Uremic Serum Subfraction Inhibits Apolipoprotein A-I Production by a Human Hepatoma Cell Line


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ABSTRACT
Abnormalities in lipoprotein metabolism are common in uremic patients and may represent an additional risk factor for the development of atherosclerosis. Despite the frequent occurrence of lipoprotein abnormalities, the role of various serum toxins and subfractions that contribute in uremic patients on lipoprotein metabolism is not clearly understood. This study addressed the role of uremic toxins on lipoprotein metabolism by examining the effect of a 2,000-d subfraction obtained from the serum of uremic and control subjects on the synthesis of apolipoprotein (apo) A-I in a human hepatoma cell line (Hep-G2). Serum subfractions obtained from uremic patients inhibited apo A-I synthesis and secretion by Hep-G2 cells in a dose-dependent manner as measured by [3H]leucine incorporation into apo A-I, immunoprecipitation, and ELISA. The uremic serum subfraction decreased the mRNA expression for apo A-I in Hep-G2 cells when compared with controls. These observations suggest that a complement of uremic serum can have the potential to inhibit hepatic apo A-I synthesis and may adversely influence high-density lipoprotein metabolism, thus increasing the risk for the development of atherosclerotic vascular complications in uremic patients.

Key Words: ESRD, uremic toxins, atherosclerosis, high-density lipoproteins

Although atherosclerotic cardiovascular disease is commonly observed in patients with ESRD (1), hypercholesterolemia, and increased serum low-density lipoprotein (LDL) cholesterol, conventionally accepted risk factors for the development of atherosclerosis in the general population may not be consistently found (2–4) in these patients whose serum triglycerides are often elevated and whose high-density lipoprotein (HDL) cholesterol is often decreased (2,3,5,6). In this regard, previous studies using apolipoprotein (apo) measurements in patients with chronic renal disease showed decreased concentrations of apo A-I and A-II and increased apo C-III, suggesting abnormalities in very low-density lipoprotein (VLDL) and HDL metabolism (2,7). Additionally, patients with chronic renal disease often exhibit diminished apo A-I and HDL synthetic and fractional catabolic rates, which have been thought to contribute to their lowered levels of HDL and apo A-I (9). Although the association between lipids and progressive renal disease is not clearly understood, there exist sufficient data to suggest that abnormalities in lipoproteins and apo in uremic patients may contribute to their increased risk for developing atherosclerotic cardiovascular disease.

Multiple factors have been proposed to influence the dyslipidemias seen in patients with chronic renal disease including coexisting diabetes, hypertension, liver disease, electrolyte imbalances, hemostatic abnormalities, and the potential effect of circulating serum components that accumulate in uremia. The accumulation of toxins in the serum of uremic patients has been proposed to contribute to some of the metabolic disturbances associated with chronic renal disease (9,10). Despite speculation, the role of the serum subfractions that accumulate in uremic patients in altering lipoprotein and apo metabolism and possibly contributing to the development of systemic atherosclerosis remains unclear.

In this study, the effect of uremic serum subfractions on apo A-I synthetic capacity and secretion was examined in a human hepatoma cell line (Hep-G2) that has previously been reported to possess many normal hepatic cell functions, including the synthesis of serum lipoproteins and apo (11,12). In recent years, these cells have been used extensively to study various aspects of lipoprotein metabolism. The results of this study indicated that a uremic serum subfraction could reduce the hepatic synthetic ca-
cacity of apo A-I and suggested a possible mechanism for the altered HDL metabolism seen in uremic patients.

MATERIALS AND METHODS

Tissue culture materials and media were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT). [*-H]leucine was purchased from Amersham Corporation (Arlington Heights, IL). Sephadex G-25 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Polyclonal antibody for human apo A-I was obtained from Medix Biotech Inc. (Foster City, CA). The human hepatocellular carcinoma cell line Hep-G2 and human apo A-I DNA probe were obtained from American Type Culture Collection (Rockville, MD). All other chemicals used were of analytical grade.

Patients and Control Subjects

Five male patients with stable ESRD and five healthy male volunteers were studied. After the subjects signed informed consent, blood samples were drawn from the vascular access of the ESRD patients immediately before their regularly scheduled hemodialysis treatments; the serum was separated and used in subsequent chromatography. Samples were collected before heparinization. All of the uremic patients entered into the study were being dialyzed at this medical center for at least 6 months with a bicarbonate dialysate and a cellulose-based membrane. Patients were excluded from the study if they had unstable diabetes mellitus, nephrotic syndrome, obesity, angina, or uncontrolled hypertension or if they were receiving specific treatments including certain antihypertensive medications such as β-adrenergic blocking drugs, androgens, progestins, or a modified fat diet that are known to influence lipoprotein metabolism. None of the patients were cigarette smokers. Serum lipid profiles and apo A-I and apo B levels were determined by standard lipid research clinic methods at this medical center.

