Molecular mechanisms of altered cholesterol metabolism in rats with spontaneous focal glomerulosclerosis

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Background. Imai rats exhibit spontaneous focal glomerulosclerosis (FGS), which is marked by heavy proteinuria, severe hyperlipidemia, and progressive renal insufficiency beginning at 8 to 10 weeks of age. In an earlier study, we reported severe skeletal muscle and adipose tissue lipoprotein lipase, and very low-density lipoprotein (VLDL) receptor deficiencies, which account for elevated plasma VLDL and triglycerides in Imai rats at 34 weeks of age. In this study, we investigated key factors involved in cholesterol metabolism.

Methods. Male Imai and Sprague-Dawley control rats were fed a regular rat chow and observed from age 8 through 34 weeks. Hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, cholesterol 7α-hydroxylase, low-density lipoprotein (LDL) receptor and acyl CoA cholesterol acyltransferase (ACAT) were measured by Western blot and plasma lecithin:cholesterol acyltransferase (LCAT) protein was measured by enzyme-linked immunosorbent assay (ELISA).

Results. At 34 weeks of age, the Imai rats showed severe proteinuria, hypoalbuminemia, 60% reduction in glomerular filtration rate (GFR), elevated plasma total and LDL cholesterol and LDL/high-density lipoprotein (HDL) ratio. Imai rats showed a twofold elevation of hepatic HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis, but no significant change in cholesterol 7α-hydroxylase, the rate-limiting enzyme in cholesterol catabolism to bile acids. This was accompanied by and largely due to a threefold down-regulation of hepatic LDL receptor, which limits hepatic uptake of LDL; and a threefold up-regulation of hepatic ACAT (P < 0.01), which augments esterification of hepatocyte free cholesterol, thus, limiting cholesterol-mediated feedback regulation of cholesterol synthesis and catabolism. Moreover, plasma LCAT concentration was severely depressed (by fourfold) in Imai rats. This abnormality can impair HDL-mediated cholesterol transport from extrahepatic tissues to the liver.

Conclusion. The study revealed marked abnormalities of the key proteins involved in regulation of hepatic cholesterol metabolism. These abnormalities can account for severe dysregulation of cholesterol metabolism in Imai rats with spontaneous FGS, which closely resembles FGS in humans.

Imai rats were originally derived from the mating of a male Sprague-Dawley rat that exhibited spontaneous renal disease and hyperlipidemia while consuming a low-fat basal diet [1]. Male Imai rats develop a progressive focal glomerulosclerosis (FGS), which presents with mild proteinuria at 5 to 6 weeks of age progressing to frank proteinuria and hyperlipidemia by 8 to 10 weeks of age, and significant renal insufficiency by 30 to 40 weeks of age [2]. The severity of the disease is considerably less and the onset of the disease is significantly delayed in female animals [1]. The histologic and biochemical features of FGS in the Imai rats closely resemble those of progressive FGS in humans [2, 3].

Severe nephrotic proteinuria in the Imai rats is accompanied by marked elevation of plasma cholesterol, triglycerides, low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) and phospholipid concentrations [1, 2]. In a recent study, we found marked down-regulations of lipoprotein lipase and VLDL receptor in adipose tissue and skeletal muscle of 34-week-old Imai rats with severe nephrotic proteinuria and moderate renal insufficiency [4]. These findings helped to elucidate the molecular mechanism of hypertriglyceridemia and elevation of plasma VLDL in this model. The observed down-regulations of lipoprotein lipase and VLDL receptor in 34-week-old Imai rats exhibiting severe nephrotic syndrome and moderate renal insufficiency paralleled the findings of our earlier studies in rats with puromycin-induced nephrotic syndrome [5, 6] and those with chronic renal insufficiency induced by 5/6 nephrectomy [7–9].

