CASE REPORT

Hepatic Iron Overload Associated With a Decreased Serum Ceruloplasmin Level in a Novel Clinical Type of Aceruloplasminemia

SATOSHI KONO,* HITOSHI SUZUKI,* KAZUO TAKAHASHI,† YOSHITOMO TAKAHASHI,* KENTARO SHIRAKAWA,* YOHKO MURAKAWA,† SHUHEI YAMAGUCHI,† and HIROAKI MIYAJIMA*

*The First Department of Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan; and †the Division of Neurology, Hematology and Rheumatology Faculty of Medicine, Shimane University, Shimane, Japan

Background & Aims: **Aceruloplasminemia is a novel hereditary iron overload disease caused by a mutation in the ceruloplasmin gene and characterized by a complete deficiency of serum ceruloplasmin and iron accumulation in the liver and brain.** ***Methods:*** **We herein studied a novel clinical type of aceruloplasminemia in which a low amount of ceruloplasmin was detected in the serum of a patient. The patient presented with an asymptomatic hepatic iron overload, retinal degeneration, and diabetes mellitus. Magnetic resonance imaging of the liver and basal ganglia showed T2-hypointensity signals associated with parenchymal iron accumulation because of an absence of the ferroxidase activity in ceruloplasmin.** ***Results:*** **A gene analysis showed a novel G969S mutation in the ceruloplasmin gene. A biochemical analysis of the patients' serum and a biogenesis study of G969S mutant ceruloplasmin using mammalian cell culture system resulted in the synthesis and secretion of only apoceruloplasmin without any ferroxidase activity.** ***Conclusions:*** **This novel clinical type of aceruloplasminemia should therefore be considered in the differential diagnosis of unexplained hemochromatosis, which is associated with a decrease in the serum ceruloplasmin level.**

Ceruloplasmin is a multicopper oxidase, with 6 atoms of copper incorporated in it, which plays a role in the mobilization and oxidation of iron from the tissue stores associated with the subsequent incorporation of ferric iron into transferrin.[1] Ceruloplasmin is mainly synthesized in hepatocytes and is secreted into the plasma as a holoceruloplasmin with 6 atoms of copper incorporated during biosynthesis. The failure to incorporate the copper in ceruloplasmin results in the secretion of an unstable apoceruloplasmin, which is devoid of any oxidase activity and then rapidly degrades in the plasma. In Wilson's disease, the dysfunction of a copper-transporting ATPase disturbs the copper transport in the secretory pathway, thus resulting in a marked reduction in the serum concentration of ceruloplasmin.[2] Under normal circumstances, serum ceruloplasmin does not cross the blood-brain barrier. In the brain, most of the ceruloplasmin is located on the surface of astrocytes in a glycosylphosphatidylinositol (GPI)-anchored form.[3] Ceruloplasmin plays a major role in the mobilization of iron in the central nervous system.[4] Aceruloplasminemia is a novel iron overload disease caused by a complete absence of ceruloplasmin ferroxidase activity resulting from mutations in the ceruloplasmin gene.[5] The diagnosis of aceruloplasminemia in a symptomatic individual relies on the demonstration of the complete absence of serum ceruloplasmin and some combination of the following: a low serum copper concentration, low serum iron concentration, high serum ferritin concentration, and increased hepatic iron concentration. The diagnosis is strongly supported by the characteristic magnetic resonance imaging (MRI) findings of abnormal low intensities reflecting iron accumulation in the liver and brain including basal ganglia, thalamus, and dentate nucleus on both T1- and T2-weighted images. Aceruloplasminemia was initially described as familial apoceruloplasmin deficiency because this disease is characterized by a complete absence of holo- and apoceruloplasmin in the serum by immunoblot analysis.[6] The pathogenesis of aceruloplasminemia is an impairment in the iron efflux from tissue to the serum because of the absence of any ferroxidase activity in the ceruloplasmin. Clinically, the disease consists of hepatic iron overload, anemia, retinal degeneration, diabetes mellitus, and adult-onset neurologic symptoms including cerebellar ataxia, involuntary movement,

Abbreviations used in this paper: GPI, glycosylphosphatidylinositol; TEPA, tetraethylenepentamine.

