Ester Transfer Protein by Human Hepatocarcinoma Cell Line, HepG2

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We have examined the synthesis, secretion, and functional and physical characteristics of a lipid transfer protein synthesized by a human hepatocellular carcinoma line. We found that this protein shares immunochemical determinants and many other properties with the lipid transfer protein, LTP-I, which has been purified from human plasma. We conclude that the human liver cell line, HepG2, synthesizes and secretes LTP-I. Thus, hepatocytes may be the source of LTP-I in human plasma.

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Although the majority of the cholesteryl ester (CE) resides in the low density and very low density lipoproteins (LDL, VLDL) of human plasma, most of plasma CE is generated on the high density lipoprotein (HDL) fraction by the lecithin:cholesterol acyltransferase (LCAT) enzyme from free cholesterol and phospholipid (PL). Since the substrate on which this LCAT reaction occurs is primarily the HDL, the observed distribution of CE among other plasma lipoproteins reflects the movement of CE from HDL to VLDL and LDL. This phenomenon, described in 1965,1 is now believed to result from the action of a single protein, designated lipid transfer protein-one (LTP-I), which has been purified from both human2,3 and rabbit plasma.4 In addition to promoting CE exchange and transfer, this protein also mediates the exchange and net mass transfer of triglyceride (TG) and PL between the plasma lipoproteins.5 A second transfer protein in human plasma, LTP-II, facilitates the exchange and transfer of only PL between plasma lipoproteins.5 The two transfer proteins can be distinguished by several properties: LTP-I, Mr approximately 63 kilodaltons, transfers CE, TG, and PL between the plasma lipoproteins, exhibits no affinity for heparin, and is stable to heat (56°C, 1 hour); LTP-II, Mr approximately 66 kilodaltons, transfers only PL and binds to heparin; virtually all of its activity is destroyed by heating (56°C, 1 hour).

In situ evidence which implicates LTP-I in a fundamental role in plasma sterol homeostasis is rapidly accumulating. LTP-I cholesteryl ester transfer activity modulates the LCAT reaction.2 It has been suggested that this may be due to the relief of CE product-inhibition of LCAT by transfer of CE out of HDL, since the enhancement of cholesterol esterification by VLDL is dependent upon transfer of CE from HDL to VLDL.5 LTP-I is postulated to play a role in reverse cholesterol transport6 of cholesterol from extrahepatic tissues by transferring LCAT-derived CE from HDL to other lipoproteins for uptake by the liver.7 Excess cholesterol in these lipoproteins is ultimately excreted by the liver into the biliary as unesterified cholesterol or bile acid.8 LTP-I may also mediate transport of cholesteryl ester directly to and from cells as suggested by recent in vitro models of CE efflux from cellular and extracellular components.9 and by preliminary data on the transfer of CE from macrophages.10

Efforts to understand the function and regulation of the cholesteryl ester transfer protein have increased interest in the tissue source(s) of LTP-I. It is perhaps intuitive that the hepatic parenchymal cell would be the source of a plasma transfer protein intimately involved in lipoprotein sterol metabolism. This expectation seems to be supported by reports of LTP-I transfer activity in concentrated rabbit liver perfusate.11,12 On the other hand, there has been no direct demonstration of LTP synthesis or secretion by hepatocytes or any liver cell line. Furthermore, our demonstration of synthesis and secretion of the cholesteryl ester transfer protein by human monocyte-derived macrophages13 suggests an alternative explanation: Since Kupffer cells, the resident macrophages of the liver,14 may account for 10% of liver cells, 11 it is conceivable that these cells were the source of lipid transfer activity detected in rabbit hepatic perfusate.

The HepG2 human hepatocarcinoma cell line has generated enthusiasm as a human hepatocyte model,16 and has been validated as a model for the human hepatocyte metabolism of many hepatic proteins. These cells secrete the hepatic lipoproteins17-21 and the LCAT22 and TG-lipase23 enzymes. We therefore investigated the HepG2 cell line for potential synthesis and secretion of the human plasma lipid transfer proteins.

Methods

Materials

The human hepatocellular carcinoma lines, HepG2 and Hep3B, were purchased from American Type Culture Col-
lection (Rockville, Maryland). All tissue culture reagents were from Gibco (Santa Clara, California). T-75 culture flasks were from Corning (Seattle, Washington) and the six-well culture plates were from Flow Laboratories (McLean, Virginia). Aprotinin and cycloheximide were from Sigma (St. Louis, Missouri). All isotopically-labelled lipids were purchased from New England Nuclear (Boston, Massachusetts). Most column matrices were from Pharmacia (Piscataway, New Jersey); CNBr-activated Sepharose used for immunoaffinity purification of goat antihuman LTP-I was obtained from Sigma. All centrifugation materials and equipment were by Beckman (Palo Alto, California).

