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Copper
Menkes syndrome
skin fibroblasts

^{64}Cu Metabolism in Menkes and Normal Cultured Skin Fibroblasts

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Summary

The amount of radioactivity accumulated in normal skin fibroblasts cultured in the presence of ^{64}Cu increased during the first few hours of incubation, and reached a plateau after 18 hr. Fibroblasts from patients with Menkes syndrome continued to accumulate ^{64}Cu and after 48 hr, the mutant cells contained over 3 times more radioactivity than the normal cells. Normal skin fibroblasts grown in the presence of ^{64}Cu for 24 hr and chased for 6 and 24 hr with ^{64}Cu-free medium released 78% and 91% of the radioactivity, respectively, whereas Menkes fibroblasts grown under similar conditions released only 22% and 51%, respectively. The amount of radioactivity incorporated by fibroblasts increased with increasing concentrations of ^{64}Cu in the culture medium, but the Menkes fibroblasts incorporated more ^{64}Cu than normal fibroblasts. A slight reduction in the incorporation and efflux of ^{64}Cu was seen with several metabolic inhibitors, but there was no difference between normal and mutant cells. Treatment of labeled cells with trypsin did not affect the amount of ^{64}Cu picked up by the cells. Most of the radioactive copper incorporated by the cells was not precipitated by trichloroacetic acid or phosphotungstic acid, although the percentage of precipitated ^{64}Cu was consistently higher in normal than in Menkes fibroblasts. Most of the ^{64}Cu was bound to a molecule with a molecular weight of about 10,000, whereas a small fraction, proportionally higher in normal cells, was bound to a large molecular weight component(s). The amount of ^{64}Cu bound to the small molecular weight species was significantly greater in Menkes fibroblasts than in normal cells. Menkes fibroblasts were more sensitive to high nonphysiologic levels of nonradioactive copper than were normal cells. These findings demonstrate pronounced metabolic differences between normal and Menkes fibroblasts and indicate the need for further studies before proper treatment of this disease can be instituted.

Speculation

Copper picked up by cultured skin fibroblasts is preferentially bound to a molecule with a molecular weight of about 10,000. The increased copper accumulation in Menkes fibroblasts may be caused by the deficiency of an enzyme that catalyzes the cleavage of this bond, by the presence of an increased amount of this small molecular weight molecule in the mutant cells, or by an increased binding capacity of this molecule for copper.

Menkes kinky hair syndrome is characterized clinically by progressive central nervous system involvement with severe mental retardation, pili torti, arterial intimal abnormalities, bone lesions, and hypothermia (1, 9). Light microscopic and electron microscopic abnormalities have been found in the cerebellar cortex of the patients (6). The disease is inherited as an X-linked recessive trait. The frequency of the Menkes syndrome in an Australian population has been estimated to be 1 in 35,000 live births (1). The plasma copper and ceruloplasmin are very low (1). The conversion of iv administered copper into ceruloplasmin is normal, although oral administration of copper fails to restore normal plasma copper levels (2). The duodenal mucosa, however, has been found to contain significantly greater concentrations of copper in such patients than in normal control subjects (3). Therefore, it seems that the defect does not involve the uptake of copper from the intestinal lumen but rather the intracellular handling or transport across the serosal cell membrane (3). The copper concentration of liver is very low although the copper concentration of erythrocytes is either high or normal (2). It appears that the copper concentration of whole brain is normal (10).

Cultured skin fibroblasts obtained from hemizygous patients and heterozygotes for Menkes syndrome have shown intense metachromasia (3). Subsequently, it was found that the copper concentration in Menkes cultured skin fibroblasts (5) and the ^{64}Cu uptake by these cells (4, 7) and by cultured amniotic fluid cells from an affected fetus (7) were greater than in control cultures. For these reasons we decided to investigate further copper metabolism of cultured skin fibroblasts.