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and cognitive dysfunction.[7] These clinical symptoms reflect the sites of iron deposition. Genetic testing has allowed the demonstration of more than 20 mutations in the ceruloplasmin gene in 25 affected patients and family members all over the world.[1,5,8] The prevalence of aceruloplasminemia was estimated to be approximately 1 per 2 million in nonconsanguineous marriages.[9] The initial clinical clue for making a diagnosis of aceruloplasminemia is to prove a complete deficiency of serum ceruloplasmin. However, we herein report a novel clinical type of aceruloplasminemia that showed severe iron accumulation in both the liver and central nervous system, despite the detection of serum ceruloplasmin.

Case Report

A 66-year-old Japanese woman was investigated for iron refractory anemia of 3 years duration. At age 50 years, the patient developed insulin-dependent diabetes mellitus. Her parents were second-degree cousins, but there was no family history of anemia, liver dysfunction, or neurological diseases. The mild anemia (hemoglobin 9.8 g/L) was accompanied with a low serum iron concentration (22 μg/dL) and a high level of serum ferritin (970 μg/dL). Bone marrow aspirate revealed mild dyserythropoiesis with iron accumulation in the reticuloendothelial cells. The serum ceruloplasmin level decreased to 7.2 mg/dL (normal, 21–37 mg/dL) as measured by nephelometry. The serum copper level decreased to 10 μg/dL (normal, 62–128 μg/dL). Her neurologic examination showed no cerebellar ataxia, involuntary movement, or dementia. An ophthalmoscopic examination showed retinal generation with several small yellowish opacities, which were scattered over grayish atrophy of the retinal pigment epithelium in both eyes. Her visual acuity was not disturbed. Fluorescein angiography demonstrated window defects corresponding to the yellowish opacities. These findings differed from diabetic retinopathy. MRI showed an abnormal hypointensity on the T2-weighted images of the liver (Figure 1*A*) as well as in the basal ganglia and the cerebellum in the brain (Figure 1*B*). A liver biopsy showed a normal architecture. Few portal inflammatory cells were observed, without piecemeal necrosis or cholangitis. Perls' stain showed severe iron accumulation revealing abundant hemosiderosis in the hepatocytes distributed evenly within the lobule (Figure 1*C*). A quantitative measurement of the hepatic iron and copper content showed that the hepatic iron content was 1564 μg/g dry weight (control, <700) and the hepatic copper content was 11.3 μg/g dry weight (control, <14). Informed consent for the gene analysis and molecular study was obtained from the patient according to guidelines of the institution.

