

Molecular Studies of Ceruloplasmin Deficiency in Wilson's Disease

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Abstract

Deficiency of serum ceruloplasmin is a characteristic biochemical abnormality of Wilson's disease, although the mechanism of this finding is unknown. Ceruloplasmin messenger RNA (mRNA) levels were therefore examined in five patients with Wilson's disease and five controls with other types of hepatic disease. Northern and dot blot hybridizations showed that detectable ceruloplasmin mRNA was present in all of the patients with Wilson's disease, including one patient with no detectable serum ceruloplasmin. However, the ceruloplasmin mRNA levels in the Wilson's disease patients were only 33% that of controls ($P < 0.001$). In contrast, albumin mRNA levels in the Wilson's disease patients averaged 161% that of controls. In an attempt to better delineate the level of gene expression responsible for this decrease in ceruloplasmin mRNA, the nuclear run-on assay was used to analyze transcriptional rates. The amount of ceruloplasmin gene transcription in four Wilson's patients was decreased to 44% that of three controls. These results indicate that the diminished serum ceruloplasmin levels in patients with Wilson's disease are due at least in part to a decrease in ceruloplasmin gene transcription.

Introduction

Wilson's disease is an inborn error of copper metabolism that results in abnormal copper deposition in several organs. This increase in tissue copper can cause abnormalities in several systems, but the most common clinical manifestations are signs of hepatic and neurological dysfunction. The exact mechanism of these abnormalities in copper metabolism is unknown despite extensive study (1). A characteristic bio-

chemical finding of this disease is a deficiency in the serum of the copper-binding protein ceruloplasmin. ~ 95% of all Wilson's disease patients have low or absent ceruloplasmin levels (1–3), and 20% of heterozygotes for the disease have low levels as well (2). Other liver diseases with increased hepatic copper have normal or elevated concentrations of ceruloplasmin (1).

The basis for the diminished ceruloplasmin levels in Wilson's disease and their effect on the pathophysiology of this disorder is obscure. Although several studies have described electrophoretic and chromatographic differences in the ceruloplasmin of patients with Wilson's disease (4–6), the finding of several normal polymorphisms of this protein makes the significance of these differences unclear (7, 8). Despite the diagnostic value and possible pathogenetic role of ceruloplasmin in Wilson's disease, little has been done to investigate the molecular basis of ceruloplasmin deficiency in this disease. Recently the human ceruloplasmin gene was cloned from a liver complementary DNA (cDNA) library (9), and in the present study we employed this clone pHCP1 in an attempt to define the molecular basis for the deficiency of ceruloplasmin in Wilson's disease.

Methods

RNA extraction and RNA-DNA hybridization studies. Total RNA was extracted from liver samples from five patients with Wilson's disease and from five patients with other forms of liver disease. We selected patients with other types of hepatic disease as more suitable controls rather than patients with histologically normal livers to control for the effects of chronic liver disease on protein synthesis. Although all patients enrolled in this study had significant chronic liver disease requiring either percutaneous biopsy for evaluation (two in each group) or transplantation, the Wilson's disease patients had perhaps somewhat less severe disease, as indicated by statistically lower bilirubin levels and a trend towards higher serum albumin levels. Clinical information about these patients is shown in Table I. The control patients had autoimmune chronic active hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis. The liver samples were obtained from unused portions of percutaneous biopsies performed for clinically indicated reasons or from livers removed at the time of transplantation. These specimens were quick frozen and stored for periods ranging from days to years at -70°C . The RNA isolation was performed using a modification of the Chirgwin procedure (10) as we have previously described (11). The frozen samples were homogenized in 3.5 ml of 4 M guanidine thiocyanate solution (11). After removing the cellular debris by low-speed centrifugation, the RNA was pelleted through a cesium chloride gradient. The RNA obtained was redissolved in 10 mM Tris, pH 7.4, 1 mM Na_2EDTA , precipitated three times with 0.1 M sodium acetate and ethanol, and then quantitated by

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A₂₆₀ spectrophotometry. When enough total RNA was available poly (A⁺) RNA was prepared by oligo(dT) columns as previously described (12). The amounts of RNA isolated were correlated with the protein and DNA contents of the homogenized samples and there were no differences in each of these ratios between groups.

