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Genetics

Menkes disease: A biochemical abnormality in cultured human fibroblasts

(copper/kinky-hair disease/X-linked inheritance)

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ABSTRACT **Cultured skin fibroblasts from patients with Menkes disease, an X-linked disorder involving a defect in copper metabolism, were analyzed for copper concentration by means of atomic absorption spectrophotometry. These cultures consistently exhibited elevated copper concentrations (mean = 335.5 ng of copper per mg of protein) when compared to control fibroblast cultures (mean = 59.2 ng of copper per mg of protein). External factors that could influence the copper content of cultures were found not to affect the differences in copper concentration between control and Menkes cells. Furthermore, Menkes cells could be differentiated from cultured fibroblasts of controls, of presumed heterozygotes, and of Wilson's disease patients by copper concentration. These observations led to the conclusion that the increased copper content of cultured Menkes cells was characteristic of Menkes disease, resulting from the expression of the genetic abnormality. This provides a genetic marker, a defect in metal metabolism demonstrated in human fibroblasts, that should prove valuable in both the diagnosis of Menkes disease and in the study of the fundamental defect of this genetic disorder.**

Menkes kinky-hair disease is an X-linked, recessively inherited disorder characterized by severe mental retardation, abnormalities in hair structure, bone and connective tissue degeneration, hypothermia, and low serum copper ceruloplasmin concentrations. These symptoms have been attributed to copper deficiency (1), since copper is known to have a role in the formation of myelin, keratin, melanin, elastin, collagen, and ascorbic acid metabolism as well as in electron transport (2). We report the discovery of a genetic marker in cultured skin fibroblasts from patients diagnosed with Menkes disease. The marker is expressed as increased copper concentrations in the Menkes cultures.

MATERIALS AND METHODS

Skin fibroblast cultures were established from biopsies of three typical Menkes patients utilizing methods previously described (3). An additional Menkes culture, GM 220/227, was obtained from the Human Genetic Mutant Cell Repository in Camden, N.J. The remaining Menkes cultures were obtained through the generosity of several colleagues. Control cultures established from both the cell repository and skin biopsies of individuals seen by our department were grown under conditions identical to those of the Menkes cultures. Although some of the control cultures were from patients with other genetic disorders, none were related to known abnormalities in copper metabolism.

Menkes and control cultures were incubated in 5% CO_2 at 37° in Eagle's minimum essential medium (GIBCO) supplemented with 15% fetal calf serum and antibiotics. Following three washes in isotonic buffer solutions of low and established copper content, harvested cells were suspended in deionized water and sonically disrupted. The protein content was measured on a Turner Fluorometer utilizing fluorescamine (Roche), similarly as in previous reports (4). Copper concentration was determined by atomic absorption spectrophotometry employing a carbon-rod atomizer (Perkin-Elmer model 403). The absorbance at 324.7 nm of 20 μl of samples were equated to copper concentrations of aqueous standards. Since the protein content is proportional to culture size, the copper concentration was related to the culture protein content to account for variations in culture size. This allowed comparison of copper content among different cultures, which was expressed as ng of copper per mg of protein.

RESULTS

The mean copper concentrations and 95% confidence limits of fibroblast cultures are presented in Table 1. These values represent the mean of the average concentration for each culture. The difference in copper concentration between Menkes and controls is characteristic for Menkes cells.

We investigated factors which could influence the difference in copper concentration between the Menkes and control cultures. The copper concentration of media and various

Table 1. Copper concentrations for fibroblast cultures

Culture	Copper concentrations (ng of copper per mg of protein)	
	Mean	Range
Menkes (n = 8)	335.5	230.7–440.3
Control (n = 29)	59.2	23.0–95.4
Amniotic (control) (n = 7)	25.2	10.0–40.8

Multiple measurements (at least three) of individual cultures were averaged. These means were then averaged in the appropriate category to obtain the means listed above. The absorbance at 324.7 nm for 20 μl of sonicated cell suspensions was measured on a Perkin-Elmer model 403 atomic absorption spectrophotometer equipped with a graphite furnace. The absorbance was converted to copper concentrations (ng of copper per ml) by relating to aqueous standard solutions (0–600 ng of Cu per ml). The copper concentrations were then divided by the protein concentrations of the cultures, measured fluorometrically with fluorescamine (Roche), to allow for comparative analyses among cultures. This gave the copper concentration in terms of ng of copper per mg of protein. The range was statistically defined by the 95% confidence limits about the mean.

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culture solutions was monitored, and while some variations were observed for these solutions, no effect upon copper concentration of the fibroblasts was detected. The average copper concentration for the media was 54.1 ng of copper per ml. The copper concentration of the media was varied from 38 to 85 ng of copper per ml by the addition of copper sulfate. Subcultures from a single Menkes and a single control line were incubated in this medium for 4 days before copper analyses. Despite the variation in medium copper concentration, there was very little variation in culture content of control (45–60 ng of copper per mg of protein) and Menkes (272–307 ng of copper per mg of protein) cultures. Furthermore, no correlation between the medium copper concentration and culture copper levels existed for either control or Menkes cultures.