MATERIALS AND METHODS

Skin fibroblasts derived from four patients with the Menkes syndrome (12) and from five normal donors were used in the study (13). Cultures were between the 6th and 12th passage. Cells were cultured in RPMI 1640 medium supplemented with 16% fetal calf serum, 1% 200 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The concentration of copper in the fetal calf serum used was 0.22 μg/ml. Fibroblasts were transferred to 75-cm^2 flasks and 24 hr later, while at growth phase, were incubated in the medium described above to which had been added 2 μg/ml (2.45 μCi/μg) carrier-free ^{64}Cu(NO$_3$)$_2$ (New England Nuclear, Boston, MA). At specific intervals the culture flasks were washed with isotonic saline and the cells lysed with 1% sodium dodecyl sulfate. The ^{64}Cu content in the lysates was determined by Cerenkov counting in a Packard Tri-Carb liquid scintillation spectrometer (model 3375, Packard Instruments Corp., Downers Grove, IL). The counts per min of all of the samples from 0–24 hr were determined after 24 hr had elapsed from the start of the experiment. Samples of greater than 24 hr of incubation were measured relative to a ^{64}Cu standard and adjusted to the 24-hr determinations. Since the ^{64}Cu decayed with the same rate in both background and culture samples, this did not lead to any sampling error in these measurements. For chase experiments, fibroblasts were labeled as above for 24 hr. The radioactive medium was then removed, and the cultured cells washed and incubated with unlabeled medium. The cells were harvested 6 and 24 hr later and the radioactivity was determined as above.

Fibroblasts at growth phase were incubated with 1.0 mM 2,4-dinitrophenol, 2.5 mM NaF, 1.0 mM NaN_3, or 5 μg/ml cytochalasin B for 1 hr. Subsequently, ^{64}Cu was added to the medium and the cells were incubated for another 24 hr. Cells also were labeled with ^{64}Cu for 24 hr and were chased in the presence of the above inhibitors for 24 hr.

Menkes and normal fibroblasts were cultured in the presence of specific concentrations of nonradioactive copper and the morphologic characteristics of the cultures were observed under an inverted microscope at specific intervals.

Gel filtration of cell lysates was performed at room temperature on a Sephadex G-75 or G-100 column, 0.9 × 60 cm, equilibrated with 0.1 M Tris-HCl buffer, pH 6.5. Protein in the fractions was measured as optical density at 280 nm.

Protein in the cell lysates was measured by the method of Lowry *et al.* (8). All cultures and determinations were carried out in duplicate.

RESULTS

In normal cells, the amount of ^{64}Cu accumulated increased during the first few hours of incubation, and after 18 hr reached a plateau that was maintained for the duration of the experiment. The accumulation of ^{64}Cu by the Menkes fibroblasts, however, did not reach a steady state but rather continued to increase. After 48 hr the mutant cells contained over three times more intracellular ^{64}Cu than the normal cells (Fig. 1*A*). Six and 24 hours after replacement of ^{64}Cu-containing medium by nonradioactive medium the normal cells released 78% and 91% of the radioactivity, respectively, whereas the Menkes cells released only 22% and 51%, respectively (Fig. 1*B*). The amount of ^{64}Cu incorporated by normal and Menkes fibroblasts increased linearly with increasing concentrations of copper, but the mutant cells incorporated more radioactivity than normal fibroblasts (Fig. 2).

A slight reduction in the incorporation and efflux of ^{64}Cu was observed with several metabolic inhibitors (Table 1). After exposure of the fibroblasts to the inhibitors, cells remained alive, as indicated by the ability of similarly treated control fibroblasts to form confluent cultures after removal of the inhibitors from the culture medium. In order to ascertain the amount of extracellular ^{64}Cu binding to the cells, normal and mutant fibroblasts labeled with radioactive copper were harvested by scraping, washed with saline, and then treated with 0.25% trypsin for 15 min. No reduction in the amount of ^{64}Cu accumulated by the cells was seen after exposure to trypsin.

Fibroblasts cultured in the presence of ^{64}Cu for 24 hr were harvested by scraping, washed, lysed by freezing and thawing 10 times, and centrifuged at 1800 × *g* for 15 min. The percentage of the radioactivity present in the supernatant fraction from Menkes fibroblasts was higher (84%, range 81–87%) than in normal cells (64%, range 60–71%). When the supernatant fractions from Menkes and normal fibroblast lysates were treated with 6% trichloroacetic acid (TCA), 90–97% and 64–77% of the radioactivity remained in suspension, respectively. Similarly, when the pellets of the lysed Menkes and normal cells were treated with TCA, approximately 80% and 60% of the counts were solubilized and found in the supernatant, respectively. Precipitation with 4% phosphotungstic acid in 2 N HCl gave similar results.

