Cytokines Decrease Apolipoprotein Accumulation in Medium From Hep G2 Cells

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Abstract Cytokines, important biochemical mediators of inflammation, cause a rapid fall in the plasma concentration of cholesterol in vivo. One mechanism by which cytokines may cause acquired hypercholesterolemia is by decreasing the hepatic synthesis and secretion of apolipoproteins. To test this hypothesis, we incubated Hep G2 cells with human recombinant tumor necrosis factor-α, interleukin-1β, and interleukin-6. Each of the cytokines resulted in a dose-related reduction in the concentrations of apolipoprotein (apo) A-I, apoB, and lecithin:cholesterol acyltransferase (LCAT) activity in the medium after 24 hours of incubation. The effect of cytokines on apolipoprotein accumulation was not affected by preincubation of Hep G2 cells with fatty acids. Cytokines decreased the concentration of cellular apoA-I mRNA in a dose-related fashion but did not affect cellular concentrations of apoB mRNA. The concentrations of triglyceride and cholesterol were also reduced in the medium of cells incubated with cytokines. Total cell sterol synthesis rates were calculated by [14C]acetate incorporation. Cells incubated with interleukin-6 had a 31% increase in sterol synthesis rate but a 41% decrease in sterol secretion. These data suggest that these cytokines can decrease the hepatic synthesis and/or secretion of apolipoproteins and that this may explain, in part, the acquired hypercholesterolemia seen during acute and chronic inflammation. (Arterioscler Thromb. 1994;14:8-13.)

Key Words • cytokines • apolipoproteins • Hep G2 cells

Acute and chronic inflammation cause hypercholesterolemia in humans and nonhuman primates.1-7 Many of the effects of inflammation on lipoprotein metabolism appear to be mediated by cytokines.8-17 Injection of interleukin-2, colony stimulating factor, or interferon results in hypercholesterolemia in humans,11,13,14 whereas tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) cause a rapid fall in plasma cholesterol as well as the concentrations of apolipoprotein (apo) A-I and apoB in nonhuman primates.8,12,15

The effects of cytokines on lipoprotein metabolism are complex, and the mechanisms by which cytokines cause hypercholesterolemia have not been studied extensively. However, at least two changes in lipoprotein metabolism appear to be important in the development of acquired hypercholesterolemia from inflammation in primate species. First, the metabolism of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) particles is altered by inflammation; concentrations of both particles fall rapidly after injection of lipopolysaccharide and cytokines. Data from Schectman et al11 suggest that inflammation causes a decrease in LDL production rates, as injection of interferon into normocholesterolemic humans reduced the LDL-apoB production rate but did not change the fractional catabolic rate. Second, injection of lipopolysaccharide and cytokines into nonhuman primates results in a significant reduction in the cholesterol ester content of HDL and LDL that is preceded by a rapid fall in the plasma concentration of lecithin:cholesterol acyltransferase (LCAT), suggesting that acute inflammation may result in lower production of cholesterol esters in plasma.8,12,15

One mechanism by which cytokines and thus inflammation could cause acquired hypercholesterolemia is by decreasing the hepatic synthesis and secretion of apolipoproteins and LCAT. Cytokines increase the hepatic synthesis and secretion of acute-phase proteins such as C-reactive protein and α-1-acid glycoprotein and decrease the hepatic synthesis and secretion of anabolic proteins such as albumin and transferrin.18-20 Thus, we hypothesized that cytokines decrease apoA-I, apoB, and LCAT secretion from the liver, their major source of production in primates.

To address this question, we incubated Hep G2 cells with three cytokines, TNF-α, IL-6, and interleukin 1β (IL-1β), to determine their effects on apolipoprotein accumulation and LCAT activity in medium. These cytokines were chosen for study because they have demonstrable effects on lipoprotein metabolism in vivo.10,12,15 Hep G2 cells are a transformed human hepatoma cell line that retains many of the functions of normal human liver such as synthesis of albumin, lipoproteins, and other liver-specific proteins.21-29 Previous studies indicate that incubation of these cells with cytokines results in increased secretion of acute-phase proteins and decreased secretion and mRNA levels of albumin and other anabolic proteins.18-20 In these experiments we tested the effects of these cytokines on the accumulation of apoA-I, apoB, triglyceride and cholesterol mass, and LCAT activity in cell culture medium and cellular concentrations of mRNA for apoA-I and apoB.
Methods

Materials

Minimal essential medium (MEM) with modified Earl salts without phenol red was purchased from Flow Laboratories, McLean, Va. Dulbecco’s phosphate-buffered saline (PBS), fetal bovine serum (FBS), trypsin, l-glutamine, and tissue culture–grade sodium bicarbonate were purchased from Gibco Laboratories, Grand Island, NY.Essentially fatty acid–free bovine serum albumin (BSA) was purchased from Sigma Chemical Company, St Louis, Mo. Concentration cones with a molecular-weight cutoff of 25 000 were purchased from Amicon, Danvers, Mass. [α-32P]dCTP, [14C]acetate, [14C]cholesterol, and [3H]cholesterol were purchased from DuPont–New England Nuclear, Wilmington, Del. Recombinant human TNF-α, IL-6, and IL-1β were purchased from R&D Systems, Minneapolis, Minn.