The present study was designed to discern the molecular basis of hypercholesterolemia, elevation of plasma LDL and LDL to high-density lipoprotein (HDL) cholesterol ratio in Imai rats with spontaneous FGS. Accordingly, we examined hepatic expressions of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (the...
rate-limiting step in cholesterol synthesis), cholesterol 7α-hydroxylase (the rate-limiting step in cholesterol catabolism to bile acids), LDL receptor (the principal pathway of cholesterol clearance) and acyl CoA:cholesterol acyltransferase (ACAT) (the main enzyme responsible for intracellular esterification of cholesterol) in male Imai rats with FGS and age-matched male Sprague-Dawley control rats. In addition, plasma immunodetectable lecithin:cholesterol acyltransferase (LCAT), which is the main enzyme responsible for esterification of cholesterol in the plasma and maturation of HDL was measured. We studied the Imai rats at 34 weeks of age at which point severe nephrotic syndrome and moderate renal insufficiency were present. It is of note that earlier studies from our group and other investigators had explored the effects of nephrotic syndrome and chronic renal insufficiency separately. However, little is known about the effects of the combination of the two conditions, which frequently occurs in advanced stages of FGS and many other glomerulopathies in animals and humans. Investigation of the Imai rats at 34 weeks of age afforded the opportunity to explore this issue.

METHODS

Animals

Imai hyperlipidemic rats (Biological Research Laboratories, Central Research Division of Takeda Chemical Industries, Osaka, Japan) were bred in the animal facility of Saga Medical School (Saga, Japan). Six male Imai rats and six male Sprague-Dawley control rats were monitored for 34 weeks. They were fed a regular rat chow (containing 24.8% protein, and 0.08% cholesterol) and housed in a climate-controlled space with 12-hour light, 12-hour dark cycles. All animals were allowed access to food and water ad libitum. Arterial pressure was measured by a tail plethysmograph (Harvard Apparatus, Cambridge, MA, USA). A 24-hour urine collection was obtained for determinations of urinary protein and creatinine excretion using metabolic cages. At the conclusion of each observation period, animals were anesthetized with intraperitoneal injections of pentobarbital (50 mg/kg) and euthanized by exsanguination using cardiac puncture. Liver was harvested and frozen in liquid nitrogen immediately and stored at −70°C until processed. In addition, plasma was separated and used for measurements of lipids, creatinine and albumin concentrations, as well as LCAT protein abundance.

Routine biochemical determinations

Serum total cholesterol, triglyceride, creatinine, and albumin concentrations were measured by a synchro CX3 autoanalyzer (Beckman Instruments, Inc., Fullerton, CA, USA). Urinary protein concentration was determined by a kit purchased from Wako Pure Chemical Industries, Tokyo, Japan.

Creatinine clearance was calculated from urinary and plasma creatinine values using the standard formula. Plasma HDL, LDL, and VLDL levels were determined by ultracentrifugation method as described by Lasser et al [10].

HMG-CoA reductase protein determination

Frozen rat liver tissue was homogenized in 1 mL of 20 mmol/L Tris-HCl (pH 7.5) buffer containing 2 mmol/L MgCl₂, 0.2 mol/L sucrose, 5 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 10 μg/mL aprotinin, and 3 μg/mL pepstatin A. The crude extract was centrifuged at 12,000 × g at 4°C for 10 minutes to remove tissue debris. The supernatant was processed for determination of HMG-CoA reductase. Protein concentration of the supernatant was determined by a bicinchoninic acid (BCA) protein assay kit (Pierce, Inc., Rockford, IL, USA). HMG-CoA reductase protein abundance in the liver extract was quantified by Western blot analysis as described by Ness, Chambers, and Lopez [11]. Briefly, aliquots containing 100 μg proteins were fractionated on 4% to 20% Tris-glycine gel (Novex, Inc., San Diego, CA, USA) at 120 V for 2 hours. After electrophoresis, proteins were transferred to Hybond-ECL membrane (Amersham Life Science, Inc., Arlington Heights, IL, USA). The membrane was incubated for 1 hour in blocking buffer [1× Tris-buffered saline (TBS), 0.1% Tween-20 and 7% non-fat milk] and then overnight in the same buffer containing 1:7500 polyclonal anti-HMG-CoA reductase antibody (generously provided by Professor G.C. Ness, Department of Biochemistry and Molecular Biology, University of South Florida, Tampa, Florida). Membrane was washed four times for 10 minutes in 1× TBS, 0.1% Tween-20 prior to a 2-hour incubation in blocking buffer plus diluted (1:10,000) horseradish peroxidase– linked antirabbit immunoglobulin G (IgG) (Amersham Life Science, Inc.). The washes were repeated before the membranes were developed with chemiluminescent agents (ECL; Amersham Life Science Inc.) and subjected to autoluminography for 3 minutes.

Determination of cholesterol 7α-hydroxylase protein

Cholesterol 7α-hydroxylase protein abundance in the liver tissue preparation was determined by Western blot analysis using a rabbit antirat cholesterol 7α-hydroxylase antibody as described in our earlier studies [12]. The antibody employed in this assay was generously provided by Professor John Y.L. Chiang (Northeastern Ohio University College of Medicine, Rootstown, OH).

