Prenatal and Postnatal Diagnosis of Diseases of Copper Metabolism

WAI-YEE CHAN, Ph.D. and OWEN M. RENNERT, M.D.

Departments of Pediatrics, Biochemistry and Molecular Biology,
University of Oklahoma Health Sciences Center,
Oklahoma City, OK 73190

ABSTRACT

Menkes' kinky hair disease can be successfully diagnosed both prenatally and postnatally using cultured skin fibroblasts derived from the patient or amniotic fluid cells from the affected fetus. Determination of intracellular copper concentration under normal and copper loaded conditions, as well as examination of the kinetics of copper retention may be necessary for diagnosis. At present, cell culture techniques have been proven to be applicable for postnatal diagnosis of Wilson's disease. More investigation is necessary to determine whether or not the present method for prenatal diagnosis of this disease is possible.

There are two well established genetic metabolic disorders of copper, namely Menkes' Kinky Hair Disease (Trichopoliodystrophy) and Wilson's Disease (Hepatolenticular Degeneration). Menkes' disease is caused by defective absorption and defective utilization of copper resulting in copper deficiency. Wilson's disease, on the other hand, is caused by excessive deposition of copper in organ systems resulting in copper toxicosis.

Menkes' Kinky Hair Disease

CLINICAL ASPECTS

Menkes' disease is a sex-linked recessive disease first described in 1962 in a series of related male infants. Patients have distinctive facial appearance, characteristic spirally-twisted hair (pili torti) and a rapidly progressive central nervous system degeneration that begins during the first four to six weeks of life. Symptoms of this disorder include failure to thrive, infantile seizures, profound psychomotor retardation, hypothermia, and inanition. The life expectancy of affected infants is four years or less. Recently, Henkin and Grover reported clinical heterogeneity among the Menkes' patients with a less severe form identified as having normal serum dopamine-β-hydroxylase. This less severe form of Menkes' disease has been observed in other institutions including the authors'
in institution. This disease was initially described in Caucasians; however, reports documented its occurrence also in blacks and orientals.

In 1972, Danks et al reported hypocupremia, hypoceruloplasminemia, and depressed tissue copper level in infants with Menkes' disease. Their observation of increased intestinal mucosal copper levels and characteristic metachromasia in primary fibroblast explants suggested a generalized defect in membrane copper transport and that affected infants might be treated parenterally. Subsequent attempts to treat affected infants with parenteral copper (inorganic salts or organic complexes) proved to be of no objective clinical benefit even though there are reports of partially successful treatment.

The poor clinical response to copper infusion therapy has been attributed to the presence of irreversible damage at the time of institution of treatment. Molekaer demonstrated the presence of characteristic hair abnormalities in an infant during the first day of life. Heydorn et al have demonstrated the abnormal accumulation of copper in multiple tissues of an abortus with kinky hair syndrome. This observation is substantiated by subsequent reports and by the observations of Grover et al of increased amniotic fluid copper, increased serum copper, excess urinary copper but decreased liver copper and serum dopamine β-hydroxylase levels in one and three day old neonates.

**DIAGNOSIS**

In table I are shown the laboratory findings in Menkes' disease patients. Decreased brain and liver copper content and increased kidney and intestinal mucosal copper content are pertinent findings in Menkes' disease patients. Decreased urinary and serum copper and serum ceruloplasmin levels are easily detectable. However, these findings are not specific for Menkes' disease. Hypocupremia and hypoceruloplasminemia are demonstrable in a number of disease states. Besides their limited specificity, these tests can only be performed postnatally. Since there is no successful therapy for Menkes' disease and its clinical course is irreversible, a test to detect the disorder early or preferably prenatally is desirable. Recent research has led to the development of a tissue culture method which can be used for both prenatal and postnatal diagnosis of Menkes' disease.

Cultured skin fibroblasts derived from Menkes' disease patients exhibited abnormal copper metabolism. These ob-

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Laboratory Data in Menkes' Kinky Hair Disease</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Normal</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum copper</td>
<td>23.37 ± 10.17* µg/100 ml</td>
</tr>
<tr>
<td>Serum ceruloplasmin</td>
<td>6.17 ± 3.07 mg/100 ml</td>
</tr>
<tr>
<td>Urinary copper</td>
<td>18.4 ± 4.16 µg/24 hr.</td>
</tr>
<tr>
<td>Intestinal mucosal copper</td>
<td>57.22 ± 12.67 µg/g dry weight</td>
</tr>
<tr>
<td>Liver copper</td>
<td>13.5 ± 5.17 µg/g dry weight</td>
</tr>
<tr>
<td>Brain copper</td>
<td>0.75 ± 0.07 µg/g wet weight</td>
</tr>
<tr>
<td>Kidney copper</td>
<td>37.1 ± 19.85 µg/g wet weight</td>
</tr>
</tbody>
</table>