Cell Culture

The human hepatocarcinoma cell lines, HepG2 and Hep3B, were grown in minimal essential medium (MEM) with Hank's salts, 10 mM HEPES, 6.8 mM glucose, penicillin and streptomycin, and nonessential amino acids, supplemented with 10% heat-inactivated fetal bovine serum (FBS), and incubated at 37°C under 5% CO2/95% air atmosphere. The media were replaced with fresh MEM/FBS every three days, and the cells were subcultured weekly: 1:3 for HepG2 and 1:6 for Hep3B. The cells were utilized for experiments within 10 days of plating upon reaching approximately 90% confluency, corresponding to approximately 2 x 10⁷ cells/T-75 flask.

For timecourse secretion studies, cells were grown in six-well plates and allowed to reach approximately 90% confluency before use in experiments. At T₀ all growth media were aspirated, cell layers were washed twice with 1.5 ml serum-free MEM (SF-MEM), and replaced in 1.0 ml fresh SF-MEM for incubation at 37°C, 5% CO₂/95% air for varying lengths of time to condition media. These initial washes were assayed for residual apoprotein A-I and lipid transfer activity. At each respective time-point, media were harvested from each of six wells from a single six-well culture plate. Harvested media were centrifuged for 1 minute in a "Beckman 12" microfuge, and washed twice with Hank's buffer, and cell viability was estimated by trypan-blue exclusion. For cell protein determination, cell layers were then harvested by trypsinization, pelleted by centrifugation at 5000 g for 1 minute in a "Beckman 12" microfuge, and washed twice with Hank's buffered saline. The resulting pellet was dissolved in 0.1 N NaOH for protein determination. To examine the effect of the inhibition of cellular protein synthesis on LTP secretion, cycloheximide was made up to 1.0 mM stock in absolute ethanol and was diluted 1:200 and 1:2000 with SF-MEM for use. Control cultures were treated in parallel with identical dilutions of ethanol; the final concentration of ethanol in all instances was < 0.5%.

For all other analyses of HepG2-secreted lipid transfer activity, cells were grown in 75 cm² flasks and the cells were utilized for experiments upon reaching approximately 90% confluency as above. HepG2 culture media were harvested following a 24-hour incubation and immediately brought to 4°C, filtered (0.45 μm, Millipore, Bedford, Massachusetts), and brought to 0.05% NaN₃, and 0.5 IU/ml Aprotinin and 1 mM EDTA, pH 7.4. All subsequent chromatographic steps were carried out at 4°C.

Preparation of Labelled Lipoprotein Substrates

The ³⁴C-cholesterol ester (CE)-HDL₃, ³²P-triglyceride (TG)-HDL₃, and ³²P-phosphatidylcholine (PC)-HDL₃ donors were prepared as described previously.² The approximate specific activities for these (CE)-HDL₃, (TG)-HDL₃, and (PC)-HDL₃ donor lipoproteins were 2,500 dpm/μg CE (assuming a molecular weight of 650 for CE), 18,000 dpm/μg TG, and 8,000 dpm/μg PC, respectively.

Radioassays of Lipid Transfer Activity

All assays were performed at the optimal lipoprotein-donor/lipoprotein-acceptor (d/a) ratio for each HDL₃ donor and d < 1.063 (or d < 1.006) g/ml acceptor lipoprotein to yield maximal transfer activity for that substrate preparation as determined with a given amount of cholesteryl ester transfer activity partially purified from human plasma.

The cell medium harvested during the timecourse experiments was frozen and stored at −70°C for analysis at the close of the experiment to minimize the potential systematic error. A typical transfer assay incubated 50 to 200 μl HepG2 conditioned medium (CM) with 200 μl donor/acceptor substrate, 4°C for 10 to 20 hours at 37°C. To determine the amount of nonfacilitated, spontaneous transfer of CE label between donor and acceptor lipoproteins, the negative controls included substitution of the HepG2 CM sample with an equal volume of serum-free MEM incubated with d/a substrate at 37°C, an equal volume of HepG2 CM test sample incubated with d/a substrate at 4°C, or an equal volume of the final (SF-MEM) cell wash from the start of the experiment incubated with d/a substrate at 37°C. Aliquots of partially purified LTP from human plasma, "PS-pool," (eluted from phenyl-Sepharose CL-6B with H₂O, purified approximately 250-fold) were stored at −70°C for use as a positive reference standard; the activity of this standard was observed to remain stable over the course of these investigations.