Cell Culture

Hep G2 cells were grown at 37°C in 25-cm² or 75-cm² flasks containing 0.1 to 0.2 mL/cm² MEM supplemented with 10% FBS under a humidified atmosphere of 95% air and 5% CO2. The cells were split 1:4 weekly, and the medium was renewed once a week. For some experiments, cells were seeded on 60-mm or 100-mm dishes. On day 7, when the cells were nearly confluent, they were washed twice with PBS (pH 7.4) to remove traces of FBS. Cells were then incubated in MEM with and without cytokines for up to 24 hours. In other experiments, cells were incubated for 24 hours with 50 μmol/L docosahexaenoic acid (DHA), palmitic acid, or linoleic acid complexed to 75 μmol/L BSA before incubation with cytokines. The culture medium was collected and centrifuged at 2000 rpm for 20 minutes at 4°C to remove cell debris. EDTA (1 mg/mL) was added to the medium, and the pH was adjusted to 7.4. Culture medium was concentrated 10- to 20-fold by using Amicon cones. The concentrations of apoA-I, apoB, triglycerides, cholesterol, and LCAT activity in medium at 12, 16, 24, and 48 hours from cells used in these experiments were linear over time and similar to results reported in other studies.

Biochemical Analyses

Cholesterol and triglyceride levels were measured in the concentrated medium by enzymatic methods. Cells were digested with 1N NaOH, and cell protein was determined by a modified Lowry procedure using BSA as the standard. ApoA-I and apoB were measured in concentrated medium by enzyme-linked immunosorbent assay as described. LCAT Activity

LCAT activity was measured by a modification of our procedure using an exogenous substrate. Recombinant substrate particles consisting of egg yolk lecithin, [14C]cholesterol, and human apoA-I (80:5:1 molar ratio) were made by the cholate dialysis method. The standard reaction mixture (0.5 mL total volume) consisted of recombinant particle substrate (1.0 mg [14C]cholesterol), 2% BSA, 10 mmol/L β-mercaptoethanol, and 150 μg concentrated culture medium (as a source of LCAT) in 10 mmol/L tri(hydroxymethyl)aminomethane, 14.0 mmol/L NaCl, 0.01% EDTA, and 0.01% NaN3, pH 7.4. Incubations were conducted for 2 hours at 37°C, after which the free and esterified [14C]cholesterol were extracted, separated, and quantified as described.

Sterol Synthesis

Cellular synthesis and secretion of cholesterol were estimated by measuring [14C]acetate incorporation into sterols saponified with tetramethylammonium hydroxide–isopropanol, both from cell extracts and medium, as described. Briefly, cells were incubated as described above for 21 hours. [14C]acetate (3 μCi) was added per plate, and the cells were incubated for an additional 3 hours. Cellular lipids were extracted by using the Bligh-Dyer technique; [3H]cholesterol was added as an internal standard. Cellular extracts were dried under nitrogen and saponified. Sterols were extracted and reconstructed by using chloroform. Thin-layer chromatography plates were spotted with terbutylsiline bands in the cholesterol ester and free cholesterol regions were scraped and counted in a scintillation counter. For measurements of secretion, lipids were extracted from 500-μL aliquots of medium by using the Bligh-Dyer technique. Samples were applied to thin-layer chromatography plates as described above. Sterol synthesis and secretion are expressed as micromoles [14C]acetate incorporation into sterols per milligram per plate protein.

Isolation and Quantification of Cellular RNA

Immediately after removal of the culture medium, cells were collected from the dishes by adding 0.5 mL 0.25% trypsin solution. The cells were transferred to a 15-mL conical tube, and the dishes were washed two additional times with 2 mL cold PBS. Cells were pelleted by centrifuging at 2000 rpm for 10 minutes at 4°C, washed with 3 mL cold PBS, pelleted again, and finally suspended in 200 μL 0.9% NaCl and 0.01% EDTA, pH 7.4. The cell suspension was sonicated on ice, and an aliquot was immediately removed and added to a prechilled tube containing 4 mol/L guanidine isothiocyanate. Total cellular RNA was purified from the cell extract. Purified RNA was dissolved in diethyl pyrocarbonate–treated water, and the concentration was determined by the absorbance at 260 nm. The integrity of the purified RNA was determined by formaldehyde–agarose gel electrophoresis. Cellular apoA-I, apoB, and glyceraldehyde-3-phosphate dehydrogenase (G3PD) mRNA abundances were quantified by using a DNA-excess solution hybridization assay.