Determination of LDL receptor protein

Hepatic LDL receptor protein abundance was determined in the plasma membrane preparation by Western
Determination of ACAT-2 protein

Hepatic ACAT-2 protein abundance was quantified by Western analysis using a polyclonal antibody against ACAT-2 as described in our recent study [14]. The antibody employed in these experiments was a generous gift from Professor Lawrence L. Rudel, Department of Biochemistry and Comparative Medicine, Wake Forest University, Winston-Salem, North Carolina.

Determination of plasma LCAT protein

Plasma LCAT protein concentration was measured in triplicate by enzyme-linked immunosorbent assay (ELISA) method using rabbit antihuman LCAT antibody as described by Wang et al [15]. This antibody was generously supplied by Professor John S. Parks, Wake Forest University, Winston-Salem, North Carolina. Briefly, 10 uL of plasma were diluted in binding buffer (1/11003) and incubated at room temperature for 3 hours. The plates were washed 3 times with washing buffer (1/11003) and blocked for 3 hours at room temperature with control group. A significant inverse correlation was found between liver tissue LDL receptor protein abundance and serum total cholesterol concentration in the study animals (r = 0. 7, P < 0.05). How-ever, HMG-CoA reductase to cholesterol 7α-hydroxylase ratio (which reflects the balance between hepatic cholesterol biosynthesis and catabolism) was markedly increased in the FGS group as compared to that in the control group.

Hepatic LDL receptor protein data

Data are given in Figure 3. The FGS group showed an approximately 2.5-fold reduction in hepatic tissue LDL receptor protein abundance as compared to the normal control group. A significant inverse correlation was found between liver tissue LDL receptor protein abundance and serum LDL cholesterol concentration in the study animals (r = −0. 7, P < 0.05).

Hepatic ACAT-2 results

Data are shown in Figure 4. Liver-specific ACAT-2 protein abundance was nearly 2.5-fold higher in the FGS group as compared to the normal control group. Hepatic ACAT-2 abundance was inversely related to liver free cholesterol (r = −0.74, P < 0.01) concentration.

Table 1. Serum concentrations of total triglycerides, very low-density lipoproteins triglycerides (VLDL-TG), albumin, and creatinine, creatinine clearance, urinary protein excretion and systolic blood pressure in normal Sprague-Dawley control rats and 34-week-old Imai rats with focal glomerulosclerosis (FGS)

<table>
<thead>
<tr>
<th></th>
<th>CTL (N = 6)</th>
<th>FGS (N = 6)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride mg/dL</td>
<td>73.1 ± 10.2</td>
<td>218.1 ± 15.2</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>VLDL-TG mg/dL</td>
<td>63.0 ± 10.2</td>
<td>194.4 ± 16.3</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Albumin g/dL</td>
<td>3.70 ± 0.1</td>
<td>2.80 ± 0.1</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Creatinine mg/dL</td>
<td>0.54 ± 0.29</td>
<td>1.05 ± 0.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine clearance mL/min · g kidney weight</td>
<td>0.54 ± 0.03</td>
<td>0.20 ± 0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urinary protein mg/24 hours</td>
<td>35.2 ± 5.5</td>
<td>467.0 ± 27.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood pressure mm Hg</td>
<td>116.7 ± 5.9</td>
<td>187.5 ± 12.6</td>
<td>&lt;0.001</td>
</tr>
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</table>

Values are mean ± SEM.
Plasma LCAT data

Results are depicted in Figure 5. Plasma LCAT protein concentration was approximately fourfold lower in the FGS group than that in the normal control group. Plasma LCAT protein concentration was inversely related to serum LDL cholesterol to HDL cholesterol concentration ratio ($r = -0.72$, $P < 0.05$) and serum free cholesterol concentration ($r = -0.84$, $P < 0.01$).

DISCUSSION

Severe nephrotic proteinuria in our Imai rats with FGS was accompanied by marked elevation of serum total cholesterol and LDL cholesterol concentrations. Earlier studies have pointed to increased LDL synthesis [16] and depressed LDL clearance [17] as the cause of increased plasma LDL cholesterol in nephrotic syndrome. However, the molecular mechanisms responsible for these phenomena were not known.