After the sample incubations, two methods were employed for separating the labelled-HDL₃ donor lipoproteins from the acceptor lipoproteins. In the first method, separation of HDL₃ donor from d < 1.006 g/ml acceptor lipoproteins was achieved by ultracentrifugation in a Beckman Ti 42.2 rotor (230 μl sample volume) at 40,000 rpm, 48 hours, 4°C. Following ultracentrifugation, sample tubes were flash-frozen in liquid nitrogen, and the d < and > 1.006 g/ml fractions were obtained by tube slicing. Each fraction was counted in its entirety to obtain the percent of labelled lipid transferred from the HDL₃ donor to the d < 1.006 g/ml fraction when incubated at 37°C with HepG2 CM. Alternatively, labelled HDL₃ donor lipoproteins were separated from d < 1.063 g/ml acceptor lipoproteins by precipitating d < 1.063 g/ml acceptor lipoproteins with 50 kDa dextran sulfate-MgCl₂ as described previously.² Aliquots of the donor supernatants were taken for calculation of labelled substrate lipid transferred. For both methods the transfer activity was defined as the percentage of transfer of la-
belled substrate from donor lipoprotein to acceptor lipoprotein mediated by a specific protein or protein complex present in cell culture media or in human plasma. All calculations took into account the small amount of spontaneous, nonfacilitated transfer by subtracting endogenous transfer activity in the controls from total transfer observed in sample assays. Nonfacilitated transfer of neutral lipid substrates under these assay conditions was <3%. 2

Determining Heat Stability of HepG2 LTP-I Activity

Aliquots of HepG2 medium were mixed 1:1 (vol/vol) with 10% bovine serum albumin and incubated at either 37°C or 56°C for 60 minutes, after which triplicate aliquots were taken for the assay of radiolabeled CE-, TG-, or PC-transfer activity as described above.

Chromatography of HepG2 Lipid Transfer Activity

Hydrophobic Matrix Chromatography

Hydrophobic-interaction chromatography was carried out on phenyl-Sepharose as described2 for plasma-derived LTP-I, modified to include 1 M urea at all steps as follows: HepG2 CM collected as above after a 24-hour exposure to HepG2 were adjusted to 2.0 M NaCl/1.0 M urea with the addition of solid salt, and by dilution with 8.0 M urea solution. This sample was passed through a 8 × 0.5 cm phenyl-Sepharose CL-6B column equilibrated with 2.0 M NaCl, 1.0 M urea, 10 mM Tris (pH 7.4), at a flow rate of approximately 20 ml/hr. Aliquots of the nonbinding volume were assayed for cholesteryl ester transfer activity and compared to starting material to determine the percentage of applied activity which remained bound to phenyl-Sepharose. The column was then washed with several column-volumes of 2.0 M NaCl, 0.01 M Tris (pH 7.4) to remove any residual nonbinding material. After absorbance at 280 nm of the nonbinding volume returned to baseline, the column was washed with several column volumes of 0.15 M NaCl, 1.0 M urea, 10 mM Tris (pH 7.4) to lower the ionic strength of the eluant without eluting the transfer activity. The HepG2 lipid transfer activity was finally eluted with 1 M urea in distilled, deionized H2O. The 1 ml fractions were collected and 50-µl aliquots were assayed for CE transfer activity. An aliquot of human plasma was chromatographed as above to determine whether the presence of this concentration of urea affected the elution profile of LTP-I CE transfer activity from phenyl-Sepharose and for a qualitative comparison of HepG2 CE transfer activity and plasma CE transfer activity elution profiles.

Chromatofocusing

Chromatofocusing of the LTP-I activity secreted by HepG2 in vitro and of partially purified LTP-I from human plasma was carried out on a 0.5 × 6 cm column of PBE-94 chromatofocusing gel, with a 1 cm overlay of G-25 to evenly disperse the sample. The column was eluted with 25 mM histidine-HCl buffer (pH 5.8) until the eluant pH indicated equilibration. An aliquot of HepG2 CM or plasma sample was dialyzed against the starting buffer (pH 5.8) and was applied to the column. Cholesteryl ester transfer activity was eluted with a pH gradient running from pH 5.5 to 3.9 at a flow rate of 18 cm hr⁻¹. To determine the pl of transfer activity in the presence of urea, chromatofocusing was carried out as above, with 1 M urea present in all buffers and samples. Fractions of 600 µl were collected, their pH was recorded, and 1 M Tris (pH 7.4) was added to a final concentration of 10 mM to equilibrate all samples before assay; aliquots were assayed in triplicate for CE- and PC-transfer activity. A sample of plasma PS-pool was chromatographed as above, with and without 1 M urea, for comparison.

Heparin-Affinity Chromatography of HepG2 Neutral Lipid Transfer Activity

An aliquot of CM collected after a 24-hour exposure to HepG2 was diluted 1:3 with distilled, deionized H2O to lower its ionic strength to equal that of 50 mM NaCl; it was applied to a heparin-Sepharose CL-6B column equilibrated with 50 mM NaCl, 10 mM Tris (pH 7.4). Aliquots of the starting material and of the nonbinding volume were assayed for CE-, TG-, and PC-transfer activity to determine the percentage of the activity that remained bound to heparin-Sepharose.