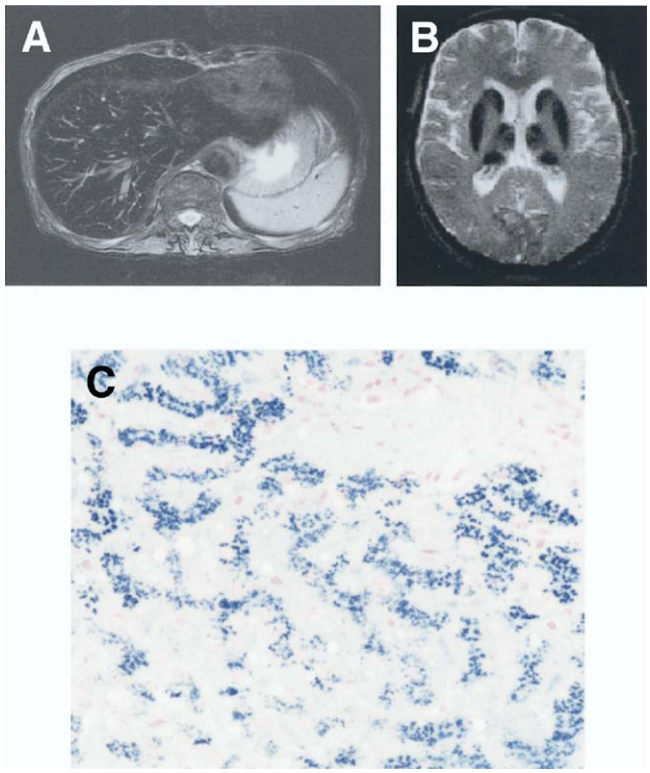

Figure 1. T2-weighted magnetic resonance images of the patient. (*A*) T2-weighted axial image of the liver showed signal attenuation consistent with iron accumulation. (*B*) T2-weighted axial image of the brain showed signal attenuation of the basal ganglia. (*C*) A liver biopsy specimen stained with Perls' stain showing iron in the hepatocytes (original magnification, ×200).

Methods and Results

Genomic DNA was extracted from peripheral blood leukocytes and was amplified by polymerase chain reaction (PCR). The PCR products were directly sequenced by the previously described method.[10] The sequence analysis revealed a novel missence mutation G969S in exon 17 in the ceruloplasmin gene (Figure 2). The patient was homozygous for the G969S mutation. The HFE gene region of DNA samples was amplified by PCR.[11] Restriction enzyme analysis was performed using *Sna*BI for C282Y and *Bcl*I for H63D. The patient had neither a C282Y nor an H63D mutation. An immunoblot analysis and an oxidase staining of patient's serum were performed as described previously.[12,13] The immunoblot analysis of the serum using anti-human ceruloplasmin antibody demonstrated only a band of apoceruloplasmin and an absence of holoceruloplasmin (Figure 3). The oxidase stain of the serum with *p*-phenylenediamine showed no oxidase activity (Figure 3). We thus hypothesized that G969S mutant ceruloplasmin was secreted to serum in the form of apoceruloplasmin, which lacked any ferroxidase activity because the mutant protein could not be incorporate copper. To elucidate

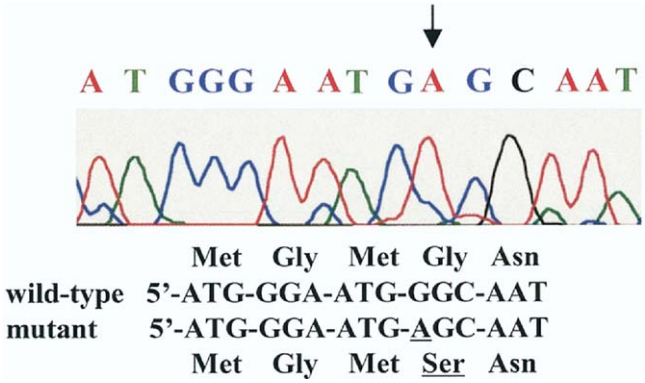

Figure 2. The identification of a novel G969S missence mutation in the ceruloplasmin gene. A direct sequence analysis of PCR product of exon 17 in the ceruloplasmin gene revealed a homozygous missence mutation caused by a G to A substitution at nucleotide 2962, thus resulting in an amino acid alternation at 969 (Gly969Ser).

the molecular pathogenesis of the novel clinical type of aceruloplasminemia, a gene expression study of the G969S missense mutation was performed. Chinese hamster ovary (CHO) cells that had no endogenous ceruloplasmin synthesis were transfected with complementary DNA (cDNA) encoding either secreted and GPI-anchored wild-type or G969S human ceruloplasmin because we had previously studied the biogenesis of the mutants.[14,15] The transfected CHO cells were analyzed by immunoblot analyses using anti-human ceruloplasmin antibody. The immunoblot analyses of both secreted and GPI-anchored wild-type ceruloplasmin showed the presence of 2 bands of 135 kilodaltons and 85 kilodaltons (Figure 4*A*, lanes 1 and 5), respectively. We previously showed these 2, 135-kilodalton and 85-kilodalton bands to be, respectively, apoceruloplasmin and holoceruloplasmin.[13–15] Treatment with 200 μmol/L $CuCl_2$ in the media increased the expression of ceruloplasmin (Figure 4*A*, lanes 2, 4, 6, 8). An immunoblot analysis of a media sample of the secreted form of the G969S mutation revealed the mutant to be synthesized and secreted as only apoceruloplasmin, even though the transfected CHO cells were incubated in media containing an excessive amount of