Statistical Analysis

The results of experiments are presented as the mean±SD. ANOVA with post hoc analysis was used to test differences among cells incubated with different cytokines or varying concentrations of cytokines.

Results

The effects of TNF-α, IL-1β, and IL-6 on the concentrations of apolipoproteins, LCAT, and lipids in the medium are presented in the Table. There was a consistent 50% decrease in the concentration of apoA-I and a 50% to 60% decrease in the concentration of apoB in the medium after incubation with each of the three cytokines. Similarly, triglyceride and cholesterol concentrations and LCAT activity decreased in the medium of cells incubated with the cytokines.

The effects of increasing cytokine concentrations on apolipoprotein concentrations and LCAT activity in the media are shown in Fig 1. There was a dose-related decrease in accumulation of apoA-I, apoB, and LCAT in the medium. The cytokines had no effect at concentrations below 0.01 ng/mL (data not shown).

We examined the interactions of three fatty acids, linoleic acid, DHA, and palmitic acid, with TNF-α on apolipoprotein secretion. Fatty acids increase apoB and triglyceride secretion from Hep G2 cells, and they may affect the in vivo response to cytokines. Linoleic acid, DHA, and palmitic acid increased apoB concentrations 1.4, 2.4, and 2.6 times control, respectively, from the Hep G2 cells. However, when TNF-α was added, there was a significant 40% to 50% decrease in the accumulation of apoB in the medium in cells incubated with each of the fatty acids. None of these fatty acids affected the accumulation of apoA-I, nor did...
they modify the effect of TNF-α on apoA-I accumulation.

The effect of cytokines on steady-state cellular mRNA levels for apoA-I, apoB, and G3PD were measured. Each of the three cytokines resulted in a 50% reduction in cellular apoA-I mRNA concentrations after 24 hours of incubation (Fig 2). In contrast, there was no significant effect of these cytokines on the concentration of mRNA levels for apoB or G3PD. Furthermore, similar to the effect seen on apoA-I accumulation in the medium, apoA-I mRNA levels decreased in a dose-dependent fashion when incubated with IL-6 (Fig 3). Dose-response experiments with the other cytokines were not done.

Cholesterol synthesis and secretion were estimated by 

\[ ^{14}C \text{acetate incorporation into sterols over 3 hours.} \]

In cells incubated with IL-6, total \[^{14}C \text{acetate incorporated into all sterols was 31 \% higher than controls (Fig 4).} \]

In contrast, cholesterol secretion was decreased by IL-6; \[^{14}C \text{acetate levels in sterols were 41 \% less in medium from cells incubated with IL-6 than from controls.} \]

**Discussion**

Acute and chronic inflammation cause acquired hypocholesterolemia in humans and nonhuman primates. Cytokines appear to be important mediators of the effects of inflammation on lipoprotein metabolism, as injection of several different cytokines results in a rapid fall in serum concentrations of total plasma cholesterol, apoA-I, apoB, and LCAT and a reduction of LDL-apoB production rates. Thus, we hypothesized that at least one mechanism of acquired hypocholesterolemia from inflammation is decreased hepatic secretion of lipoproteins and LCAT due to the direct effect of cytokines on the liver. To test this hypothesis, we incubated Hep G2 cells with TNF-α, IL-1β, and IL-6 to test the effects of cytokines on the accumulation of apolipoproteins and LCAT activity in medium. The results showed that incubation with any of these three cytokines caused less accumulation of apoA-I, apoB, triglyceride and cholesterol mass, and LCAT activity in medium. These data support the hypothesis that hepatic secretion of apolipoproteins, and presumably lipoproteins, is decreased during inflammation due to an effect of cytokines on the liver in vivo.

The plasma concentrations of both HDL cholesterol and apoA-I decrease in response to inflammatory stimuli in humans and nonhuman primates. Similarly, hepatic apoA-I secretion and mRNA concentrations decrease in mice that are given an acute inflammatory stimulus. Our data are consistent with these reports, as we observed a dose-related decrease in the accumulation of apoA-I from human hepatoma cells incubated with TNF-α, IL-1β, and IL-6. Several cytokines inhibit transcription of the genes for albumin and other anabolic proteins, and our data suggest that the same may be true for apoA-I, as steady-state apoA-I mRNA concentrations were lower after incubation with cytokines. Although our results are consistent with an effect...
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Fig 2. Bar graph showing effects of the cytokines tumor necrosis factor-α (TNF), interleukin-1β (IL-1), and interleukin-6 (IL-6) on cellular mRNA concentrations. Cells were incubated for 24 hours with cytokines. Each bar represents the mean±SD of four culture dishes. The mean±SD concentrations (picograms mRNA per nanogram cellular mRNA) of mRNA in the control experiments were apolipoprotein A-I (APO AI), 54±8; apolipoprotein B (APO B), 209±45; and glyceraldehyde-3-phosphate dehydrogenase (G3PD), 9±2.