Chromatographic Isolation of an Inhibitor of LTP-I Activity from HepG2

HepG2 CM and heparin-Sepharose gel were prepared as described above for heparin-affinity chromatography. Approximately 50 ml of HepG2-CM (diluted 1:3 with ddH2O) were applied to a 0.75 × 8 × 8 cm heparin-Sepharose column at approximately 20 ml/hr. The nonbinding volume was collected and the column was washed with 50 mM NaCl, 10 mM Tris (pH 7.4) until optical absorption at 280 nm returned to baseline. The column was then eluted with a gradient generated from 50 mM to 500 mM NaCl, 10 mM Tris (pH 7.4), and 1.0 M urea. The optical density at 280 nm was determined for each 3 ml fraction collected, and fractions comprising the single protein peak were pooled, dialyzed against TBS, and tested for inhibitory activity against HepG2 and partially purified plasma CE transfer activity.

LTP-I Inhibitor Assay

Inhibitory activity against cholesteryl ester transfer activity was taken to indicate inhibition of LTP-I. The inhibition assay used was similar to that described previously. 26 Briefly, donor and acceptor lipoproteins (d/a) were incubated with samples containing either HepG2 culture medium or partially purified plasma lipid transfer activity (PS-pool) as sources of positive CE transfer activity. Aliquots of the test sample (bound protein which was eluted from heparin-Sepharose and dialyzed against TBS) were added to tubes of this d/a-transfer activity mixture in varying amounts. The final volumes of all tubes were equilibrated by addition of TBS. Percent inhibition was calculated as the percent decrease in apparent CE-label transferred from HDL₃ donor lipoproteins to d < 1.063 g/ml acceptor lipoproteins in incubations containing test samples, as compared to incubations containing no inhibitory activity (TBS).
Isolation and Characterization of Anti-LTP-I

LTP-I was purified from plasma to homogeneity (as determined by SDS-polyacrylamide electrophoresis) by sequential chromatographic isolation on phenyl-Sepharose, diethylaminoethyl-Sepharose, carboxymethyl-cellulose, heparin-Sepharose, and hydroxyapatite-BioGel, and finally eluted from a 2 M urea gel. This sample was used for the initial inoculation of a goat, with a booster injection administered 3 weeks later. The goat was bled prior to inoculation and 1 week after the booster shots. Both samples were brought to d = 1.21 g/ml by addition of solid KBr and ultracentrifuged at 48,000 rpm, 4°C, for 40 hours. Any endogenous goat LTP-I activity which might have yielded spurious activity in the neutral lipid transfer assay was removed from d > 1.21 g/ml goat plasma by hydrophobic-interaction chromatography on a phenyl-Sepharose CL-6B column equilibrated with 2 M NaCl. The nonbinding pool was pooled, dialyzed vs. TBS, and assayed for CE transfer activity to confirm that it contained no residual LTP-I activity in a transfer assay using human HDL₃ as donor, and human d < 1.063 g/ml lipoproteins as acceptor.

Western blot analysis of this polyclonal antibody preparation revealed the presence of contaminant antibodies that recognized human serum albumin (HSA). These were subsequently removed by passage of the antibody preparation over HSA-Sepharose (Sigma fraction-V, 17.6 mg total). This sample was used for immunoblot analysis.

Further analysis of this antibody preparation revealed the following characteristics:

1) This affinity-purified anti-LTP-I does not immunoprecipitate radiolabelled apoproteins A-I, A-II, D, E, or LCAT.
2) This antibody does not interfere with recognition of these antigens by their respective antibodies in radioimmunoassay.
3) Western immunoblot analysis of plasma, partially-purified LTP-I (PS-pool), apoproteins A-I, A-II, D, E, LCAT, and HSA demonstrate no immunoreactivity of this antibody with A-I, A-II, D, E, LCAT, or HSA.
4) In addition to its immunoprecipitation of LTP-I activity, this antibody has the ability to directly inhibit LTP-I activity when incubated with LTP-I that has been purified to homogeneity, as determined by SDS-PAGE.