Steady-state mRNA levels were determined by Northern and dot blot hybridizations. For Northern blots 10 µg of total RNA or 1 µg of poly (A⁺) RNA per specimen were denatured in 0.5 M glyoxyl, 50% dimethyl sulfoxide, and 10 mM phosphate buffer, electrophoresed in a 1% agarose gel, transferred to a GeneScreen filter (New England Nuclear, Boston, MA) and baked for 2 h at 80°C. After prehybridization (11), the filters were hybridized using standard stringent conditions for GeneScreen as described by the manufacturer which include 50% formamide, 0.04% polyvinyl-pyrrolidone, 0.04% bovine serum albumin, 0.04% ficoll, 0.75 M NaCl, 0.075 M Na citrate, 1% sodium dodecyl sulfate, and calf thymus DNA. A ceruloplasmin cDNA clone (9) that was labeled to a specific activity of 2–5 × 10⁸ cpm/µg DNA with [³²P]deoxycytidine triphosphate by primer extension (13) was added to the hybridization buffer. The filters were washed under stringent conditions, exposed to x-ray film, and the developed film scanned by densitometry as previously described (11). The filters were subsequently stripped and rehybridized with a human albumin probe HSAF-47 (14) (kindly provided by Dr. R. Lawn, Genentech Inc., San Francisco, CA), and subjected to the same autoradiographic process. For dot blots RNA was diluted serially in 3% formaldehyde, spotted on GeneScreen, baked, prehybridized, hybridized, and autoradiography performed (11).

Transcriptional rate analysis. Nuclear run-on assays were performed on pretransplant surgical liver specimens employing minor modifications of the methods we previously described (15). Liver nuclei from patients with Wilson's disease and from controls were isolated according to the method of Clayton and Darnell (16). These nuclei were then labeled for 15 min with high-specific activity [³²P]-uridine triphosphate (UTP)¹ (16), and the labeled RNA transcripts were isolated by lysis of the nuclei in a hypotonic solution, followed by DNA digestion with RNase-free DNase (100 U/ml), proteinase K digestion, phenol extraction, and ethanol precipitation. We routinely obtain 2–10 × 10⁶ cpm of labeled transcripts from 1–3 g of frozen liver tissue. Between two ethanol precipitations, the labeled RNA was precipitated in 10% TCA to remove the unincorporated UTP.

The labeled RNA was then hybridized with at least a 10-fold excess of human ceruloplasmin and albumin cDNAs bound to nitrocellulose membrane as described by Hofer and Darnell (17). In addition pBR322 and a mouse cDNA of arginine transfer RNA (provided by Dr. J. Darnell, Jr., Rockefeller University, New York, NY), were used as controls. Although arginine transfer RNA is not an ideal control because it is not transcribed by RNA polymerase II, it can still be used to indirectly equate transcription between various samples because its transcriptional rate is more or less constant in a variety of cells (16). Blots were prepared as follows: the cDNAs were denatured, applied to nitrocellulose, prehybridized, and then hybridized with equal amounts of the labeled nuclear RNA. After hybridization the filters were washed extensively and digested with RNase A (10 µg/ml) (16). Filters were then exposed to Kodak XAR-5 film at –70°C, and densitometric scanning was performed.

Before studies employing frozen human samples, experiments were performed using rat livers to determine whether the freezing of the tissue affected the nuclear run-on assay. Nuclei were prepared from either fresh livers or from livers quick-frozen and stored for several months at –70°C. Although the nuclei from frozen tissue incorporated somewhat less radioactivity, there was no difference in the relative transcriptional rates of individual genes between the fresh and frozen livers.

1. Abbreviation used in this paper: UTP, uridine triphosphate.

Results

As demonstrated in Table I, the patients with Wilson's disease had slightly better liver function than did the patients with other forms of liver disease, with somewhat higher serum albumin levels (but not statistically different) and significantly lower bilirubin values. The Wilson's disease patients had an increased albumin mRNA content compared with control patients (161±69% SEM of control) when the albumin mRNA dot blots were analyzed by densitometry (Fig. 1). This difference was not statistically significant, but it did reflect the trend toward higher serum albumin levels in the Wilson's disease patients. When RNA was subjected to Northern blot analysis and hybridized with a ceruloplasmin cDNA clone, the two known human ceruloplasmin mRNA species (9) were present in all five patients with Wilson's disease (Fig. 2). This finding occurred despite low ceruloplasmin levels in four patients and no detectable serum ceruloplasmin in the fifth patient. However when dot blots were scanned by densitometry the ceruloplasmin mRNA levels in patients with Wilson's disease were significantly decreased (33±7.7% SEM of control, *P* < 0.001), in distinct contrast to the findings for albumin mRNA which codes for another secreted protein (Fig. 1). To ensure that control patients' ceruloplasmin mRNA levels were not elevated over Wilson's disease patients merely due to a variation in acute phase reactants, we also probed the RNA with α₁-acid glycoprotein (18). In contrast to our ceruloplasmin data, steady-state levels of mRNA for this acute-phase reactant were not different in the four Wilson's disease patients studied as compared with three controls (114±48% SEM of control).

