Caeruloplasmin isoforms in Wilson’s disease in neonates

G F E Chowrimootoo, H Scowcroft, Carol A Seymour

Abstract

**Aim**—To investigate the neonatal diagnosis of Wilson’s disease from caeruloplasmin isoforms in cord blood.

**Methods**—Serum caeruloplasmin isoforms were measured in 5–10 ml cord blood from 10 fresh umbilical cords using sodium dodecyl polyacrylamide gel electrophoresis (SDS PAGE) and western blotting and analysed by densitometry. Total caeruloplasmin concentrations were determined by nephelometry and caeruloplasmin oxidase by p-nitrophenyldiamine.

**Results**—Although total caeruloplasmin concentrations are reduced in neonates, the plasma isoform was significantly reduced or absent in patients with Wilson’s disease. Sera from healthy neonates and from those with Wilson’s disease had reduced biliary isoforms.

**Conclusion**—Identification of caeruloplasmin isoforms may be a marker for Wilson’s disease in neonates.

(Arch Dis Child Fetal Neonatal Ed 1998;79:F198–F201)

Keywords: caeruloplasmin isoforms; Wilson’s disease; copper excretion

Although copper is an essential trace metal in the human body, and a component of many enzyme systems, dietary copper intake exceeds requirements. Copper homeostasis is controlled by biliary excretion of copper into the bile rather than by intestinal absorption.1 Wilson’s disease is an inherited disorder of copper metabolism, with impairment of biliary copper excretion, resulting in copper accumulation in the liver and consequent damage. There is also a variable reduction in, or absence of, circulating caeruloplasmin, the major copper transporting protein in plasma, which has until recently been difficult to explain in terms of the pathophysiology of this disease.2

Analysis of the Wilson’s disease gene now mapped to chromosome 13q 14.3, has shown a 54% homology with the ATP7A gene that is associated with the genetic copper deficiency disorder, Menke’s disease. Thus there may be common copper transporting proteins. The association between the membrane bound copper proteins and the circulating transporter, caeruloplasmin, is now of interest.

Fetal copper metabolism is different from that of the adult,3, 4 with the fetal liver tolerating up to 20 times the adult liver copper concentration without damage. This apparent similarity to Wilson’s disease has led to the suggestion that in this disease there is a failure to change from the neonatal mode of copper metabolism to the adult mechanism.5, 6 In both Wilson’s disease and the normal fetus biliary copper excretion is greatly reduced, with low plasma copper and absent or low plasma caeruloplasmin concentrations. Hepatic copper accumulation in both situations seems to result from the biliary rather than altered plasma caeruloplasmin concentration as hereditary aceruloplasminaemia is not associated with hepatic copper accumulation or liver damage.2

Caeruloplasmin, a single chain glycoprotein (132 kiloDaltons) synthesised in the liver, is found in the $\alpha$-globulin fraction of mammalian plasma. Caeruloplasmin oxidase is a measure of its functional activity as an antioxidant. Irrespective of a reduction in, or absence of, circulating caeruloplasmin, this protein is synthesised in the liver in the normal way in Wilson’s disease, and there are two molecular isoforms,7 one predominating in bile (125 kiloDaltons) and the other in plasma (132 kiloDaltons). The biliary form is always absent in Wilson’s disease bile and may be important in copper excretion. Iyengaar et al8 first noted an absence of cross reacting material to caeruloplasmin antibodies in Wilson’s disease bile, while the bile of normal subjects contained enough copper in caeruloplasmin molecules to account for copper balance regulation. They proposed that the Wilson’s gene may concomitantly affect the appearance of caeruloplasmin in the blood and into the bile, thus simultaneously accounting for both defects. However, the precise mechanism by which the caeruloplasmin isoforms are transported to plasma or to bile is still uncertain. Our studies suggest that caeruloplasmin with its copper transporting function may be involved in biliary copper excretion, by binding to the putative copper transporter protein, which is defective in Wilson’s disease.9

**Methods**

Cord blood (5–10 ml) was obtained from 10 fresh umbilical cords (full term gestation) using sterile 27 mm gauge needles. Blood was placed immediately in a sterile tube (without additives) and allowed to clot at 4 °C, before centrifuging for 15 minutes at 6000 rpm on a bench centrifuge. Other venous blood samples were collected from healthy subjects (age range 25–42 years) or known Wilson’s disease patients (age range 28–47 years) using standard procedures during routine clinical investigations. All samples were stored at −20°C for up to three months. These studies had the approval of the local ethics committee.

Bis/Acrylamide for SDS-PAGE was purchased premixed as Protogel from National Diagnostics. Affinity purified polyclonal sheep
anti-human caeruloplasmin antibody (peroxidase conjugate) was obtained from Serotec, Oxford. Hybond-C Super and enhanced chemiluminescence (ECL) western blotting detection reagents were provided by Amersham International, Hertfordshire, UK. All other chemicals were from either Sigma, Poole, Dorset, or BDH, Dagenham, Essex, UK.

Serum samples were subjected to 7.5% SDS-PAGE, as described before.10 The gel was blotted on to a Hybond-C super membrane using a semi-dry blotter (BioRad Transblot-SD) for 90 minutes at constant current of 1 mA/cm².

The blot was blocked in 5% milk solution (containing 50 mM Tris/150 mM NaCl and 0.02% NaN₃ pH 7.4) for 2 hours and incubated for 3 hours with horseradish peroxidase-conjugated sheep anti-human caeruloplasmin. The blot was washed several times in Tris/NaCl buffer and caeruloplasmin was detected using the ECL method described by Amersham. Serum caeruloplasmin concentrations were measured on all samples using a standard laboratory nephelometric method.11

For densitometric analysis, blots were scanned on a Microtek ScanMaker IISP scanner and analysed using NIH image Blots 1.52 (from National Institute of Health Research Services Branch, Bethesda, MD), as described before.12 Caeruloplasmin oxidase activity was calculated in terms of oxidase units, where one oxidase unit = (Abs₄₀min - Abs₁₀min) × 1000.