Immunoinhibition of HepG2 Lipid Transfer Activity

This immunofinity-purified goat antihuman LTP-I preparation was subsequently used for immunoinhibition assays, as described by Abbey et al. Briefly, equal aliquots of HepG2 CM were incubated for 20 hours at 4°C with increasing volumes of immune serum while maintaining a constant final volume (total incubation volumes were 2.4 ml: 2 ml CM incubated with increasing amounts of antisera, from 10 to 400 µl; samples were qs to 2.4 ml with TBS). After an end-over-end, 18-hour pre-incubation of cell CM with antisera at 4°C, samples were centrifuged at 1000 g for 20 minutes to precipitate antibody-antigen complexes, and 100 µl aliquots of the supernatant were assayed for LTP-I activity. Control incubations were run in parallel and substituted pre-immune goat serum subjected to the same treatment, or TBS, for the immune serum. Conditioned media from Hep3B cells were treated, as for HepG2-CM, for determination of anti-LTP-I immunoprecipitation of cholesteryl ester transfer activity.

Radioimmunoassay of Apoprotein A-I

Apoprotein A-I was labelled by the iodine monochloride method. The radioimmunoassay (RIA) buffer contained 1% BSA and 1 mM EDTA (pH 7.4) in TBS. All samples and standards were diluted in RIA buffer to which 0.04% Tween 20 was added. Each assay tube contained 100 µl of sample, standard, or buffer, 100 µl of affinity-purified, rabbit antihuman A-I sera diluted in RIA buffer. After an overnight incubation at 4°C, 100 µl of normal rabbit serum and 300 µl of sheep antirabbit IgG serum were added and tubes were incubated again; the precipitates were washed and counted in a similar method to that of Albers et al.

Results

The viability of HepG2 and Hep3B cells in vitro was found to exceed 85% in all cases and to average greater than 95% for all experiments. Even though cells were seeded in medium supplemented with 10% fetal bovine serum, which is considered to have virtually no cholesteryl ester transfer activity, we determined the extent to which this small amount of exogenously applied LTP-I could account for the observed activity in HepG2 conditioned medium. Of the total amount of cholesteryl ester transfer activity and apoprotein in cell growth medium, less than 6% cholesteryl ester transfer activity, and less than 1% of the A-I mass, were detectable in the first wash of serum-free medium, and no residual CE transfer activity nor apoprotein A-I mass were detectable in the second wash. Thus, in all experiments described here, the cell layers were washed three times with serum-free minimal essential medium (SF-MEM) before making final aliquots of SF-MEM for HepG2 incubation.

CE-, TG-, and PC-transfer activities were assayed by the methods of Albers et al.² by use of both ultracentrifugation and dextran-sulfate/MgCl₂-precipitation to separate labelled donor HDL from acceptor VLDL. The results obtained by these two methods were found to agree within 10%. Therefore, separation of donor and acceptor for most assays was carried out by the method of dextran precipitation for the sake of convenience.

The secretion of CE transfer activity into HepG2 serum-free culture medium was observed to be linear for at least 24 hours (Figure 1A), with 28.7% transfer (%T/200 µl/20 hr incubation) secreted by the cells during the first 24 hours in vitro. Freezing HepG2 CM at −70°C over the course of these experiments did not diminish detectable lipid transfer activity. The transfer of radiolabelled cholesteryl ester by HepG2 CM collected at 24 hours exhibited a linear, dose-dependent increase in activity (Figure 2A), and also
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Figure 1. Time course of LTP-I (A) and apoprotein A-I (B) secretion by HepG2 in culture. Serum-free HepG2 culture media were harvested at 6, 12, 24, and 48 hours for assay of cholesteryl ester transfer activity and radioimmunoassay of apo A-I mass. LTP-I activity is expressed as % labelled cholesteryl ester transferred during a 20-hour assay incubation, by 200 µl HepG2 conditioned media collected at the times indicated on the abscissa. Data points represent the mean ± SD from five dishes, with LTP-I activity and apo A-I mass assayed in sextuplicate and triplicate, respectively.

showed a linear increase in activity during incubation with substrate donor and acceptor lipoproteins (Figure 2B). The level of CE transfer activity in HepG2 CM between experiments was variable, with the 24-hour HepG2 culture medium exhibiting a mean of 14.0 ± 6.4% T/100 µl/20 hr incubation (mean ± SD, n = 5; range = 5–19.5% T). The levels of transfer of the different lipid substrates were similarly variant, and depended in large part on each respective donor/acceptor lipoprotein substrate preparation as has been reported by others.31

The detection of apo A-I mass in unconcentrated HepG2 culture medium revealed a pattern of secretion with time similar to that observed for CE transfer activity (Figure 1B), and very similar to that reported by Chen et al.22 The detectable rate of apo A-I secretion was 5.7 mg/g of cell protein per day, not accounting for endocytosis or degradation of secreted protein. This is in close agreement with the rate of secretion (4.5 mg/g/day) recently reported by Thrift et al.17

The secretion of CE transfer activity was inhibited in a dose-dependent manner by cycloheximide and was completely blocked by 5.0 µM cycloheximide (Figure 3A) suggesting that active protein synthesis is required for the appearance of LTP-I transfer activity in HepG2 CM. Apoprotein A-I secretion followed a similar pattern of dose-dependent inhibition (Figure 3B). There were no differences in cell viability between cycloheximide-treated and ethanol-treated controls.