In an attempt to determine the level of gene expression responsible for this decrease in steady-state levels of ceruloplasmin mRNA, we adapted the nuclear run-on assay to analyze transcriptional rates in human liver samples for the first time. Fig. 3 is a representative nuclear run-on assay demonstrating a marked decrease in the transcription of ceruloplasmin in a Wilson's disease patient in comparison with a control with primary biliary cirrhosis. Densitometry scanning of all the assays and normalization of the results for the *t*-arginine control demonstrated that the amount of ceruloplasmin gene transcription in four Wilson's disease patients was decreased when compared with that of three controls (44±14% SEM of control; Fig. 4). There was no significant change in the transcription of albumin mRNA between the groups (Figs. 3 and 4).

Table I. Clinical Data for Patient Population Studied

	Controls	Wilson's disease
Number	5	5
Age (mean yr)	27.4	29.4
Sex (M:F)	2:3	1:4
Albumin (g/dl)*	2.8±0.3	4.0±0.5
Bilirubin (mg/dl)†	9.9±2.9	2.4±1.2
Ceruloplasmin (mg/dl)‡	57.9±12.9	3.8±1.2

Mean±SEM for all values. * NS † *P* < 0.05. ‡ *P* < 0.001.

^{||} Values available for only three patients.

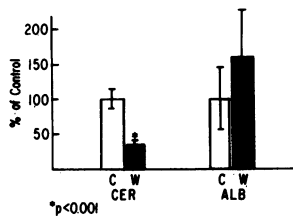


Figure 1. Bar graph representation of the steady-state mRNA content for ceruloplasmin (*CER*) and albumin (*ALB*) in control (*C*) and Wilson's disease (*W*) patients. Each bar represents the mean of five patients \pm SEM. mRNA content in each patient was quantitated by means of densitometric analysis of autoradiograms of dot blots hybridized with the appropriate [32 P]-labeled probe as described in Methods. Results are expressed as percentage of control values.

Although patients with other forms of chronic liver disease were selected as controls for the reasons discussed, the possibility existed that ceruloplasmin mRNA content in our controls was greater than normal. Therefore our patients' ceruloplasmin steady-state levels and transcriptional rates were also compared with that of a normal liver (an unused donor transplant liver). The normal liver had both a greater ceruloplasmin mRNA steady-state level and a higher ceruloplasmin gene transcriptional rate than all of the control and Wilson's disease patients.

Discussion

Ceruloplasmin is a single-chain polypeptide of mol wt 132,000 D which exists in serum as a holoprotein bound to six or seven copper atoms per molecule (1). This protein is present in the serum of all mammals, but its multiple functions are not well understood although it is thought to be important in copper transport and homeostasis (1). Nearly all Wilson's disease patients have abnormally low or absent serum levels of the holoprotein at some time in their disease (1), but the basis for the deficiency is unknown. Most authorities believe that the ceruloplasmin protein in Wilson's disease patients is structurally normal, although there is controversy on this point (1). For example some investigators have found a reduction in the amine oxidase activity of ceruloplasmin obtained from patients with Wilson's disease. It is felt that this reduced activity is due to a structural defect (2, 19, 20). Finally the livers of patients with Wilson's disease have been shown by Graul et al.

Discussion

to contain apoceruloplasmin (21), raising the possibility that the formation of the holoprotein is inhibited. However other studies have shown apoceruloplasmin to be almost completely absent in patients with Wilson's disease (22).

We have investigated the basis of ceruloplasmin deficiency in Wilson's disease using the techniques of molecular biology. We found significant quantities of ceruloplasmin mRNA to be present in all five patients with Wilson's disease studied despite the absence of detectable serum holoceruloplasmin in one of these patients and marked deficiency in the other four. However when compared with patients with other forms of hepatic dysfunction known to be associated with increased hepatic copper levels, the Wilson's disease patients had only one-third as much ceruloplasmin mRNA as was present in the livers of the controls. Using a nuclear transcriptional assay we found that these reduced steady-state levels were due, at least in part, to a decrease in ceruloplasmin gene transcription. Such a defect would explain our experimental findings and also provide an explanation for the absent serum ceruloplasmin levels found in some patients.

It has been firmly established that Wilson's disease is an autosomal recessive disorder (1), yet the specific mechanisms that yield the abnormalities of copper metabolism in this disease are unknown. There appear to be two defects in copper metabolism that have been established in this disorder: a reduction in biliary copper excretion and a reduced rate of incorporation of copper into apoceruloplasmin (3). Because a deficiency in the serum levels of the holoceruloplasmin has been a major biochemical finding in this disease, it has been postu-

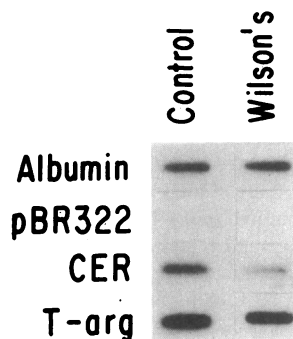


Figure 3. Autoradiogram of a representative nuclear run-on assay analyzing the transcriptional rates of genes in a Wilson's disease patient and in a control with primary biliary cirrhosis. Nascent-labeled nuclear RNA transcripts from liver nuclei of the two patients were hybridized with 5 μ g of specific cDNA probes bound to a nitrocellulose filter as described in Methods. Autoradiography was then performed. pBR322 was used to determine background activity and arginine transfer RNA was used as a control of total transcription.