Isolation of Uremic Serum Subfractions

A Sephadex G-25 column (16 x 75 mm) was prepared in a standard manner with phosphate-buffered saline (PBS). The void volume of the column was approximately 40 mL. Serum samples (2 mL) obtained from either normal subjects or uremic patients were applied to the column and eluted with PBS at a flow rate of 0.6 mL/min. Four-minute subfractions were collected, and the absorbance at 280 nm of each subfraction was measured. Subfractions 28 to 48 were pooled, lyophilized, reconstituted in 10 mL of PBS, and used as uremic serum subfractions in experimental incubations with Hep-G2 cells. The reconstituted serum subfractions were diluted with culture medium 10 times before their use.

Cell Culture

A human hepatoma cell line, Hep-G2, was grown in T-25 flasks with 5 mL of high-glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1% glutamine-penicillin-streptomycin, and 1% Fungizone (Irvine Scientific, Santa Ana, CA). Cells were grown in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air. Subcultures were made from confluent stock cultures by trypsinization in PBS containing 0.5 mM EDTA.

Studies on Secretion of Apo A-I

Hep-G2 cells were plated in 35-mm culture petri dishes at a concentration of 4 x 10⁵ cells/dish in 3 mL of Dulbecco's modified Eagle's medium and grown for 3 to 4 days until they attained 75 to 80% confluence. The studies examining the dose response of uremic serum subfractions on apo A-I secretion were performed by the incubation of Hep-G2 cells with various amounts of the uremic serum subfractions (50 to 300 μL) at 37°C for 72 h. The effect of incubation time on apo A-I secretion was examined by the incubation of cells with 200 μL of the uremic serum subfraction at 37°C for varying times (4 to 72 h). At the termination of the incubation, culture media from each flask were removed, and the cell monolayer was washed with PBS and digested with 0.5 mL of 1 N NaOH for protein measurement [13]. A 50-μL sample of culture media was assayed for apo A-I by an ELISA [14] with an apo A-I-specific monoclonal antibody developed and characterized in the Cholesterol Center Lipid Laboratory at this medical center [14]. This monoclonal antibody against human apo A-I showed no cross-reactivity with human apo A-II, LDL, VLDL, or albumin or with bovine apo A-I (personal communication, Dr. M.L. Kashyap). The concentration of apo A-I was expressed as micrograms per milligram of cellular protein. The ELISA used was linear over a concentration range of 0.5 to 50 μg/mL with a correlation coefficient = 0.995. The aliquots of media used in the assays performed for this study corresponded to a concentration of 2 to 30 μg/mL, values within the linear range of the assay. Pooled normal human plasma served as a source for the apo A-I standard that was calibrated against the Centers for Disease Control and Prevention (CDC)-reference plasma by a CDC-lipid/apo standardization program at the University of Cincinnati.

De Novo Synthesis of Apo A-I

Studies examining the effect of various doses of the uremic serum subfraction and the time of incu-
bation on apo A-I synthesis by Hep-G2 were performed as described earlier, except that these incubations contained 5 μCi/mL of L-[4,5-3H]leucine. The de novo synthesis of apo A-I was performed by the measurement of the incorporation of radiolabeled leucine into apo A-I secreted into the media (15). In brief, 100 μL of the medium was added to an Eppendorff tube containing 200 μL of PBS and 100 μL of antihuman apo A-I (1:50 dilution). The polyclonal anti-apo A-I did not cross-react with human LDL, VLDL, or albumin, or with fetal bovine serum as determined by an immunodiffusion method. After a 48-h incubation at 4°C, 100 μL of rabbit immunoglobulin G (1:10 dilution) was added to each tube and incubated for 24 h at 4°C. The precipitated apo A-I immune complexes were separated by centrifugation, the pellets were washed five times with PBS containing 0.1% sodium dodecyl sulfate (SDS) and dissolved in 500 μL of 0.5 N NaOH, and a 400-μL aliquot was used to measure the radioactivity. The incorporation of [3H]leucine into apo A-I was expressed as counts per minute per total trichloroacetic acid–precipitable protein from culture media.