Hypercholesterolemia in our nephrotic rats with spontaneous FGS was accompanied by an approximately twofold increase in the liver tissue abundance of HMG-CoA reductase. The endoplasmic reticulum-bound enzyme, HMG-CoA reductase is the rate-limiting factor in cholesterol biosynthesis. Although this enzyme is expressed in all tissues, it is most abundantly expressed in the liver, which plays a critical role in regulation of plasma cholesterol level [18]. Thus, marked up-regulation of hepatic HMG-CoA reductase in the FGS group can contribute to hypercholesterolemia in these animals. This supposition is supported by the observed correlation between hepatic HMG-CoA reductase and serum cholesterol in
the study animals. In an earlier study, we found up-regulation of hepatic HMG-CoA reductase mRNA abundance and enzymatic activity in rats with puromycin-induced nephrotic syndrome [19]. The present study revealed up-regulation of immunodetectable HMG-CoA reductase protein in spontaneously occurring nephrotic syndrome caused by a glomerulopathy, which closely resembles FGS in humans.

Despite severe hypercholesterolemia, hepatic tissue cholesterol 7α-hydroxylase protein abundance in our FGS animals was virtually identical to that of the normocholesterolemic control rats. Cholesterol 7α-hydroxylase is a unique cytochrome P450 enzyme, which is the first and the rate-controlling step in cholesterol conversion to bile acids. Consequently, this enzyme plays a critical role in cholesterol catabolism. The observed up-regulation of HMG-CoA reductase, which is the determinant of cholesterol biosynthesis [18], coupled with a lack of rise in cholesterol 7α-hydroxylase, which is the determinant of cholesterol catabolism, can support the induction and maintenance of hypercholesterolemia in the FGS animals. The failure of the nephrotic liver to raise cholesterol 7α-hydroxylase expression in rats with spontaneous FGS is consistent with our earlier studies of animals with puromycin-induced proteinuria [12].
Elevation of serum total cholesterol and LDL cholesterol concentration in our FGS rats was associated with an approximately 2.5-fold reduction in hepatic LDL receptor protein abundance. LDL receptor-mediated hepatic uptake of LDL represents the predominant pathway of clearance of plasma cholesterol. In fact, hereditary LDL receptor deficiency results in severe hypercholesterolemia and premature atherosclerotic cardiovascular disease [20]. It is, therefore, intuitive that acquired LDL receptor deficiency observed in our nephrotic rats with FGS could have contributed to the associated elevation of LDL and total cholesterol concentrations. This viewpoint is supported by the strong inverse correlation between hepatic LDL receptor and plasma total and LDL cholesterol levels founds in the study animals. This phenomenon provides the molecular basis for the previously reported impairment of LDL clearance in nephrotic syndrome [17]. Down-regulation of hepatic LDL receptor protein abundance found in the nephrotic rats with spontaneous FGS is consistent with the results of our earlier study in rats with puromycin-induced nephrotic syndrome [13], thus, pointing to the relevance of the findings to nephrotic syndrome regardless of the underlying cause.

Nephrotic proteinuria in our 34-week-old Imai rats was accompanied by moderate renal insufficiency. This may raise the question as to the possible contribution of renal insufficiency in the pathogenesis of the observed dysregulation of the above cholesterol regulatory proteins in the liver of our FGS rats. In an earlier study of rats with chronic renal insufficiency induced by 5/6 nephrectomy, we found no significant change in hepatic expression of either HMG-CoA reductase, cholesterol 7α-hydroxylase, or LDL receptor [21]. The latter study tends to exclude the possible role of renal insufficiency in the pathogenesis of the above abnormalities.

Liver tissue ACAT-2 protein abundance was nearly 2.5-fold greater in our Imai rats with FGS than that found in the control group. ACAT is a membrane-associated enzyme, which is primarily localized in the endoplasmic reticulum. ACAT catalyzes intracellular esterification of cholesterol and formation of cholesterol ester in nearly all mammalian cells [22]. Two isoforms of ACAT have, thus far, been identified. These include ACAT-1, which is expressed in most tissues, and ACAT-2, which is primarily expressed in the liver and intestine [23–26]. Esterification of free cholesterol by ACAT has been shown to enhance production and secretion of VLDL by the liver [27–29]. Thus, up-regulation of hepatic ACAT-2 in our rats with spontaneous FGS may contribute to elevation of plasma VLDL in these animals. Expression of HMG-CoA reductase is inhibited, while that of cholesterol 7α-hydroxylase is up-regulated by free cholesterol in the hepatocyte [30, 31]. Thus, the observed up-regulation of HMG-CoA reductase and the lack of rise in cholesterol 7α-hydroxylase seen in the FGS rats may be, in part, due to up-regulation of ACAT-2, which helps to lower cellular free cholesterol concentration by catalyzing the formation of cholesterol ester. This is compounded by LDL receptor deficiency, which limits the influx of cholesterol into the hepatocyte.