*Means ± S.D. calculated from data obtained from references.*
servations have been reported by other laboratories. In table II are shown the intracellular copper content of fibroblast cultures derived from Menkes' patients, heterozygotes, and normal controls (ng copper per mg soluble cell protein) obtained in our laboratory. Cultures with passage number between six to 13 were used. Confluent cultures were kept at 37°C for at least four days before intracellular copper content was determined. Higher cellular copper concentrations were observed when the cells were lysed by sonication* as contrasted to cell disruption by repeated, rapid freezing and thawing (up to seven cycles). The intracellular copper level is markedly influenced by the culture medium in which the cells have been grown. In table II are presented results obtained when cells were cultured in Dulbecco's modified Eagle's medium enriched with 20 percent fetal bovine serum. Higher intracellular copper levels were obtained when cultures were grown in Eagle's minimum essential medium with 20 percent fetal bovine serum. This effect of culture medium on cellular copper content also has been reported by Goka et al. It is advisable to confirm the difference in intracellular copper level between a normal and an unknown cell line by growing the cultures in medium enriched with copper. The difference in cellular copper content between Menkes' cells and normals is more pronounced when the cells are stressed with copper below the toxic level. In table II are shown the effect of the addition of 6 μg of copper per ml of medium on the intracellular copper content of Menkes' disease and normal control cells. This procedure is useful when the patient cell line does not show as big a difference from normal as expected.

Heterozygous carrier fibroblast cultures always demonstrate intermediate intracellular copper content as contrasted to normal and homozygous patients (table II). Heterozygous fibroblast cultures contain two clonal populations, one of the normal phenotype and one of the Menkes' disease phenotype. Each cloned culture exhibited the appropriate intracellular copper content. The abnormal kinetics of copper metabolism of heterozygous cultures is accounted for by the two clonal populations. These data indicate that the abnormal intracellular copper level in Menkes' disease fibroblasts represents expression of the genetic defect by cultured fibroblasts.

Cultured skin fibroblasts from Menkes' disease patients or amniotic fluid cells from affected fetus have been reported to show abnormal copper uptake kinetic. This observation has been disputed by some investigators. The consistent abnormality of copper metabolism kinetics in cultured fibroblasts is retention by or efflux from the cells. Menkes' disease fibroblasts have increased retention or decreased efflux of copper when examined in pulse-chase experiments. The experiment is performed by pulse labelling cultures for 16 to 24 hours in media enriched with copper-64. After the pulse labelling period, the cultures were washed with prewarmed media containing no added copper and incubated with standard culture medium for various time periods. Cells were harvested and the retained intracellular copper-64 (copper-64 radioactivity per mg cell protein) was ex-

---

* Branson Cell Disruptor 200.

**TABLE II**

Intracellular Copper Content of Fibroblast Cultures (ng/mg Cell Protein)

<table>
<thead>
<tr>
<th>N</th>
<th>Basal Media*</th>
<th>Media with 6 μg Cu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control 4</td>
<td>10.55 ± 4.30†</td>
<td>67.10 ± 2.31</td>
</tr>
<tr>
<td>Heterozygote 2</td>
<td>22.5 ± 2.95</td>
<td>97.10 ± 2.50</td>
</tr>
<tr>
<td>Menkes' disease 3</td>
<td>36.14 ± 1.06</td>
<td>338.95 ± 1.08</td>
</tr>
</tbody>
</table>

*Culture medium is Dulbecco's modified Eagle's medium with HEPES buffer and 20 percent FBS.
†Means ± S.D.
pressed as the percentage of intracellular copper-64 observed at the beginning of the chase. The results of a representative experiment are presented in figure 1. Menkes’ disease cultures always retain more copper than controls. Heterozygous cultures have an intermediate retention curve. This is a definitive test for Menkes’ disease.

Prenatal diagnosis of Menkes’ disease has been reported using the cell culture method. Cultured amniotic fluid cells demonstrated an elevated intracellular copper level in amniotic cells from Menkes’ disease fetuses. In table III are presented the copper levels of amniotic fluid cell cultures from normal and affected fetuses. Abnormal kinetics of copper metabolism has been observed in amniotic fluid cell cultures. Aborted fetus diagnosed by the cell culture method showed abnormal tissue distribution of copper confirming the validity of this method for prenatal diagnosis of Menkes’ disease. Abnormal amniotic fluid copper level was reported in a pregnancy with a fetus affected with Menkes’ disease. Whether or not this can be used for diagnosis of this disease needs further confirmation.

Thus, Menkes’ disease can be diagnosed both prenatally and postnatally by examining the cellular concentration and the kinetics of retention of copper in cultured skin fibroblasts or amniotic fluid cells. Whether or not amniotic fluid copper concentration is also useful as a diagnostic index for Menkes’ disease needs further confirmation.