Northern Blot Analysis

Hep-G2 cells were incubated with various aliquots of normal or uremic serum subfractions for 72 h at 37°C. After incubation, the media were removed and cell monolayers were washed three times with PBS. Total RNA was isolated from Hep-G2 cells by the protocol of Chomczynski and Sacchi (16). In brief, Hep-G2 cells were homogenized with 4 M guanidinium thiocyanate, total protein and DNA were extracted with acid phenol, and the RNA was precipitated with isopropanol. After being washed with ethanol, the samples were dried under vacuum centrifugation and the amount of RNA was quantitated by the measurement of the absorbance at 260 nm with a spectrophotometer. Twenty micrograms of total RNA was loaded into individual wells of a 1.2% agarose gel containing formaldehyde, and electrophoresis was performed (17). The RNA from the gel was transferred onto MS template nylon membranes (Fisher Scientific, Tustin, CA) with a trans-blotting cell (Bio-Rad, Richmond, CA). The nylon membrane was UV-linked with a Stratalink (Stratagene, La Jolla, CA). The membrane was hybridized with a 32P-labeled human apo A-I cDNA probe (PAL-113; insert size, 0.6 kilobases) with random hexanucleotide primers. The membranes were washed three times for 30 min: first, in 2× saline–sodium citrate (SSC) with 0.1% SDS at 25°C; second, in 0.5× SSC with 0.1% SDS at 25°C; and third, in 0.5× SSC with 0.1% SDS at 55°C. Autoradiography was performed by exposure of the blots to Kodak x-ray film (Eastman Kodak, Rochester, NY) with intensifying screens at −70°C. Blots were then rehybridized with a human [32P]glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Clontech, Palo Alto, CA) probe as a housekeeping enzyme to assess RNA quantity and integrity. Quantitative analysis of apo A-I mRNA message was performed by densitometric scanning of the blots and normalization with the respective GAPDH message.

Statistical Analysis

Data presented are the mean ± SE. All incubations were performed in quadruplicate. Statistical significance was calculated by use of the t test for unpaired data or analysis of variance when appropriate.

RESULTS

Serum lipid profiles of the five ESRD patients were performed and showed total cholesterol levels of 197 ± 9 mg/dL, LDL cholesterol levels of 132 ± 6 mg/dL, HDL cholesterol levels of 36 ± 3 mg/dL, and apo A-I levels of 137 ± 11 mg/dL. Although total cholesterol and LDL cholesterol levels in these patients appeared normal, HDL cholesterol was reduced when compared with normal levels reported by the reference laboratory.

Sephadex G-25 chromatography of uremic serum showed a broad peak from Fractions 28 through 45. On the basis of the use of calibrator molecular weight markers, the molecular weight of the components of this fraction was between 500 and 2,000 d (Figure 1) and was similar to that previously reported by others who have isolated subfractions from uremic serum using Sephadex G-25 chromatography (18,19). The inset in Figure 1 shows an enlargement of a portion of the chromatogram from Fractions 25 to 50 indicating a broad peak in uremic serum that was not present in normal serum.

The incubation of various amounts of the uremic serum subfractions for 72 h with Hep-G2 cells showed a dose-dependent decrease in apo A-I secretion in the media as measured by an ELISA. A significant reduction in apo A-I secretion by Hep-G2 cells was noted at 100 μL of the uremic serum subfraction, and the maximum effect was observed at 200 μL of the subfraction. The incubation of Hep-G2 cells with uremic serum subfractions at these doses (50 to 300 μL) for 72 h did not alter the morphology or the viability of cells as assessed by trypan blue exclusion criteria. At any dose (50 to 300 μL), the addition of a similar subfraction obtained from normal serum did not show any changes in apo A-I secretion by Hep-G2 cells when compared with a PBS control (Figure 2).

The effect of time of incubation on apo A-I secretion by Hep-G2 cells in the presence of 200 μL of the uremic serum subfraction was examined (Figure 3). Apo A-I secretion increased in a time-dependent
Figure 1. Representative Sephadex G-25 chromatogram of normal (dashed line) and uremic (solid line) serum. The column was calibrated with standard molecular weight markers. Fractions (2.4 ml) were quantitated by the measurement of the optical density at 280 nm. Fractions 28 to 45 corresponded to a molecular weight of 500 to 2,000 kDa. The inset shows an expanded portion of the chromatogram from Fractions 25 to 50 indicating the presence of a broad peak only in the uremic serum.

