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Genetic Expression of Wilson's Disease in Cell Culture: A Diagnostic Marker

Abstract. *Wilson's disease fibroblasts have an elevated intracellular copper concentration as compared to cultured control cells. A decreased ratio of copper to protein was observed in cytoplasmic protein (or proteins) having a molecular weight ≥ 30,000 in Wilson's disease cells. The results of this culture study indicate its potential importance in the early unequivocal diagnosis of this disorder.*

Wilson's disease (hepatolenticular degeneration) is an autosomal recessively inherited metabolic dysfunction of copper metabolism (*1*). It is caused by excessive deposition of copper in various organs and, if untreated, the disease is invariably fatal. However, all the clinical manifestations can be prevented if the disease is diagnosed before functional impairment occurs (*2*). The onset of symptoms is usually in late childhood or early adulthood. The classical symptoms are Kayser-Fleischer rings, neurologic dysfunction, liver cirrhosis, and hypoceruloplasminemia. These symptoms may be absent in childhood. This, coupled with the absence of a simple noninvasive test, make early diagnosis of this disorder difficult (*3*).

The basic biochemical defect of Wilson's disease is still unclear. Abnormalities of the structure and metabolism of ceruloplasmin have been suggested (*4*). However, no confirmatory evidence has been found. Several investigators have suggested that metallothionein from the liver of patients with Wilson's disease has an abnormally high copper affinity (*5*); others have isolated a low-molecular-weight copper binding protein and found it to have a different quantity of bound copper as compared to normal liver (*6*). We now report that the genetic abnormality of Wilson's disease is expressed in cultured skin fibroblasts derived from these patients. Thus, cultured fibroblasts may serve (i) as an in vitro model for studying the pathogenesis of this disorder and (ii) as a noninvasive method for early diagnosis.

Skin fibroblast cultures were established from biopsies of typical Wilson's disease patients. Additional cultures were obtained from the Human Genetic Mutant Cell Repository (GM 32, 33). Cell cultures from individuals matched according to age and sex were used as controls. Both patients and controls included males and females ranging in age from 9 to 20 years. Cells were grown in 75-cm^2 plastic culture flasks containing 12 ml of Eagle's minimum essential medium with Hanks balanced salt solution, fetal calf serum (10 percent), and antibiotic-antimycotic (1 percent) solution and adjusted to *p*H 7.2 with 7.5 percent sodium bicarbonate solution. The cultures were incubated in a humidified environment of 5 percent carbon dioxide at 37°C. Only confluent cultures with a passage number between 6 to 13 were used. Confluent cultures were harvested with a rubber policeman, and the cells were suspended with 5 ml of deionized distilled water. Cells were lysed by rapidly freezing and thawing the suspension five times in Dry Ice and acetone. The lysate was centrifuged at 10,000*g* for 20 minutes. The supernatant was assayed for copper and cadmium with a Perkin-Elmer model 703 atomic absorption spectrophotometer equipped with a Perkin-Elmer model HGA-500 graphite furnace (*7*). Protein concentration of the lysates was determined by the method of Lowry *et al.* (*8*).

Table 1. Intracellular content of copper and cadmium in cultured fibroblasts. The results are expressed in nanograms (means ± standard deviation) of metal per milligram of soluble cell protein. The numbers in parentheses denote the number of different cultures (each from a different patient or control) tested.

Fibroblasts	Copper	Cadmium
Control (8)	101 ± 17	21.2 ± 5.0
Wilson's disease (7)	280 ± 19	20.5 ± 3.1

Wilson's disease cultured fibroblasts were morphologically normal and had a growth rate similar to control cells in basal medium. Completed media had a copper content of 25.4 ± 2.8 ng/ml and a cadmium content of 1.76 ± 0.14 ng/ml. The intracellular copper concentration of Wilson's disease cultured cells was significantly higher (approximately threefold) than that of normal cells (Table 1). The intracellular cadmium in both cell types was comparable. This observation indicates that the genetic abnormality of Wilson's disease, that is, elevated accumulation of copper, is expressed in cultured fibroblasts.

To examine the distribution of intracellular copper in Wilson's disease cultured cells, we fractionated cell lysates by gel filtration on a Sephadex G-50 column (2.5 by 60 cm) equilibrated with demineralized 0.02*M* tris-acetate (*p*H 8.6). Proteins were eluted from the column with the equilibrating buffer at a flow rate of 24 ml/hour. Proteins eluted were monitored by measuring the absorbance at 220 nm of the fractions (25 drops per fraction) with a Beckman ACTA III spectrophotometer. The copper concentration of the column fractions was determined by flameless atomic absorption spectroscopy at 324.7 nm. The fractionation procedures were carried out at 4°C (Fig. 1). A number of copper peaks are present in lysates from Wilson's disease cells but not in control lysates. These peaks had a very low absorbance at 220 nm. Only two copper peaks showed sig-

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nificant absorbance at 220 nm, namely, the void volume peak corresponding to proteins of molecular weight ≥ 30,000 and the wash peak. The amount of copper in the wash peak is increased in Wilson's cells, indicating that there is more free copper or copper bound to low-molecular-weight compounds in these cells. In the void volume peak, the ratio of copper to protein for the Wilson's disease cell lysate is 0.45 ng of copper per microgram of protein while that for controls is 2.04 ng/μg. This decrease in the copper-to-protein ratio in the void volume peak of Wilson's disease cell lysates may be due to a decreased quantity of bound copper or to increased protein concentration in the void volume region.