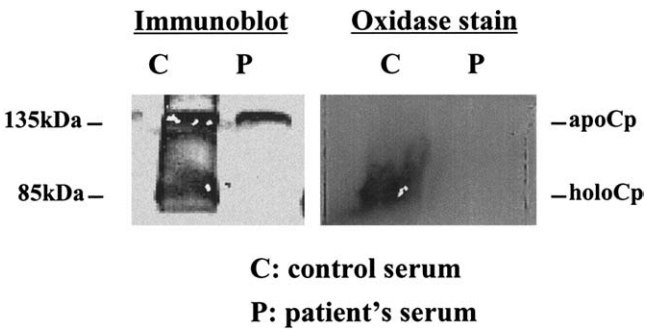

Figure 3. An immunoblot analysis of the patient's and the control serum by anti-human ceruloplasmin antibody. The immunoblot analysis of the patient's serum detected a 135-kilodalton band corresponding to apoceruloplasmin. The oxidase activity stain of the patient's serum with *p*-phenylenediamine showed no staining.

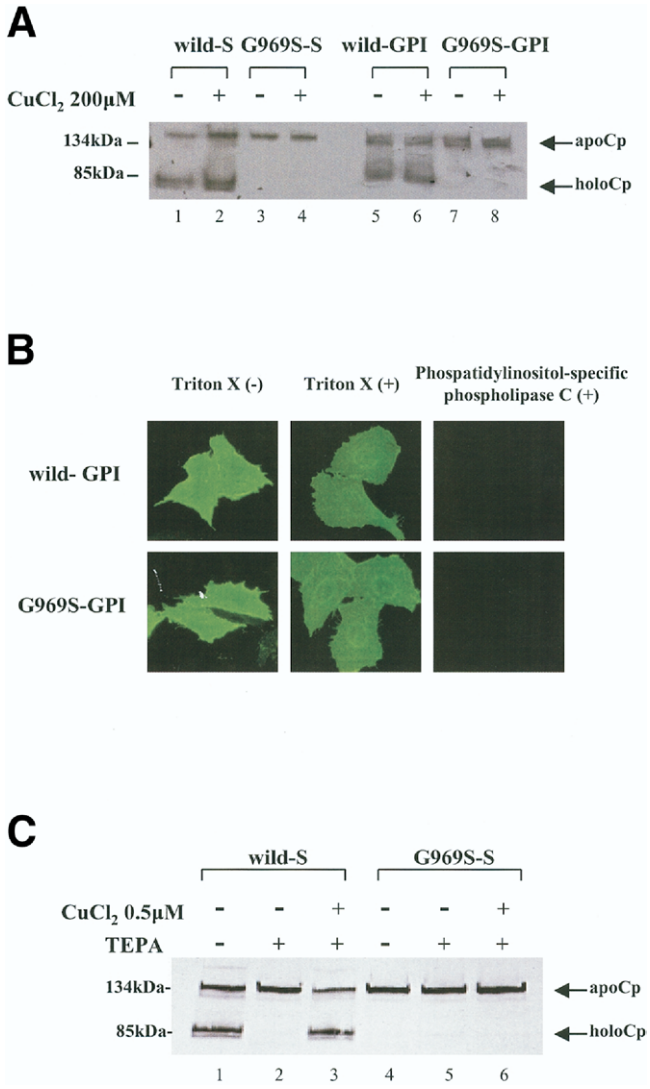

Figure 4. A biogenesis study of G969S missense mutation. (*A*) Immunoblot analysis of the secreted form of ceruloplasmin in the media and the GPI-anchored form of ceruloplasmin in the cell lysates from the CHO cells transfected with wild-type or G969S mutant ceruloplasmin. In some experiments, cells were incubated with 200 μmol/L $CuCl_2$ in culture media for 48 hours prior to the immunoblot analysis. Media and cell lysate samples were analyzed by nonreducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using antibody to human ceruloplasmin. (*B*) Indirect immunofluorescence localization of the GPI-anchored form of G969S ceruloplasmin. The CHO cells transfected with either wild-type or G969S ceruloplasmin were stained with anti-human ceruloplasmin antibody. In some experiments, cells were stained either in the absence of membrane permeabilization or after treatment with phosphatidylinositol-specific phospholipase. (*C*) An in vitro copper incorporation study of wild-type apoceruloplasmin and G969S mutant. CHO cells were transfected with the secreted form of either the wild-type or G969S mutant and then incubated in the copper chelator tetraethylenepentamine (TEPA). The secreted form of ceruloplasmin in the media was collected and incubated with pH 5.6 sodium acetate and 0.5 μmol/L $CuCl_2$. Ceruloplasmin was detected by immunoblot analysis following nonreducing SDS-PAGE. Secreted form of wild-type ceruloplasmin and G969S mutant ceruloplasmin represent wild-S and G969-S, respectively. GPI-anchored form of wild-type ceruloplasmin and G969S mutant ceruloplasmin represent wild-GPI and G969S-GPI, respectively.