Wilson’s Disease

CLINICAL ASPECTS

Wilson’s disease was recognized as a clinical entity in 1912 by Wilson. It is inherited in an autosomal recessive mode. Clinical and biochemical studies indicate that the disorder might be genetically heterogeneous. The classical form of the disease is characterized by degenerative changes in the brain, particularly in the basal ganglia, chronic liver disease, functional renal disturbances, and the Kayser-Fleischer ring of the cornea. However, patients manifesting all these characteristics are rare. The patients suffer from the deleterious effects of excess tissue copper. Laboratory findings include

![Figure 1. Retention of copper-64 radioactivity by normal, heterozygous and Menkes' disease cultured fibroblasts.](image)

**TABLE III**

Intracellular Copper Content of Amniotic Fluid Cell Cultures

<table>
<thead>
<tr>
<th></th>
<th>ng Cu/mg Cell Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>46,XY - Controls</td>
<td>53</td>
</tr>
<tr>
<td>46,XY - Affected</td>
<td>10</td>
</tr>
<tr>
<td>46,XY - Unaffected</td>
<td>14</td>
</tr>
</tbody>
</table>

*All data from Horn, N. and Heydon, K.: Prenatal detection of Menkes' disease and copper distribution in affected fetuses. Excerpta Medica Int'l Congress Series, 426:56-57, 1977. Control cultures were obtained from normal individuals. Affected and unaffected cultures were derived from at-risk pregnancies from families with a history of Menkes' disease.
excess accumulation of copper in liver, brain, kidney, and cornea, but with low serum ceruloplasmin levels, elevated non-ceruloplasmin bound copper, increased urinary copper, and decreased fecal copper. As the disease progresses, the most significant observations are the slowing down of copper clearance from blood and the hepatic uptake of copper, as well as the gradual increase of non-ceruloplasmin plasma copper and urinary copper.

The age of onset of symptoms of Wilson's disease is highly variable. Even though symptoms may appear as early as four years of age, most patients develop symptoms in adolescence or early adulthood. The recognition of the homozygous abnormal patients during the presymptomatic phase is difficult. This is due partly to the variable and nonspecific mode of presentation and clinical course of Wilson's disease in children. During this state, many of the characteristic biochemical abnormalities are absent or not fully developed. Some of the characteristic biochemical features may be present in heterozygous carriers. Thus, the diagnosis of this disorder in presymptomatic pediatric patients is difficult.

There are no clinical phenotypic characteristics of the heterozygote for Wilson's disease. Measurement of serum copper and ceruloplasmin as well as urinary copper is found to be inadequate for detecting the heterozygous state. Besides kinetic measurement of administered radioactive copper-64, whole body counting method, or liver biopsy, there is no simple, direct, non-invasive test whereby the diagnosis of the Wilson's disease carrier state can be made safely and reliably.

Wilson's disease is fatal if not treated. However, administration of a copper chelating agent such as D-penicillamine can alleviate all the symptoms observable in Wilson's disease. This treatment is most effective if the drugs are administered early. Delayed treatment, usually the result of delayed diagnosis, can cause irreversible neurological damage. Thus, there is a need for a simple, specific non-invasive method whereby homozygous patients with Wilson's disease can be diagnosed early in life, preferably prenatally.

**Diagnosis**

The existing laboratory diagnostic methods for Wilson's disease are far from perfect. Presence of Kayser-Fleischer corneal rings and elevated hepatic copper are present in other forms of liver disease besides Wilson's disease. Kayser-Fleischer-like rings have been reported to be present in patients with progressive intrahepatic cholestasis of infancy and childhood, primary biliary cirrhosis, and chronic active hepatitis with cirrhosis. Increased liver copper accumulation have been found in primary biliary cirrhosis, prolonged large duct biliary obstruction, chronic active liver disease, cirrhosis associated with $a$-1-antitrypsin deficiency, and several other forms of cirrhosis. Measurement of serum copper and ceruloplasmin and urinary copper do not always permit unequivocal diagnosis. Examination of handling of radiocopper suffers from the disadvantages of requiring sophisticated instrumentations as well as the danger of exposing the patient to copper-64 or copper-67, which are strong gamma emitters. Recent research in Wilson's disease has led to the development of an in vitro diagnostic method which might be applicable both prenatally and postnatally.