Results

As expected both molecular forms of caeruloplasmin (132 kDa and 125 kDa) were present in sera from normal adult subjects (fig 1, lanes 2–4). By comparison, cord samples showed substantial amounts of 132 kDa caeruloplasmin (fig 1, lanes 5–7) similar to adults but with a greatly reduced 125 kDa caeruloplasmin expression and a profile similar to that of sera of patients with Wilson’s disease (fig 1, lanes 8–9). Densitometric analysis of these western blots is summarised in table 1.

Table 1. Densitometric analysis of molecular forms of caeruloplasmin in serum samples from normal adult cord and Wilson’s disease samples

<table>
<thead>
<tr>
<th>Molecular forms of caeruloplasmin</th>
<th>Normal adult</th>
<th>Cord samples</th>
<th>Wilson’s disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>132 kDa</td>
<td>171.6 (6.5)</td>
<td>127.8 (8.3)</td>
<td>19.8 (17.7)</td>
</tr>
<tr>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>161.2–179.5</td>
<td>117–139.1</td>
<td>0.3–41.9</td>
<td></td>
</tr>
<tr>
<td>125 kDa</td>
<td>159.8 (14.4)</td>
<td>47.6 (26.8)</td>
<td>2.1 (17.7)</td>
</tr>
<tr>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>137.5–170.7</td>
<td>19.5–90.1</td>
<td>0–8.1</td>
<td></td>
</tr>
</tbody>
</table>

Molecular forms of caeruloplasmin are shown in OD units in sera from normal controls, cord blood and Wilson’s disease samples. Densitometry was as previously described.11 Data are shown as mean (SEM), with number of samples and range below.

Table 2. Caeruloplasmin oxidase activity in normal adult cord and Wilson’s disease serum samples

<table>
<thead>
<tr>
<th>Sample (number)</th>
<th>Average oxidase activity (units*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (4)</td>
<td>210.8 ± 49.3</td>
</tr>
<tr>
<td>Cord blood (9)</td>
<td>187.4 ± 26.0</td>
</tr>
<tr>
<td>Wilson’s disease (4)</td>
<td>7.8 ± 2.8</td>
</tr>
</tbody>
</table>

* Caeruloplasmin oxidase activity is expressed as units/2 mg protein.
In summary, using western blotting of cord blood, we have shown that the reduced plasma caeruloplasmin in neonates is due to reduced expression of biliary (125 kiloDalton) caeruloplasmin which is also defective in Wilson’s disease. In both situations hepatic copper accumulation occurs which is later compensated in neonates without Wilson’s disease in the six months after birth by synthesis of caeruloplasmin and unloading of hepatic copper.

The presence of oxidase activity in umbilical cord sera and impairment of biliary copper excretion in neonates further supports the original proposal of Iyengaar et al and our concepts that caeruloplasmin has a major role in biliary copper excretion. It also supports our finding that caeruloplasmin in Wilson’s disease incorporates copper normally. The clear difference in expression of caeruloplasmin between cord and Wilson’s disease blood samples by western blotting may be useful in the diagnosis of Wilson’s disease in the first 3 to 6 months after birth, where we would expect absent or undetectable concentrations of both isoforms. This would be feasible as western blotting requires only a few microlitres of serum and could be made even more user friendly by extraction from filter paper, thus avoiding previous sampling and diagnostic problems. This finding is being developed further using Guthrie card blood spots to pick up pre-symptomatic Wilson’s disease. This would enable the diagnosis of Wilson’s disease to be made before substantial tissue damage.

### Table 3 Caeruloplasmin measurement in cord and normal adult serum

<table>
<thead>
<tr>
<th>Sample (number)</th>
<th>Mean caeruloplasmin (g/l) (SEM)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord (9)</td>
<td>0.152 (0.02)</td>
<td>0.09-0.27</td>
</tr>
<tr>
<td>Normal adult (7)</td>
<td>0.264 (0.02)</td>
<td>0.20-0.35</td>
</tr>
</tbody>
</table>

In previous studies, it is noted that caeruloplasmin in Wilson’s disease is most likely to involve biliary caeruloplasmin (transporting copper) bound in some way to the ATP7B copper transporter. A reduction in caeruloplasmin that was previously noted in neonatal serum, and in this study (table 3), is attributed predominantly to a lack of the 125 kiloDalton isoform. In the past it has been difficult to detect Wilson’s disease in neonates, as total plasma copper and caeruloplasmin are both reduced in neonates. The clear difference in expression of the 132 kiloDalton caeruloplasmin isoform in cord blood and Wilson’s disease samples, as shown by western blotting (fig 1 and table 2), could be used to distinguish Wilson’s disease in the neonatal period. Early diagnosis of Wilson’s disease would be likely to prevent complications such as haemolysis, cirrhosis, and permanent brain damage, because treatment with a chelating agent to remove copper could be commenced before any tissue damage.

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We thank Professor George Brewer, Department of Human Genetics, University of Michigan, for helpful comments, Miss Caroline Cooper for typing the manuscript, and South Thames Health Authority Research and Development Grants (CAS), for their support for this work.

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Arch Dis Child Fetal Neonatal Ed 1998 79: F198-F201
doi: 10.1136/fn.79.3.F198

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