$CuCl_2$ (Figure 4*A*, lanes 3 and 4). An immunoblot analysis of a cell lysate sample of the GPI-anchored form showed the mutant to also be synthesized as only apoceruloplasmin (Figure 4*A*, lanes 7 and 8). An indirect immunofluorescent analysis of ceruloplasmin was performed as described previously.[14] The immunofluorescent analysis of the transfected cells with and without permeabilization using Triton X-100 showed the mutant to be expressed on the diffuse cell surface (Figure 4*B*). The transfected cells were treated with phospatidylinositol-specific phospholipase C, which removes GPI linkage from the cell membrane. Both the wild-type and the mutant ceruloplasmin were not stained by the treatment with phospatidylinositol-specific phospholipase C. This finding suggested the GPI-anchored form of G969S to be expressed on the cell surface, most likely as a GPI-anchored wild-type ceruloplasmin. We examined whether the mutant protein is able to incorporate copper in vitro or not as previously described.[15] Media samples from the transfected CHO cells with either wild-type or the mutant were treated with copper chelator tetraethylenepentamine (TEPA) to reconstitute apoceruloplasmin (Figure 4*C*, lanes 2 and 5) and then were subjected to pH 5.6 sodium acetate with 0.5 μmol/L $CuCl_2$. This treatment incorporated copper into wild-type apoceruloplasmin, thus resulting in the reconstitution of holoceruloplasmin in vitro (Figure 4*C*, lane 3), whereas the mutant did not (Figure 4*C*, lane 6). This finding suggested that the conformation of the G969S mutant ceruloplasmin thus played a critical role in the copper incorporation.