Figure 2. Effect of serum subfractions from uremic and normal subjects on apo A-I secretion by Hep-G2 cells. Cells were incubated at 37°C for 72 h with varying amounts (50 to 300 μL) of uremic serum subfractions or with control buffer. At the end of the incubation, culture media were assayed for apo A-I by ELISA and expressed in terms of total cellular protein. There were no differences in apo A-I secretion between normal serum subfractions (50 to 300 μL) and control buffer; representative data of a dose of 200 μL of normal serum subfraction are shown. Statistical significance was compared with results of control buffer. n.s., not significant.

Effect of Uremia on Hepatic Apolipoprotein Synthesis

Figure 3. Effect of serum subfractions from uremic and normal subjects on apo A-I secretion by Hep-G2 cells at varying incubation times. Cells were incubated with 200 μL of serum subfractions at 37°C for 4 to 72 h. At the end of the incubation, culture media were assayed for apo A-I by ELISA and expressed in terms of total cellular protein. The five columns on the left of the figure indicate the results of incubations with uremic serum subfractions compared with a buffer control (PBS). The columns on the right show the results of normal serum subfractions and control buffer at a representative incubation time (72 h). Statistical significance was compared with results of control buffer (PBS). n.s., not significant.

when compared with PBS controls. This effect persisted at 48 to 72 h of incubation (Figure 3). The addition of normal serum subfractions to Hep-G2 cells showed no change in apo A-I secretion as compared with the respective control (Figure 3).

Experiments were designed to examine the de novo biosynthesis of apo A-I by the measurement of the incorporation of [3H]leucine into newly synthesized apo A-I secreted into the media. The data showed that the incorporation of radiolabeled leucine into apo A-I was decreased in a dose-dependent manner by Hep-G2 cells incubated with uremic serum subfractions (Figure 4). A significant decrease in the incorporation of radiolabeled leucine into apo A-I was noted by Hep-G2 cells in the presence of as little as 25 μL of the uremic serum subfractions. At higher amounts of the uremic serum subfraction (50 to 200 μL), the reduction in the incorporation of radiolabeled leucine into apo A-I was persistent, with a maximum effect noted at doses >100 μL of the uremic serum subfraction. The incubation of Hep-G2 cells with subfractions obtained from normal serum, however, had no effect on the incorporation of radiolabeled leucine into apo A-I as compared with PBS controls (Figure 4). To examine whether the reduced accumulation of [3H]-labeled apo A-I in the culture media of Hep-G2 cells incubated with the uremic serum subfractions was a result of decreased de novo apo A-I synthesis or enhanced cellular reuptake of apo A-I from the media, studies were performed to examine the intracellular accumulation of apo A-I as
determined by using cellular homogenates instead of incubation media as outlined above. In both control and uremic serum subfraction-treated cells, the intracellular distribution of apo A-I was 23% of secreted apo A-I in the media. Because the intracellular accumulation of apo A-I was comparable in both control and uremic serum subfraction-treated cells, it would be unlikely that a component of the uremic serum had any direct effect on the reuptake of apo A-I by Hep-G2 cells in these studies.

A time-dependent decrease in the incorporation of radiolabeled leucine into apo A-I was noted by Hep-G2 cells in the presence of 200 µL of the uremic serum subfractions (Figure 5). Consistent with the apo A-I secretion studies (Figure 3), the incubation of uremic serum subfractions with Hep-G2 cells for 24 to 72 h markedly lowered the apo A-I de novo synthesis, as measured by the incorporation of radiolabeled leucine into apo A-I (Figure 5). Alternatively, serum subfractions obtained from normal volunteers did not show any effect on the de novo synthesis of apo A-I by Hep-G2 cells (Figure 5).

Northern blot analysis was performed to examine the effect of uremic serum subfractions on apo A-I gene expression by Hep-G2 cells. Uremic serum subfractions significantly decreased apo A-I mRNA expression by Hep-G2 cells by 66 to 68% (Figure 6). Similar to the secretion and de novo synthesis studies, serum subfractions obtained from normal subjects had no effect on apo A-I mRNA expression by Hep-G2 cells.

To determine whether the serum subfractions altered total protein synthesis by Hep-G2 cells, aliquots of media from de novo synthesis experiments were analyzed for the incorporation of radiolabeled leucine into triglycerol-acid–precipitable proteins. The results of this study indicated that neither the uremic nor the control serum subfractions altered total protein synthesis by Hep-G2 cells (data not shown).