A minor and a major peak of radioactivity was observed when lysates from normal and from Menkes fibroblasts cultured in the presence of ^{64}Cu for 24 hr were eluted from a Sephadex G-75 column. The major peak was always significantly greater in Menkes cells than in normal cells (Fig. 3). The minor peak was eluted with the void volume. This peak also was eluted with the void volume when the gel filtration was performed with Sephadex G-100. The percentage of the radioactivity present in this first peak was 13% and 3% of the total in normal and Menkes fibroblasts, respectively. The major peak was eluted before ^{64}Cu chromatographed without protein. The molecular weight for a protein with this elution volume was estimated as 10,000.

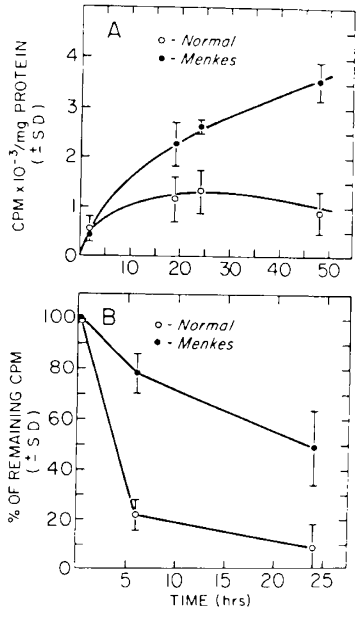

Fig. 1. ^{64}Cu accumulation (*A*) and chase (*B*) in normal and Menkes cultured skin fibroblasts. Cells were chased after being cultivated with radioactive copper for 24 hr.

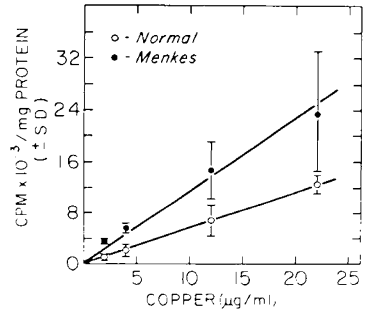

Fig. 2. Accumulation of ^{64}Cu in normal and Menkes cultured skin fibroblasts at different concentrations of radioactive copper.

Table 1. *Effect of metabolic inhibitors on ^{64}Cu accumulation and efflux by normal and Menkes cultured skin fibroblasts*[1]

	Normal cpm/mg protein		Menkes cpm/mg protein	
Inhibitor	Incorporation	Chase	Incorporation	Chase
None	1666	222	2444	822
DNP, 1.0 mM	1911	290	2667	1180
NaF, 2.5 mM	930	333	2000	1178
NaN_3, 1.0 mM	1110	178	1844	1110
CB, 5.0 μg/ml	1110	290	2422	1444

[1] DNP: 2,4-dinitrophenol; CB: cytochalasin B.

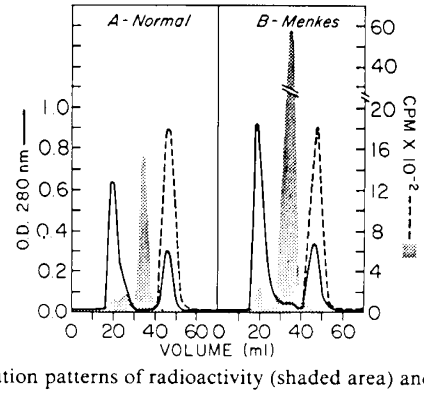

Fig. 3. Elution patterns of radioactivity (shaded area) and protein from a Sephadex G-75 column of lysates from normal and Menkes fibroblasts cultivated in the presence of ^{64}Cu for 24 hr. The elution of $^{64}Cu(NO_3)_2$(- - -) chromatographed without protein is shown.

By culturing skin fibroblasts in medium containing increasing concentrations of nonradioactive copper, it was observed that signs of cell toxicity and cell death occurred in the Menkes cells at lower concentrations of copper than in normal cells. Cultures exposed to 100 μg $CuCl_2$/ml medium for 4 days showed extensive cell death in both normal and Menkes cultures. During culturing, most of the fibroblasts became rounded and finally were detached from the surface of the culture flasks. This effect was much more prominent, however, in the Menkes cells than in the normal cells. That the detached fibroblasts were no longer viable was demonstrated by their inability to grow after these cells were washed with fresh medium and replanted in new culture flasks. After removal