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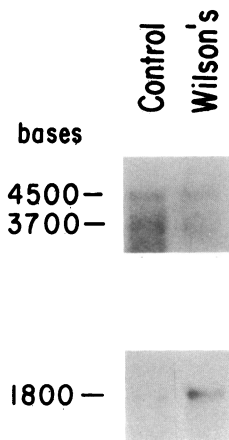


Figure 2. Representative autoradiograms of Northern blots when either a ceruloplasmin cDNA clone (*top*) or an albumin cDNA clone (*bottom*) were used as a probe. Equal amounts of poly (A⁺) RNA from a Wilson's disease patient and a control were denatured, electrophoresed, transferred to a matrix, hybridized with a [32 P]-labeled cDNA probe, and autoradiography performed as described in Methods.

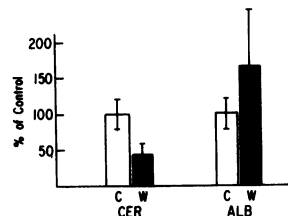


Figure 4. Bar graph summary of the results of nuclear run-on assays comparing the amounts of ceruloplasmin (*CER*) and albumin (*ALB*) gene transcription in controls (*C*) and patients with Wilson's disease (*W*). The control values are the means \pm SEM from three patients, whereas data for

Wilson's disease patients was taken from four patients. Densitometry was done on autoradiograms of nuclear run-on assays as described in Methods. Transcriptional rates for each gene are expressed as a percentage of the control values.

lated that a single gene mutation results both in ceruloplasmin deficiency and in the impairment in hepatic copper excretion. However, it has never been shown that ceruloplasmin is the principal gene product of a normal allele of the Wilson's disease gene. The fact that not all of the patients with this recessive disorder have abnormal ceruloplasmin levels speaks against ceruloplasmin deficiency being the primary defect. Recently Frydman et al. (23, 24) examined 27 serum markers in three families and found a linkage of a putative Wilson's disease gene to the esterase D locus on chromosome 13, whereas the ceruloplasmin gene is on chromosome 3. Other mechanisms proposed for the abnormal copper metabolism in Wilson's disease have included production of an abnormal copper-binding protein (25), mutation of a controller gene resulting in preservation of the fetal mode of copper metabolism (26), and abnormality in or absence of a hypothetical carrier protein required for both the biliary excretion of copper and for its incorporation into ceruloplasmin (3).

Our findings of both decreased steady-state ceruloplasmin mRNA levels and a reduced rate of gene transcription are consistent with four possible mechanisms by which ceruloplasmin deficiency may occur in Wilson's disease. First, and least likely given the results of Frydman et al. (23), the primary defect could be in the regulatory region or structure of the ceruloplasmin gene. Such a defect could then manifest itself as decreased ceruloplasmin gene transcription. Second, a mutation in another gene, such as one on chromosome 13, may result in production of a "transacting" factor that interacts with the ceruloplasmin regulatory area and inhibits its transcription. Third, the production of an abnormal protein that competes for copper could conceivably decrease ceruloplasmin transcription because copper may be needed to stimulate the transcription of this gene in the same way that several metals induce the expression of the metallothionein gene (27). Fourth, a defect in a carrier protein that presents copper for incorporation into apoceruloplasmin would be consistent with our results if apoceruloplasmin cannot exit from the cell and its increased intracellular concentration results in feedback inhibition of apoceruloplasmin transcription. Such a defect would explain our experimental findings and also provide an explanation for the absent serum ceruloplasmin levels found in some patients.

Our results seem to indicate more than a simple transcriptional defect in ceruloplasmin gene expression in Wilson's disease. The presence of ceruloplasmin mRNA in one patient with no detectable serum ceruloplasmin, along with a greater diminution in the serum protein values as compared with the steady-state mRNA levels suggest a translational or post-translational effect on ceruloplasmin gene regulation. Further analysis is necessary to better characterize the mechanisms responsible for the ceruloplasmin deficiency seen in Wilson's disease, for it appears likely that this deficiency may be significant in the pathogenesis of the disease. This study gives a direction to such an analysis by demonstrating that diminished ceruloplasmin transcription is at least partially responsible for the decreased serum ceruloplasmin levels in patients with Wilson's disease.

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