In a series of recent studies, we have demonstrated marked up-regulation of ACAT-2 in rats with puromycin-induced nephrotic syndrome [14] and rats with chronic renal insufficiency induced by 5/6 nephrectomy...
by hereditary LCAT deficiency, which is associated with reverse cholesterol transport processes is best illustrated surplus cholesterol from the peripheral cells for disposal ferred macromolecular platform for the LCAT reactions. and transesterification of fatty acid to the 3-hydroxyl lipoprotein lipase and VLDL receptor expressions [4]. liver. In the presence of cofactor, ApoA-I, the enzyme in this model revealed additive effects of nephrotic syn-

normal control group. LCAT is a 63 kD glycoprotein ever, our earlier studies of the enzymes and receptors upregulation of ACAT-2 in our Imai rats with sponta-

reductions. Dysregulation of these key cholesterol regu-

lation of hepatic HMG-CoA reductase and ACAT-2 HDL-3 or nascent HDL to the cardioprotective choles-

terol ester-rich HDL-2. The role of LCAT in this process involves generation of a chemical concentration gradient for optimal efflux of free cholesterol from the peripheral cells into the HDL particle through rapid conversion of free cholesterol to esterified cholesterol. Thus, LCAT plays an important role in HDL-mediated retrieval of surplus cholesterol from the peripheral cells for disposal in the liver, a phenomenon commonly referred to as reverse cholesterol transport.

The importance of LCAT in HDL metabolism and reverse cholesterol transport processes is best illustrated by hereditary LCAT deficiency, which is associated with a depressed HDL-2 to HDL-3 ratio, presence of cholesterol-laden foam cells in various tissues, accelerated atherosclerotic cardiovascular disease, progressive renal insufficiency, and corneal opacification [34].

Plasma HDL cholesterol is frequently reduced [35] or unchanged [17], LDL to HDL ratio is markedly elevated and maturation of HDL-3 to HDL-2 is impaired in nephrotic syndrome [36]. Similarly, plasma HDL cholesterol is depressed and HDL maturation is impaired in chronic renal insufficiency [37]. These abnormalities are highly suggestive of LCAT deficiency. In fact, marked elevation of LDL cholesterol to HDL cholesterol ratio in our FGS rats, which had severe nephrotic proteinuria and moderate renal insufficiency, was coupled with severe LCAT deficiency. In a recent study, we found marked down-regulation of hepatic LCAT mRNA abundance and significant reduction of plasma LCAT enzymatic activity in rats with chronic renal insufficiency induced by 5/6 nephrectomy [38]. In a concurrent study, we found severe reduction of plasma LCAT enzymatic activity coupled with heavy urinary losses of LCAT and normal hepatic LCAT mRNA abundance in rats with puromycin-induced nephrotic syndrome [39]. The present study revealed profound reduction of plasma LCAT protein concentration in rats with a spontaneous nephropathy presenting with severe nephrotic proteinuria and moderate renal insufficiency resembling FGS in humans.

Comparative analysis of data obtained in our earlier studies of rats with either nephrotic syndrome or chronic renal insufficiency alone with those of the 34-week old Imai rats exhibiting both conditions revealed predominant effect of nephrotic syndrome on expression of the main cholesterol-regulatory enzymes and receptors. However, our earlier studies of the enzymes and receptors involved in metabolism of triglyceride-rich lipoproteins in this model revealed additive effects of nephrotic syndrome and renal insufficiency on plasma lipids and tissue lipoprotein lipase and VLDL receptor expressions [4].

CONCLUSION

Imai rats with spontaneous FGS exhibited marked up-regulation of hepatic HMG-CoA reductase and ACAT-2 coupled with severe acquired LDL receptor and LCAT deficiencies. Dysregulation of these key cholesterol regulatory factors can account for the profound alterations of cholesterol metabolism in advanced FGS presenting with a combination of severe nephrotic proteinuria and moderate renal insufficiency.

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