As summarized in Table 1, further characterization of the lipid transfer protein secreted by HepG2 revealed it to have many physical and functional properties indistinguishable from those of human plasma LTP-I, as follows:

Immunoinhibition. CE transfer activity secreted by HepG2 was inhibited in a dose-dependent manner by preincubation with goat antihuman-LTP-I (Figure 4A). CE transfer activity secreted in vitro by another human liver cell line, Hep3B, was similarly inhibited by this antiserum (Figure 4B). Pre-immune goat serum did not inhibit secreted transfer activity, and there were no differences between CE transfer activity in samples incubated with pre-immune goat serum and those incubated with Tris-saline buffer.

Lipid Substrates. In accordance with observations on substrate transfer by LTP-I purified from human plasma, HepG2-conditioned medium promoted transfer of labelled CE, TG, and PC at approximately the same rate, with 8% 14C-HDL3-CE, 8% 3H-HDL3-TG, and 7% 3H-HDL3-PC transferred to d < 1.063 g/ml lipoproteins/100 µl CM/12 hour incubation.

Isoelectric Point. Analysis of the HepG2 CE- and PC-transfer activity profiles off chromatofocusing (Figure 5A) revealed these transfer activities to co-elute at a pH of 4.8 ± 0.1 (mean ± SD, n = 5). There was no shift in the pI of either CE or PC HepG2-secreted transfer activity when

Figure 2. Linear increase in CE transfer activity with increasing concentration of HepG2 culture medium (A) and with increasing time of incubation with donor/acceptor lipoprotein substrates (B). HepG2 conditioned media assayed for both A and B were collected following 24-hour incubation with cells. Data for both A and B are expressed as the mean of six determinations per point and the line of regression shown; symbols encompass ± SD for each respective data point.
Figure 3. Cycloheximide inhibition of HepG2 synthesis of cholesteryl ester transfer activity (A) and of apoprotein A-I mass (B). Cells were grown as for time course-secretion studies, and media replaced with SF-MEM containing 0 μM cycloheximide (•), 0.5 μM cycloheximide (○), or 5.0 μM cycloheximide (▲) and were incubated at 37°C in 5% CO₂/95% air mixture. Media were collected from separate dishes at 6, 12, and 24 hours, respectively, and assayed for CE transfer activity and apo A-I mass. Data are expressed as the percent of the total amount of LTP-I activity or apoprotein mass present at the 24-hour time point in the absence of cycloheximide (ethanol-treated controls). Data points for A and B are plotted as the mean of triplicate determinations ± SD from three and four dishes, respectively. The data-point symbols in B encompass ± their respective SD.

chromatofocused in the presence of 1 M urea (Figure 5B). This is very similar to the pl of plasma-derived LTP-I. When we chromatofocused partially purified LTP-I from human plasma, it also exhibited a pl of 4.8 for CE transfer activity, which did not shift in the presence of urea. These findings are in agreement with earlier reports on the effect of urea on the apparent pl of plasma-derived LTP-I.³

Phenyl-Sepharose Chromatography. A complete 100% of HepG2 LTP-I activity was observed to bind phenyl-Sepharose, and eluted with water containing 1 M urea (Figure 6A). Since the effect of 1 M urea on the elution profile of plasma LTP-I activity from phenyl-Sepharose was unknown, we determined whether human plasma LTP-I continued to elute in the water peak, as we had shown previously,² in the presence of urea. Chromatography of human plasma on phenyl-Sepharose in urea did not alter the elution profile of LTP-I CE-transfer activity; as with HepG2 lipid transfer activity, plasma LTP-I activity eluted with the urea-H₂O peak (Figure 6B).

Affinity for Heparin-Sepharose. As with human plasma LTP-I, no HepG2-secreted LTP-I activity was observed to bind to heparin-Sepharose. Instead, facilitated CE-, TG-, and PC-transfer activity in the heparin-Sepharose non-binding volume exhibited approximately a 40% increase over the prechromatographed, starting cell medium. This suggested that the HepG2-conditioned medium contains an inhibitor of LTP-I activity which exhibits an affinity for heparin-Sepharose. We therefore investigated the heparin-Sepharose binding material for inhibitory activity against LTP-I activity. When we subsequently eluted the column with a 50 to 500 mM NaCl, 1 M urea gradient, a fraction was obtained that inhibited CE transfer from plasma PS-pool or HepG2-conditioned medium. Under our assay conditions, the ED₅₀ of this HepG2 inhibitory fraction eluted from heparin-Sepharose was 100 μl, or approximately 25 μg of total protein, to inhibit either plasma CE transfer activity (10% transfer) or HepG2-secreted CE transfer activity (8% transfer, corresponding to 100 μg HepG2 CM incubated with transfer substrates for 12 hours at 37°C) and inhibition followed a dose-dependent pattern. Furthermore, the recovered inhibitory activity was sufficient to account for all over-recovery of LTP-I activity in the heparin-Sepharose nonbinding fraction.