Skin fibroblasts derived from both symptomatic and presymptomatic Wilson's disease patients show significantly elevated intracellular copper concentration when cultured in Eagle's minimum essential medium supplemented with 20 percent fetal bovine serum. The results are presented in table IV. In basal culture medium, the intracellular copper concentration of Wilson's disease fibroblasts was about three times that of normal. This difference between mutant and normal cells
was maintained when the medium was supplemented with copper at medium concentration of 10 μg of copper per ml (table IV). This abnormal behavior of Wilson's disease cultured fibroblast was first suspected by Goka et al.31 and was proven by our laboratory.10,12 Our observations have recently been confirmed by another laboratory.8 Even though Wilson's disease cultured fibroblasts showed elevated intracellular copper when cultured in Eagle's minimum essential medium, the difference between the mutant and normal cells diminished when Dulbecco's modified Eagle's medium was used as the culture medium. Under this circumstance, the mean intracellular copper concentration of Wilson's disease cells was still higher than that of normal cells (8.81 ng copper per mg of cell protein compared with 4.74 ng copper per mg of cell protein in normal cells), but there was significant overlap between the two types of cells.13

In this respect, Wilson's disease cultured fibroblasts behave differently from Menkes' disease cells. Fibroblasts derived from patients with other liver disease, such as primary biliary cirrhosis which resembles Wilson's disease clinically to a certain extent,63 do not show elevated intracellular copper concentration when cultured in Eagle's minimum essential medium.* Thus, elevated intracellular copper content of Wilson's disease cultured cells appears to be a phenotypic expression of the abnormal gene in this disorder.74,76 Fibroblast cultures derived from heterozygous carriers of Wilson's disease have been examined. Intermediate intracellular copper concentration between that of normal and homozygous abnormal cultures was observed. However, owing to the fact that only one heterozygous carrier has been tested, further confirmation is required. Wilson's disease is genetically heterogenous.2,15 Whether or not there is any difference in the intracellular copper concentration between the different genetic class of Wilson's disease also needs to be clarified.

Wilson's disease cultured fibroblasts also differ from Menkes' disease cells with respect to the kinetics of copper uptake. In figure 2 is shown the result of studying copper-64 uptake in cultured skin fibroblasts from patients with Wilson's disease.13 Wilson's disease cultures incorporated significantly less copper than normal control cultures for the first three

---

---

TABLE IV
Intracellular Copper Content of Culture Fibroblasts Derived from Wilson's Patients (ng/mg Cell Protein)

<table>
<thead>
<tr>
<th></th>
<th>N Basal Media*</th>
<th>Media with 10 μg Cu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>11 98.5 ± 19.2</td>
<td>259.2 ± 76.7</td>
</tr>
<tr>
<td>Wilson's disease</td>
<td>7 276.2 ± 21.4</td>
<td>703.0 ± 83.5</td>
</tr>
</tbody>
</table>

*Culture medium is Eagle's minimum essential medium with Hanks buffer and 20 percent FBS.†Means ± S.D.

---

---

* Personal observation.
hours of the experiment. The incorporation of copper reached a maximum at the third hour in normal controls and subsequently declined. Wilson's disease fibroblasts did not achieve maximum copper incorporation before the end of the experimental period (four hours). At the fourth hour, the amount of copper incorporated by Wilson's disease and normal fibroblasts was comparable. Retention of copper by normal and Wilson's disease cells, on the other hand, was indistinguishable at all times.13 This observation has been confirmed by Camakaris et al.8 Thus, Wilson's disease cultured fibroblasts exhibit abnormality only in uptake of copper and, in this respect, is different from Menkes' disease cultures.

Even though further experiments are required to establish the validity of cultured skin fibroblasts as an in vitro system for Wilson's disease, it appears that this method provides a simpler, more specific, and noninvasive way of diagnosing this disorder. No prenatal diagnosis using the cell culture system has been tried.

Discussion and Conclusion

Cultured skin fibroblasts or amniotic fluid cells can be used for both prenatal and postnatal diagnosis of Menkes' kinky hair disease. To validate the diagnosis, the intracellular copper concentration should be determined under normal and copper loaded conditions, as well as studying the kinetics of copper retention. Owing to the occurrence of a milder form of the disease,17,18 further work is necessary to identify whether or not there is any difference in copper metabolism in cultured cells derived from this milder variant and the severe type. Determination of amniotic fluid copper concentration may be a simpler and more convenient way of diagnosing the disease. However, this observation needs to be confirmed and the copper concentration of amniotic fluid from various diseases needs to be examined to establish the uniqueness of elevated copper level in amniotic fluid in Menkes' disease. The cell culture system is useful as a simpler, more specific and non-invasive method for diagnosing Wilson's disease postnatally. More work is required to extend the present method for prenatal diagnosis of the disease. Owing to the genetic heterogeneity of the disease, the similarity and difference in the metabolism of copper by fibroblast cultures among the various genetic classes must be studied. This is the prerequisite for firmly establishing the cell culture method as a diagnostic tool for this disease.

Acknowledgments

Thanks are extended to Mrs. Isabelle Lehman for typing this manuscript and Mrs. LeAnn Tease for her excellent technical assistance. This work is supported in part by a grant C11-03601 from NIH and a University of Oklahoma College of Medicine Grant Award to W.Y.C.

References