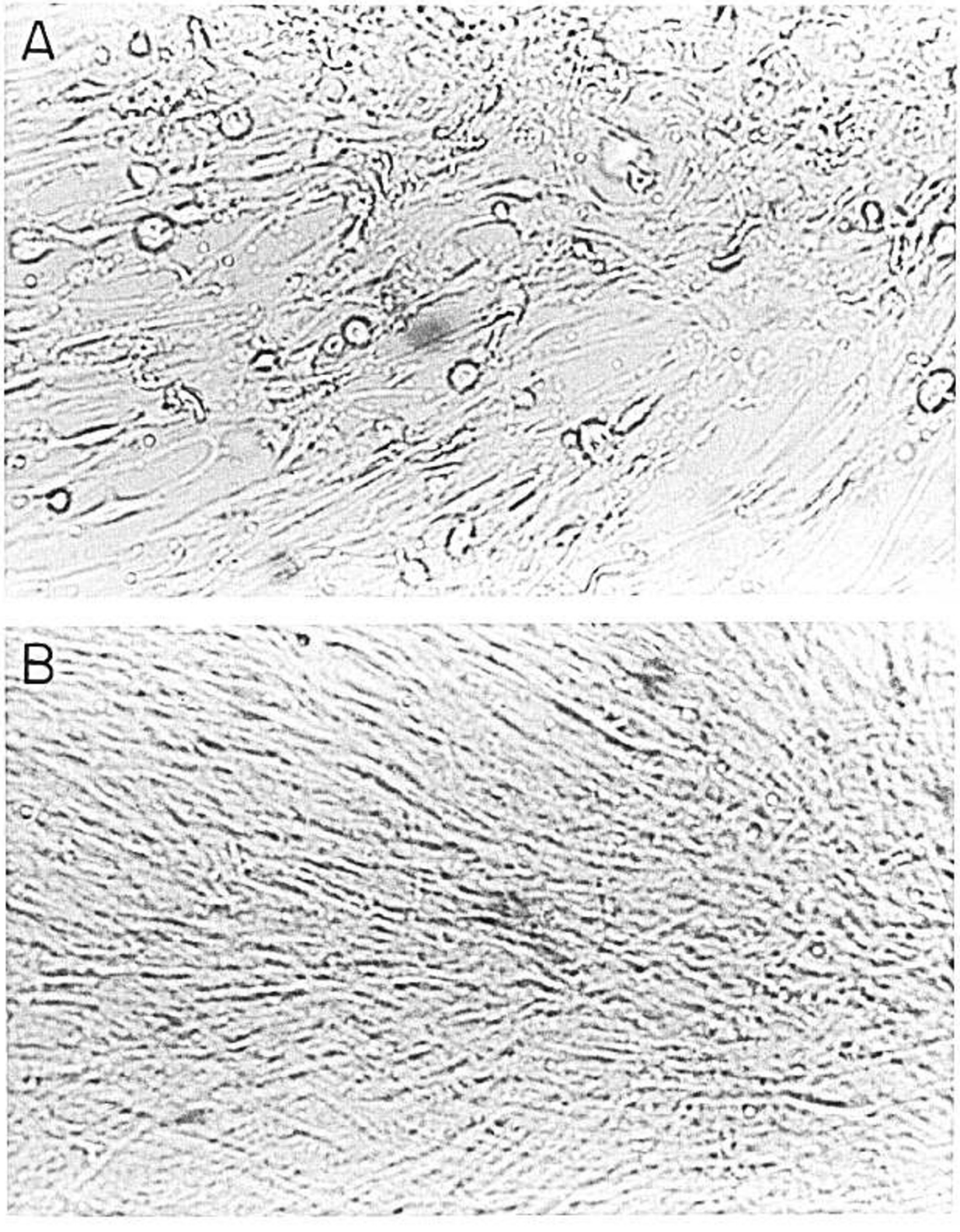

Fig. 4. Skin fibroblasts from a patient with the Menkes syndrome (*A*) and a normal subject (*B*) cultured in the presence of 60 μg/ml added nonradioactive copper for 5 days. Copper was added to the culture medium when fibroblasts were at early confluence. Rounded and detached cells are present in the Menkes culture, whereas the normal culture appears morphologically unaffected.

of the copper from the medium of the original cultures, normal cells that remained attached to the flasks recovered from the toxic effects of this element and formed confluent cultures. In contrast, cells in the Menkes cultures, that remained attached to the flasks after the exposure to copper, failed to multiply and debris of dead cells were gradually released into the culture medium during the following days. At 60 μg/ml, distinct signs of copper toxicity and cell death were apparent only in the Menkes fibroblasts (Fig. 4, *A* and *B*).