Stability

Stability with Time

The half-life of HepG2-secreted cholesteryl ester transfer activity in 0.05% NaN₃, 0.5 IU/ml Aprotinin, and 1 mM

Table 1. Summary Comparison of Physical and Functional Characteristics of HepG2-Secreted Lipid Transfer Protein with Human Plasma LTP-I

<table>
<thead>
<tr>
<th>Property</th>
<th>Human plasma LTP-I</th>
<th>HepG2 neutral lipid transfer protein</th>
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<tbody>
<tr>
<td>Immunoinhibition by goat</td>
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<td>100% inhibited</td>
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<tr>
<td>Antihuman LTP-I</td>
<td>CE, TG, PC</td>
<td>CE, TG, PC</td>
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<td>Lipids transferred</td>
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<td>4.8</td>
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<tr>
<td>pl by chromatofocusing</td>
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<td>4.8</td>
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<tr>
<td>pl by chromatofocusing in 1 M urea</td>
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<td>100% binds, elutes</td>
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<tr>
<td>Stability with time</td>
<td>Half-life = 6 days, stabilized by urea</td>
<td>Half-life = 6 days,* stabilized by urea</td>
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*Conditions of storage for half-life determination of HepG2 lipid transfer activity are as follows: conditioned medium (serum-free isotonic minimal essential medium), 0.05% NaN₃, 0.5 IU/ml Aprotinin, and 0.001 M EDTA (pH 7.4) at 4°C. CE = cholesteryl ester, TG = triglyceride, PC = phosphatidylcholine.
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Figure 4. Antihuman LTP-I immunoinhibition of cholesteryl ester transfer activity secreted by the two human hepatocarcinoma cell lines, HepG2 (A) and Hep3B (B). Equal aliquots of culture media were incubated with increasing amounts of goat antihuman LTP-I antisera, maintaining equal final incubation volumes by adding isotonic buffer. CE transfer activity with increasing antiserum (•) is expressed as the percent of control activity in the absence of added antiserum. Negative controls consisted of adding comparable amounts of pre-immune goat serum, or isotonic buffer, at three selected concentrations (○). Data points for A and B are expressed as the means ± SD for five and three determinations, respectively. Goat antihuman LTP-I and goat pre-immune sera, and LTP-I activity, were prepared and assayed as described in Methods.

Figure 5. Chromatofocusing of the cholesteryl ester and phospholipid transfer activity secreted by HepG2 in vitro without urea (A), and in 1 M urea (B).

EDTA at 4°C was approximately 6 days. With the addition of 1 M urea to HepG2 CM, 100% of the original activity remained at 60 days, 4°C. In test assays using fresh HepG2-secreted cholesteryl ester transfer activity and partially purified LTP-I from human plasma as positive controls, this concentration of urea caused a decrease in observed LTP activity of less than 10% below comparable controls containing no urea.

Thermal Stability

We compared the relative stability of CE-, TG-, and PC-transfer activities of HepG2-conditioned media and of LTP-I partially purified from human plasma. All three transfer activities (CE, TG, PC) from the cells were temperature-stable (at 56°C, less than 5% of the activity was lost in 1 hour). In contrast, PC transfer activity in plasma PS-pool decreased by approximately 50% upon heating for 1 hour, 56°C, supporting the report that approximately 50% of PC transfer activity in plasma is not due to the heat-stable LTP-I.2

Figure 6. (A) Elution of HepG2-secreted LTP-I CE transfer activity and human plasma-derived LTP-I CE transfer activity from phenyl-Sepharose in the presence of 1 M urea. (A) HepG2 CM were prepared and applied to a phenyl-Sepharose CL-6B column as described in Methods, and the column was washed with 1 M urea/1 M NaCl until optical absorbance at 280 nm returned to baseline. The ionic strength was lowered by washing (*) with 0.15 M NaCl, 1 M urea until optical absorbance returned to baseline, and LTP-I transfer activity was finally eluted with ddH2O containing 1 M urea (t). (B) An aliquot of human plasma was chromatographed as above to determine whether the presence of this concentration of urea affected the elution profile of LTP-I CE transfer activity from phenyl-Sepharose and for a qualitative comparison of HepG2 CE transfer activity and plasma CE transfer activity profiles. Data points for both A and B represent the means of triplicate determinations.
Discussion