Discussion

We herein reported a case of a 66-year-old woman who was homozygous for a novel G969S missense mutation in the ceruloplasmin gene. A common biochemical feature of previously reported patients affected with aceruloplasminemia is a complete deficiency of ceruloplasmin in the serum.[1,5,8] An immunoblot analysis of the serum of the patients showed no bands, thus indicating both apo- and holoceruloplasmin, and, thus, we initially termed this disease *familial apoceruloplasmin deficiency*.[6] The present case had an asymptomatic iron overload in the liver and brain and a one-third concentration of normal serum ceruloplasmin. In the patient's serum, only an apoceruloplasmin band was detected by an immunoblot analysis and no ferroxidase activity by an oxidase assay (Figure 3). The demonstration of a novel G969S missense mutation in the ceruloplasmin gene confirmed the diagnosis to be an atypical form of aceruloplasminemia (Figure 2). Pathogenesis of this novel form of aceruloplasminemia may be impairment of iron efflux caused by dysfunction of ferroxidase activity of the mutant ceruloplasmin because the mutant formed only apoceruloplasmin without any ferroxidase activity. As a result, the patient showed iron deposition in both the liver, pancreas, brain, and retina in which ceruloplasmin was expressed.[16] In addition to this report, until now, there have been some reports of hepatic iron overload associated with a low concentration of ceruloplasmin. Recent reports showed that patients with HFE-1 hereditary hemochromatosis had decreased ceruloplasmin level in the serum.[17,18] The ferroxidase activity in their serum was measurable.[18] The present patient showed neither a C282Y nor an H63D mutation in the HFE gene. The serum had no ferroxidase activity, even though the ceruloplasmin was detected in the serum. An abnormal MRI finding reflecting iron accumulation was detected in the brain as well as the liver. In these findings, this novel from of aceruloplasminemia differed significantly from the patients with HFE-1 hemochromatosis. Other reports showed that patients heterozygous for a mutation in the ceruloplasmin gene were associated with a hepatic iron overload and a low serum ceruloplasmin level.[10,19–21] In general, heterozygosity is usually associated with a partial ceruloplasmin deficiency, and heterozygous patients may have normal iron metabolism and no clinical symptoms. The pathologic mechanism of the symptomatic heterozygous patients remains unknown. An immunoblot analysis of the serum ceruloplasmin in heterozygous patients showed both apo- and holoceruloplasmin. However, the present patient was homozygous for the G969S missense mutation and had only apoceruloplasmin in the serum. This novel clinical form of aceruloplasminemia should be considered in the differential diagnosis of unexplained hemochromatosis. A clinical clue when making a differential diagnosis of aceruloplasminemia from hemochromatosis is to measure the serum ceruloplasmin level and confirm that the circulating ceruloplasmin is completely absent. However, this diagnostic approach is not sufficient for the diagnosis of this form of aceruloplasminemia. We should therefore consider performing a genetic analysis of the ceruloplasmin gene.

We previously revealed 2 molecular pathogeneses of missense mutant ceruloplasmin in aceruloplasminemia. A biochemical analysis of a P177R missense mutant ceruloplasmin demonstrated that the mutant failed to exit the endoplasmic reticulum (ER).[14] The finding indicated that aceruloplasminemia can result from the retention of the mutant ceruloplasmin in the ER. Another study of a G631R missense mutation resulted in the synthesis and secretion of only apoceruloplasmin.[15] The present gene expression study demonstrated that the G969S mutation also resulted in the synthesis and secretion in the form of apoceruloplasmin because the mutant may be impaired in copper incorporation into apoceruloplasmin (Figure 4*A–C*). Both G631R and G969S mutations are located at the nearby type 1 cop-