To further examine these postulations, we incubated cell lysates obtained from cells grown in nonradioactive medium with ^{64}Cu (New England Nuclear) at room temperature for 4 hours (*9*). After Sephadex G-50 chromatography, the radioactivity of each fraction was determined (Packard Auto-Gamma counter). The chromatograms are shown in Fig. 2. The large peaks of radioactive washings are due to excess ^{64}Cu not bound to protein. The ratio of radioactive copper to absorbance at 220 nm of the void volume peak is higher in the normal than in the Wilson's cells (Fig. 2). This confirmatory observation suggests decreased binding of exogenous copper by protein of molecular weight ≥ 30,000 in Wilson's disease cells.

The fact that, in Wilson's disease, the intracellular concentration of copper but not cadmium is elevated suggests that this alteration of copper metabolism is a specific phenomenon. Cultured fibroblasts derived from patients of another copper metabolic disease (Menkes' disease) also show an elevated accumulation of copper (*10–12*). The mechanism of increased copper content in Menkes' disease cells is due to impaired copper efflux (*11*), probably caused by defective metallothionein (*11–13*). Metallothionein isolated from liver of Wilson's disease patients has an increased copper affinity (*5*). Our experiments with Wilson's disease fibroblasts do not demonstrate any changes in the metallothionein. However, this peptide is present in only minute quantities in cultured cells. Our experimental conditions were not sensitive enough to detect chemical changes in metallothionein; the results, however, do show an alteration in the copper binding character of the void volume peak on Sephadex G-50 (molecular weight, ≥ 30,000). Thus, the elevated accumulation of copper in cells in the two disorders may be caused by different molecular mechanisms.

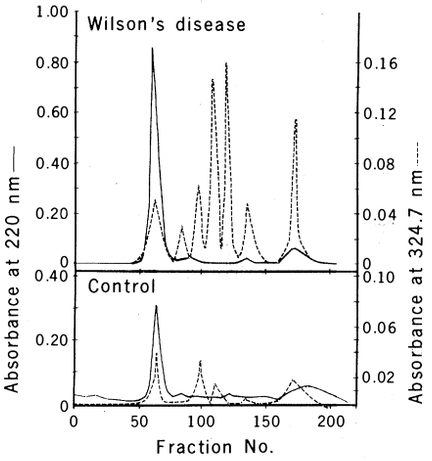

Fig. 1. Sephadex G-50 column chromatography of cell lysates. The gels were equilibrated with 0.02*M* tris-acetate (*p*H 8.6), and proteins were eluted at a flow rate of 25 ml/hour. Proteins were monitored with absorbance at 220 nm. Copper was monitored by flameless atomic absorption spectroscopy with absorbance at 324.7 nm.

Because of the limitation of patient materials, we have only studied skin fibroblasts derived from individuals ranging from 9 to 20 years of age. From the limited data (Table 1), it appears that copper accumulation is not a function of the donor's age in either patients or controls within the age span studied. In fact, the intracellular copper content of cultured cells is independent of the donor's age as indicated by the similarity of cellular copper content in our control subjects and in male infant controls (*11*) below 2 years of age. Whether the accumulation of copper in cultured fibroblasts of Wilson's disease patients is observable early in life or prenatally, however, is not clear.

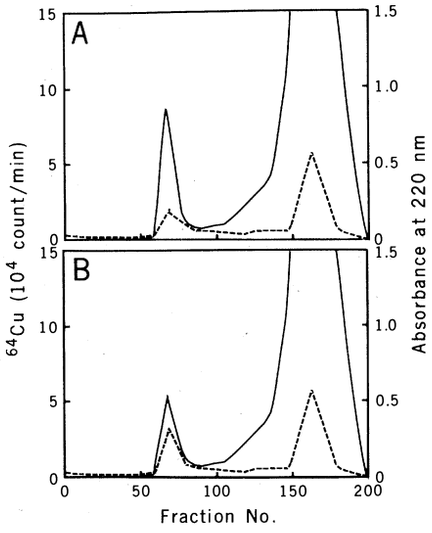

Fig. 2. Sephadex G-50 column chromatography of cell lysates after in vitro labeling with ^{64}Cu. Cell lysates were stirred with ^{64}Cu at room temperature for 4 hours and then fractionated. Column fractions were monitored for protein by absorbance at 220 nm (——) and for ^{64}Cu radioactivity (---). (A) Wilson's disease cell; (B) control cell.

Even though Wilson's disease has been known for nearly a century, no postulation about the basic biochemical defect has been proved. This is due, in part, to the relatively infrequent occurrence of the disorder and to the complexities of studying human subjects. Our results indicate that the biochemical abnormality of Wilson's disease is expressed in cultured fibroblasts derived from the patients. This abnormality provides the foundation for a simpler, controlled system for studying this disorder. Our data also suggest that cultured skin fibroblasts may be useful for prenatal or early diagnosis of the disease.

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