DISCUSSION

This study demonstrates a greater accumulation of copper and a reduced efflux of copper in cultured skin fibroblasts derived from patients with the Menkes syndrome than in fibroblasts derived from normal control subjects. Menkes fibroblasts, after incubation for 24 hr with radioactive copper, incorporated approximately twice as much ^{64}Cu than normal fibroblasts. Six hours after replacement of ^{64}Cu-containing medium by nonradioactive medium, the normal cells released approximately 4 times the percentage of the radioactivity as compared with the Menkes fibroblasts (see Fig. 1). This indicates that the normal fibroblasts actually released more copper than the mutant cells. The reduced efflux of copper in the mutant cells appears to be a metabolic abnormality closely related to the basic defect. Although in normal skin fibroblasts the incorporation of ^{64}Cu reached a plateau after 18 hr of incubation, Menkes fibroblasts continued to incorporate ^{64}Cu at least up to 48 hr. This apparent difference from the findings of Horn (7), who found that both normal and mutant cells reach a steady state by 20 hr, could result from the fact that her studies were performed with confluent rather than actively dividing cultures.

The findings of the gel filtration experiments indicate that both in Menkes and in normal fibroblasts the vast majority of ^{64}Cu is bound to a small molecule with a molecular weight of about 10,000. A small fraction of the total radioactivity, which was eluted with the void volume, appears to be bound to a larger molecule with a molecular weight over 100,000. This portion of bound copper was greater in normal cell lysates than in Menkes cell lysates. Further investigation is indicated to determine whether the difference between the proportion of copper bound to small and large molecules is of significance in the pathogenesis of the disease.

The failure of TCA to precipitate the vast majority of labeled copper suggests either that the copper is not bound to a TCA-precipitable molecule, or that the bond is broken by TCA. Phosphotungstic acid, which precipitates some glycoproteins and basic proteins not precipitated by TCA, also failed to precipitate most of the radioactivity. This is different from findings in chicks where ^{64}Cu present in the duodenal mucosa was firmly attached to a TCA-precipitable protein with a molecular weight of approximately 10,000 (11). The inability of trypsin to release ^{64}Cu both from normal and from mutant labeled fibroblasts does not support the suggestion that copper is bound at receptors on the cell surface and indicates that the intracellular location of copper should be investigated by subcellular fractionation.

The amounts of ^{64}Cu found both in normal and in abnormal fibroblasts during incorporation experiments were either diminished or, on some occasions, practically unaffected by the metabolic inhibitors used. A small reduction in the efflux of radioactivity also was seen in the presence of most of the inhibitors, but no significant difference between the two genotypes was seen. These results suggest that the process of copper transport in cultured skin fibroblasts might require metabolic energy and that the defect in Menkes disease is not related to the transport of copper across the cytoplasm membrane.

From the findings reported here, it appears that the copper deficiency present in several tissues of patients with Menkes syndrome is probably not the result only of a defect in copper transport across the serosal cell membrane of the duodenum (3). It is probably also caused by an increased retention of copper by certain cells such as connective tissue and erythrocytes. It is not known at the present time whether or not the neuronal cells in the central nervous system of patients with Menkes syndrome have an increased affinity for copper and are thereby damaged by increased copper accumulation. Cultured skin fibroblasts from Menkes patients were damaged to a greater degree than normal fibroblasts when exposed to unphysiologically high concentrations of copper. The brain damage also could be the result of copper deficiency due to a reduced supply of copper to the neuronal cells. This reduced neuronal copper supply could result from a reduced transport of copper across the intestinal wall as well as from an increased retention of copper by other tissues. A reduced transport of copper across the membrane of certain specific cells also has been hypothesized. Measurement of total brain copper in Menkes patients would not necessarily settle the question since neuronal and glial cells may differ in their retention of copper. An answer to this question is crucial for determining the proper treatment of this genetic disorder.

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12. The fibroblast strains GM-220, GM-245, GM-1057, and GM-1981 were derived from patients with Menkes syndrome and were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ.
13. The studies on human subjects were performed with their informed consent or, in the case of children, with the informed consent of their parents.

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