**DISCUSSION**

Serum lipid and lipoprotein abnormalities are common metabolic disturbances observed in patients with chronic renal disease. In addition to these abnormalities, uremic patients appear to have a higher incidence of developing atherosclerotic vascular complications (1). Because of the well-established relationships between serum lipoprotein levels and atherosclerosis (20,21) and the suggestion that atherosclerosis is accelerated in uremic patients, it appeared reasonable to examine whether serum factors that accumulate in these patients may have the ability to alter HDL and LDL metabolism.

apo A-I is a secretory protein synthesized primarily by the liver. Apart from being a structural protein for HDL, apo A-I is thought to be the initiator of reverse cholesterol transport and has been proposed to be an
accurate predictor for the development of atherosclerotic cardiovascular disease (22–27). Peripheral tissue cholesterol that is taken up by HDL and esterified is ultimately metabolized by the hepatocyte or transferred to other lipoproteins (e.g., LDL) that are also metabolized by the liver and excreted ultimately in the bile. On the basis of these and other epidemiologic studies, it was inviting to speculate that factors accumulating in uremic serum might influence lipoprotein metabolism in a way to facilitate the development of atherosclerosis in patients with ESRD.

The data presented in this investigation indicated that a subfraction isolated from uremic serum with normal or abnormal lipoprotein profiles inhibited the synthesis and secretion of apo A-I by human hepatoma cells without apparent alteration in cellular apo A-I reuptake. Further supporting experiments were performed to determine whether the uremic serum subfractions modulated apo A-I gene expression. Northern blot analysis indicated that uremic serum subfractions, at doses of 50 to 200 μL, inhibited approximately 66 to 68% of apo A-I mRNA expression when compared with controls. Taken together, these data suggested that a component present in uremic serum subfractions has the capacity to inhibit the mRNA expression of apo A-I and thus lead to lowered de novo synthesis and secretion of apo A-I by human hepatoma cells.

Although decreased concentrations of HDL are frequently seen in patients with chronic renal failure, the mechanisms responsible for this defect are not clearly understood. In vivo, HDL and apo A-I concentrations can be reduced by decreasing their rates of synthesis or by increasing their fractional catabolic rates, as previously demonstrated in patients with primary HDL deficiency (28). Although the lowered synthetic rates of HDL and apo A-I observed in uremic patients can lead to decreased levels of HDL (8), a decreased rather than an increased fractional
catabolic rate noted in these patients led to the assumption that the synthetic rate of apo A-I was an important regulator of HDL (8). Lowered activities of lipoprotein-metabolizing enzymes including lipoprotein lipase, hepatic triglyceride lipase, and lecithin:cholesterol acyltransferase (6) may have contributed to the lipoprotein abnormalities seen in uremic patients (6). Furthermore, the marked hypertriglyceridemia seen in uremic patients may accelerate the clearance of apo A-I (28,29) and also lead to an increased exchange of cholesterol from HDL to VLDL, thus resulting in the formation of cholesterol-enriched VLDL particles that have been proposed to be associated with accelerated atherosclerosis (30).

Despite these many in vivo and in vitro studies, it is not clearly understood whether the abnormalities in HDL and apo A-I metabolism in patients with chronic renal failure are the result of the presence of abnormal circulating factors in the serum of these patients or a direct consequence of other metabolic abnormalities, possibly including the severe hypertriglyceridemia often seen in these patients. This study provided direct evidence supporting the view that some component(s) found in uremic, but not control, serum inhibited apo A-I synthesis by human hepatoma cells. These in vitro observations may partly explain the decreased rate of synthesis of apo A-I observed in many patients with uremia (8) and suggest that a component present in uremic serum may, in part, have the capacity to suppress hepatic apo A-I synthesis.

Finally, these data do not establish a specific identity for any of the subfractions or toxins present in uremic serum that can reduce apo A-I synthesis. On the basis of previous studies, similar subfractions isolated from uremic serum may contain various identified compounds. The structural composition of these substances has been reported to be diverse, including peptidic substances, carbohydrates, double conjugates of glucuronic acid, guanidines, guanidino acids, amino acids, amines, phenolic acids, polyols, and ascorbic acid metabolites (31–37). Because little information is available on the effect of these substances on lipoprotein metabolism (either directly or in combination), we are unable to implicate the inhibitory effect of uremic serum components on apo A-I synthesis observed in this study to any of the previously reported substances. Further studies are warranted to isolate and characterize specific compounds associated with this uremic serum subfraction to understand their role in lipoprotein metabolism and the atherosclerotic process.

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