The principal rationale for investigating a human liver cell type for potential synthesis of LTP-I was based on the reported detection of lipid transfer activity in rabbit liver-perfusate which suggested an hepatocyte-origin.11,12

HepG2 LTP-I was observed to be secreted into serum-free culture medium in a time-dependent manner which was linear for at least 24 hours. It is possible that the apparent deviation of CE transfer activity secretion beyond 24 hours was due to the instability of the HepG2 transfer protein to the longer incubations at 37°C in culture medium. It is also possible that LTP-I synthesis or secretion was down-regulated by a feedback mechanism. However, neither of these appears to be the case, as an identical pattern of secreted activity was observed when media were collected from the same dishes in a serial fashion. That is, if activity was expressed as the cumulative from samples collected at successive timepoints, the same pattern of secreted activity with time was obtained. Thus, the apparent fall-off in secreted activity beyond 24 hours may instead reflect a lack of serum factor(s) required for synthesis or secretion of transfer activity.

Although HepG2 LTP-I activity was relatively stable for 1 hour at 56°C, it was necessary to perform most experiments soon after harvesting the HepG2-conditioned medium, because competency to transfer all three substrates (CE, TG, PC) deteriorated with time, with $t_{1/2} = 6$ days at 4°C in serum-free minimal essential medium containing 0.5 IU/ml Aprotinin, 0.05% NaN$_3$, and 1 mM EDTA (pH 7.4). As the specific activity of LTP-I (CE transfer activity per concentration of total protein) in HepG2 CM is more than 100 times greater than that of pooled human plasma, the relative instability of HepG2 LTP-I activity may reflect the relative purity of the HepG2 lipid transfer protein in medium vs LTP-I in plasma. Indeed, the observed in vitro half-life of LTP-I activity secreted by HepG2 was remarkably similar to that reported for highly purified preparations of LTP-I from human plasma.22,27 Furthermore, as with LTP-I purified from human plasma,27 HepG2 LTP-I was markedly stabilized by the presence of urea during storage at 4°C. We also observed no apparent decrease in HepG2-secreted LTP-I activity over a 60-day period when it was stored in the presence of urea at a concentration of 1 M.

Approximately 40% more HepG2 LTP-I activity was recovered in the heparin-Sepharose nonbinding fraction for all three transfer activities (CE, TG, PC) than was originally applied to the column. We interpret this phenomenon to reflect the removal of an LTP-I-inhibitory fraction from the HepG2-conditioned medium by heparin-affinity chromatography. We were able subsequently to elute an inhibitory fraction from heparin-Sepharose which inhibited LTP-I activity derived both from cells and from human plasma. While there have been several reports of an LTP-I-inhibitor isolated from plasma,26,31 there have been no reports of a cellular or tissue source for this activity. This investigation constitutes the first suggestion of the cellular source for an inhibitor of LTP-I. However, whether this inhibitor is the same protein(s) as have been isolated from plasma is unknown. We are currently pursuing further purification and characterization of cell-derived inhibitor(s) of transfer activity.

LTP-I activity in human plasma is complexed with HDL,32 and may exhibit a similar half-life ($t_{1/2}$ for apoprotein A-I = 4.5 days). Under our culture conditions, HepG2 secretes detectable LTP-I activity at approximately 4 U/hr/mg cell protein. If human hepatocytes in vivo secrete LTP-I at the rate observed for this liver cell line in vitro, hepatocytes alone can account for all LTP-I activity present in human plasma.*

In the context of the regulation of the proteins secreted by HepG2 which are important in lipoprotein metabolism, cholesterol ester transfer activity has been shown to modulate LCAT activity under conditions where buildup of cholesteryl esters on LCAT-substrate HDL is potentially rate-limiting.5 The level of LTP-I activity may in turn be regulated by an inhibitory protein that has been purified from human plasma.36,37 In the present report, we describe the partial purification of a material secreted by HepG2 in vitro which exhibits similar inhibitory activity against cholesteryl ester transfer activities derived from both human plasma and HepG2 cells.

These observations have interesting implications in the context of the postulated reverse cholesterol transport pathway.7 The principal components of this pathway are the HDL, LCAT, and the CE transfer protein, LTP-I, and B-containing lipoproteins.7 Furthermore, it has been suggested that the hepatic lipase plays a role by increasing hepatic cholesterol uptake from HDL.25,26 Thus, HepG2 synthesizes all of the proteins postulated to play a role in reverse cholesterol transport: apo A-I and A-II, LCAT,25 and LTP-I.

With the addition of LTP-I to the list of HepG2 secretory proteins that function in lipoprotein metabolism, the HepG2 cell line should serve as a valuable model of the regulation and function of LTP-I. Finally, this cell model should provide important information on the functional relationship between these key proteins of lipoprotein metabolism.

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