We also examined whether a difference in culture confluency existed between Menkes and control cultures and whether the copper content was influenced by variation in culture density. Harvesting of the cultures occurred when cell growth across the bottom surface of the containers was confluent. Both Menkes and control cultures required 8–10 days to obtain confluency. This implied not only similar growth rates, but also similar culture size at the time of analyses. Furthermore, investigations utilizing total protein content and cell counts (Coulter Counter model F) as estimates of culture size revealed little difference between cultures. The copper concentration of control cultures remained within the 95% confidence limits for over 15 passages, while the Menkes cultures seemed to possess a tendency to increase in copper content as the number of subcultures increased, magnifying the difference between the two cultures. With overly confluent cultures both control and Menkes cultures displayed a slight increase in copper concentration, with the difference remaining fairly constant.

It appears that control cultures cannot be differentiated from heterozygote cultures by copper content. Cultures from two presumed-heterozygote mothers of Menkes patients were grown and analyzed by the same procedures. The copper concentrations of these cultures fell within the statistically defined range for control values. This observation does not preclude the presence of cells expressing the abnormality, as would be predicted from the Lyon hypothesis. We would assume that when we examine cloned cells from known heterozygotes they will demonstrate both a normal and a mutant population.

We have also measured the copper content of two cultures from individuals with Wilson's disease, another genetic disorder involving copper metabolism. While the copper concentrations of these cultures were found to exceed those of controls (mean = 105.3 ng of copper per mg of protein), the Wilson cultures' copper content was distinct and much lower than the values observed for Menkes. Our experience with the cultures from patients with Wilson's disease is too limited to discuss fully any possible significance of the slightly elevated copper content, although it may be related to the abnormal protein reported by Evans *et al.* (5).

DISCUSSION

The genetic marker for Menkes disease, consistently expressed as elevated intracellular copper concentration, provides a much more specific method for the diagnosis of this genetic disorder than those previously suggested (6, 7). Since it is logical that fetal cell cultures of individuals with Menkes disease would express the genetic anomaly as increased copper concentration, prenatal diagnosis may be possible. While this hypothesis remains to be tested, data indicate that fibroblasts cultured from normal amniotic fluid consistently exhibit copper concentration values similar to, but slightly less than, those of control cultures of skin fibroblasts (Table 1).

Our findings of elevated copper concentration in Menkes cultures as a result of the genetic abnormality are consistent with the reports of Danks *et al.* (1, 6). They discovered abnormally high radiocopper levels in the duodenal mucosa following oral ingestion of ^{64}Cu by a patient with Menkes disease. This discovery led them to conclude that the cells were capable of copper uptake when copper was available, but were defective in transferring copper across the serosal membrane. The latter conclusion was based on the observation that very little radiocopper was detected in the blood stream of the Menkes patient, contrary to what was observed in the normal control. It appears that Menkes cells, in an environment of ample copper, exhibit an increased affinity for and/or retention of copper. This may be the result of a defective transport process of the cells or an unusual binding of copper within the cells and may provide an explanation for the poor results with copper supplements. The increased ceruloplasmin levels reported following injection of copper (8) may be interpreted as resulting from a separate biochemical pathway that is unique to the liver cell and not affected by the genetic defect of Menkes disease.

An additional Menkes culture, which we obtained from the Human Genetic Mutant Cell Repository, GM 245, warrants further discussion. The mean value for this culture was similar to those for other Menkes cultures, 210.0 ng of Cu per mg of protein, although an occasional single value was observed within the control range. This culture displayed abnormal growth behavior such as an erratic rate and occasional failure to reach confluent growth. We have examined the karyotype of this culture. While the karyotype is grossly normal (46, XY), the presence of widespread abnormalities such as gaps, breaks, and rearrangements was noted. Although mycoplasma screening was negative these chromosomal anomalies suggest infection, which might affect copper concentrations. We have not included data from GM 245 in this report. We were fortunate to obtain a fresh biopsy of the same individual who was the source of the subculture GM 245. Subsequent analyses of the resulting fibroblast culture reveal a mean of 314.4 ng of copper per mg of protein. Thus far, no variation in either the copper content or growth behavior, as was observed in GM 245, has been detected.

In summary, the consistently elevated intracellular concentrations of copper in skin fibroblasts cultured from patients with Menkes disease set them apart from fibroblasts cultured from control subjects, presumed heterozygotes, and patients with Wilson's disease, and provide an *in vitro* marker for this disorder. This genetic marker should prove valuable in the diagnosis and perhaps antenatal detection of Menkes disease. The Menkes culture with this marker provides a unique tool for the further elucidation of the fundamental defect in Menkes disease as well as for investigating aspects of normal copper metabolism. Preliminary uptake studies with ^{64}Cu demonstrated very striking differences between Menkes and control cultures.

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