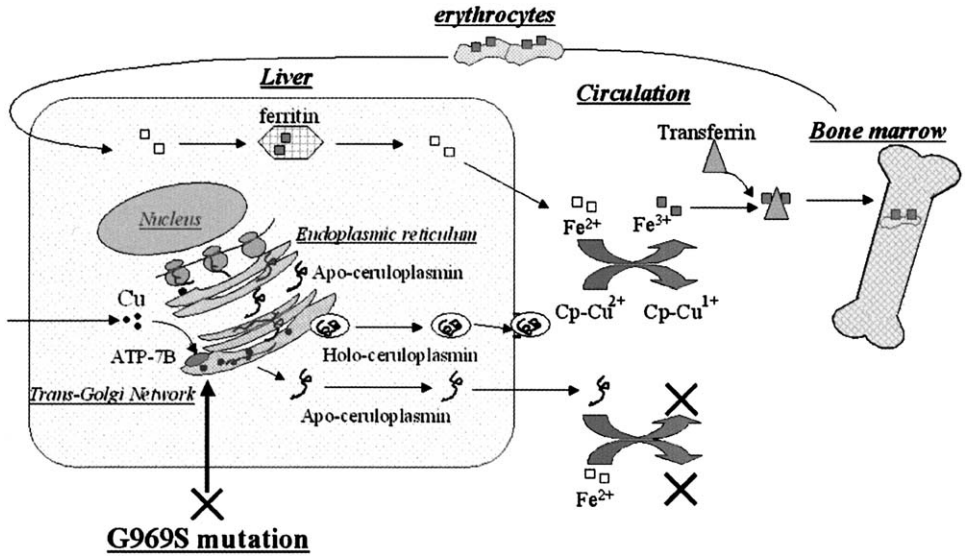

Figure 5. A model for the interaction between iron and copper homeostasis in normal subjects and in the novel form of aceruloplasminemia. In normal subjects, iron is continuously recycled between the bone marrow and hepatocytes with serum transferrin acting as a shuttle to deliver iron from hepatocytes to the bone marrow. The role of ceruloplasmin (Cp) formed as holoceruloplasmin is a ferroxidase in mediating ferrous iron oxidation and subsequent transfer into transferrin. In the normal subjects, copper enters the cell and bounds the copper chaperones which deliver the copper to ATP-7B. The ATP-7B pumps the copper into the trans-Golgi network. Ceruloplasmin is initially synthesized as apoceruloplasmin and is incorporated the copper into the apoprotein in the Golgi body resulting in formation of holoceruloplasmin prior to extracellular secretion. In the novel form of aceruloplasminemia G969S mutation may alter the intrinsic protein structure resulting in abrogation of copper incorporation into the apoceruloplasmin. The mutant ceruloplasmin may pass through the secretory pathway to secrete from the cells, however, the mutant may not possess a ferroxidase activity to oxidize ferrous iron thereby causing a dysfunction of iron efflux.

per-binding sites of His637 and His975, respectively. A site-directed mutagenesis analysis of the type 1 copper-binding site indicated the failure of copper incorporation into the apoceruloplasmin.[15] These biochemical studies showed that the type 1 copper-binding site did not affect either the protein folding for intracellular trafficking from the ER to the Golgi body or the secretion from the cell. However, the copper-binding site might play an essential role in the protein structure for copper incorporation into apoceruloplasmin. Although the patient who was homozygous for the G631R mutation had no detectable serum ceruloplasmin, even by an immunoblot analysis, the patient carrying the G969S mutation had only apoceruloplasmin in the serum. The half-life of wild-type apoceruloplasmin in the serum is very short (5 hours) in comparison with the half-life of holoceruloplasmin (5.5 days).[22,23] As a result, more than 90% of the serum ceruloplasmin is in the form of holoceruloplasmin. We speculate that the G631R mutant and wild-type apoceruloplasmin might thus be fragile in the serum, whereas the G969S mutant might be more stable. The precise biochemical mechanism by which the G969S mutant had stability in the serum remains unknown. Further study of the half-life and crystal structure of the mutant ceruloplasmin should help to elucidate the biologic function of the G969S mutant in aceruloplasminemia while clarifying the mechanism of copper incorporation into apoceruloplasmin. The G969S mutant may be synthesized and secreted with kinetics identical to that of wild-type ceruloplasmin; however, the mutation may interfere with the type 1 copper-binding site and alter the protein structure resulting in failure of copper incorporation into apoceruloplasmin. This mutant may be secreted in the form of apoceruloplasmin without any ferroxidase activity and thus cause a dysfunction of iron efflux from hepatocytes (Figure 5). We herein emphasized the importance of considering this novel clinical type of aceruloplasminemia in the differential diagnosis of disorders causing hepatic iron accumulation despite the presence of serum ceruloplasmin.

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Address requests for reprints to: Satoshi Kono, MD, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan.

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