on apoA-I transcription, changes in mRNA degradation also may be partially responsible for these effects. We measured only steady-state abundance of apoA-I mRNA, and we did not distinguish between these two regulatory mechanisms. Cytokines may also affect translation, posttranslational processing, reuptake, or secretion of apoA-I by Hep G2 cells. However, other cell systems suggest that the cellular transit time of apoA-I is rapid and that reuptake of apoA-I is minimal, making these latter mechanisms less likely.44

TNF-α, IL-1β, and IL-6 all decreased apoB accumulation in the cell culture medium but did not change apoB mRNA levels. Our results are similar to those of Delurs et al,45 who show that TNF does not affect hepatic apoB mRNA levels in mice. Thus, unlike the effect on apoA-I, cytokines do not appear to affect apoB transcription, but rather affect posttranslational processing of the apoB molecule. Our findings are consistent with other studies of apoB metabolism in Hep G2 cells that indicate that changes in apoB secretion are due to changes in the intracellular degradation of the apoB molecule. Insulin and other hormones decrease apoB secretion by increasing intracellular apoB degradation, whereas fatty acids increase apoB secretion by preventing apoB degradation.39,40,46,47 The concentration of apoB in the medium would be less if cytokines increased uptake of secreted apoB-containing lipoprotein particles. Grove et al48 indicate that IL-6 and IL-β but not TNF marginally increase LDL uptake by Hep G2 cells. Thus, increased reuptake of apoB may have contributed to the decreased apoB concentration in medium in our study.

Our results suggest that cytokines decrease the secretion rates of apoA-I and apoB and, therefore, decrease the number of lipoprotein particles from Hep G2 cells. In support of this hypothesis, we found less accumulation of triglyceride and cholesterol in the medium of cells incubated with all three cytokines and a decrease in cholesterol secretion from cells incubated with IL-6. However, cellular sterol synthesis was modestly increased by IL-6, and cytokines increase the synthesis and secretion of triglyceride by Hep G2 cells,49 although over a longer incubation time than in our experiments. Thus, it appears that cytokines may increase lipogenesis in Hep G2 cells but reduce secretion of cholesterol due to the inhibition of the secretion of apolipoproteins.

Our results appear to conflict with data that suggest that cytokines increase hepatic secretion of very-low-density lipoprotein in rats.46,47 However, caution must be used in comparing results among studies for several reasons. First, Hep G2 cells do not secrete normal very-low-density lipoprotein, and most of the lipid that is synthesized by the cells is not secreted. Therefore, studies of apoB synthesis and secretion in Hep G2 cells may not be generalizable to other systems. Second, there are consistent species differences in the effects of inflammatory stimuli and cytokines on lipoprotein metabolism. In human and nonhuman primates inflammatory stimuli as well as individual cytokines consistently cause hypocholesterolemia,1,8,11-15 whereas the acute effect in rodents of inflammation and cytokines is either an increase or no change in cholesterol levels.10,16,17,49,50 The time course of study also appears to be an important determinant of the metabolic response to cytokines, as long-term administration of IL-1β to rats results in hypotriglyceridemia and hypocholesterolemia, which is the opposite of the short-term effects of cytokines in rodents.51

LCAT activity was decreased in the medium of cells incubated with cytokines. Although we did not measure LCAT mass in our experiments, a previous report indicates28 that LCAT activity and mass are correlated in the medium of Hep G2 cells. Thus, cytokines may act to decrease hepatic secretion of LCAT during stress or inflammation. This finding is consistent with clinical data that show22-24 that patients with inflammatory diseases, liver diseases, or primary hepatic dysfunction have low levels of plasma LCAT activity. We have hypothesized that a relative LCAT deficiency contributes to acute hypocholesterolemia during inflammation. Lipoprotein particles in monkeys exposed to TNF and lipopolysaccharide have substantially less cholesterol ester content per particle (which accounts, in part, for the lower total plasma cholesterol concentration ob-
served in these animals), and the change in lipid content of the lipoprotein particles is preceded by a decrease in plasma LCAT concentration. Also, lipoprotein particles in human beings with acquired hypocholesterolemia are depleted of cholesterol ester, which contributes to the low total plasma cholesterol concentration.

In summary, TNF-α, IL-6, and IL-1β all decreased accumulation of apoA-I, apoB, triglyceride and cholesterol mass, and LCAT activity in the medium of Hep G2 cells, suggesting a decrease in the secretion of lipoprotein particles and LCAT. These data are consistent with the hypothesis that the acquired hypocholesterolemia of inflammation is due, in part, to decreased secretion of hepatic apolipoproteins and LCAT.

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