Fibroblast silver loading for the diagnosis of Menkes disease

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Abstract

Menkes disease is a genetic disorder of copper metabolism. Copper uptake and retention assays on fibroblast or amniotic fluid cell cultures have been used for pre- and postnatal diagnosis. These copper loading tests are complicated by the use of ^{64}Cu , which is not commonly available and has a very short (12.8 hours) physical half life. Besides copper, silver is also a substrate for the bacterial homologue of the Menkes transport protein. We report here that loading tests using radioactive silver (^{110m}Ag), instead of copper, can be used for the diagnosis of Menkes disease. ^{110m}Ag is commercially available and has a convenient physical half life of 250 days, which makes it suitable for use in diagnostic laboratories. Our studies support the hypothesis that reduction of divalent to monovalent copper is an essential step preceding transport.

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Menkes “steely hair” disease is a severe X linked recessive disorder of copper metabolism. Patients suffer from neurodegeneration, connective tissue abnormalities, and failure to thrive. Various copper dependent enzymes are deficient because of the malabsorption and decreased availability of copper in tissues. The disease generally leads to death in infancy or early childhood.^{1,2}

The incorporation of ^{64}Cu into cultured skin fibroblasts and amniotic fluid cells is used for pre- and postnatal diagnosis of Menkes disease.³ After identification of the defective gene in 1993, diagnostic confirmation by mutation analysis of the Menkes gene (*ATP7A*, or MNK in earlier reports) is possible. However, the spectrum of mutations is broad, 80% of mutations being accounted for by small base pair changes.^{4,5} Biochemical diagnosis remains essential, therefore, to confirm the clinical suspicion. The copper loading test requires particular expertise. ^{64}Cu is not commonly available and its physical half life is very short (12.8 hours), which greatly complicates its use, and the test is carried out in only a few specialised centres world wide.

The *ATP7A* gene product is a putative copper transporter (*ATP7A*), highly homologous to copper transporters CopA and CopB from *Enterococcus hirae*.⁴ A recent report that CopB also transports monovalent silver ions suggests that copper is transported by CopB as the

reduced monovalent cation (CuI).⁶ Also binding of copper as CuI has been shown for the in vitro expressed amino-terminal domains of *ATP7A*.⁷ There is, as yet, no direct evidence of copper transport by *ATP7A*, but recent studies have shown an increased expression of *ATP7A* in Chinese hamster ovary cells resistant to excess copper.⁸

It has been shown that *ATP7A* is predominantly localised to the trans-Golgi apparatus, where it probably serves as an import protein for copper ions to provide copper to the cuproenzymes.^{9–11} However, under conditions of copper excess a rapid shift in localisation from the Golgi to the plasma membrane is observed.⁹ This ligand regulated trafficking of *ATP7A* can explain many clinical features of patients with Menkes disease, which are the result of the deficiency of several cuproenzymes (defective Golgi import of copper ions) or defective absorption by intestinal epithelial membranes. It was shown that, besides copper ions, silver ions were also able to induce this ligand regulated trafficking.⁹ Since the bacterial homologue of *ATP7A* is able to transport copper and silver ions, we investigated whether *ATP7A* was also able to do so and consequently whether silver can replace copper in a more accessible biochemical test. ^{110m}Ag is a commercially available β and γ emitter with a physical half life of 250 days and is therefore much easier to handle in a diagnostic laboratory.

We first investigated the incorporation of radioactive silver in cultured fibroblasts (fig 1). In controls and Menkes fibroblasts a clear, time dependent incorporation of ^{110m}Ag was observed, with a higher incorporation in Menkes cells. Most of the radiolabel was released from the control cells during subsequent culturing in unlabelled medium for 24 hours (“chase”), while Menkes cells retained most radioactivity. The entire process was inhibited by copper sulphate added to the medium (data not shown). These results are comparable with previous data from ^{64}Cu loading experiments.³ Subsequently, cultured cells from 10 different Menkes disease patients were tested with this silver incorporation test. As shown in table 1, cell lines from Menkes disease patients showed a higher average level of silver incorporation during the first 16 hours as compared with controls. During the subsequent chase period, the Menkes cell lines retained high levels of radioactivity, ranging from 77 to 100% of the level before chase, compared to 14 to 48% in 10 controls. This shows that silver incorporation perfectly discriminates Menkes cells from

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Figure 1 Time dependent incorporation of ^{110m}Ag in cultured fibroblasts from a control and from a Menkes disease patient. Cells were harvested at the indicated time points ("pulse" period). After 24 hours' loading, the radioactive medium was replaced by fresh, non-silver containing medium and cells were further cultured for the indicated time ("chase" period). For details of the assay see description below table 1.

controls. Cultured amniotic fluid cells from a fetus with Menkes disease show entrapment of ^{110m}Ag after 24 hours' chase (table 1), suggesting that the silver loading test can be used for prenatal diagnosis as well.

In fig 2, the individual incorporation levels after pulse and chase are plotted for all cell strains. After the first 16 hours there is some overlap of the ^{110m}Ag levels in Menkes and control cells. However, during the subsequent 24 hours' chase all control cells rapidly release ^{110m}Ag , while all Menkes cells show no or very little efflux. Evidently the 16 hours loading levels are the result of simultaneous import and efflux processes, which are in part dependent on the rate of uptake. In contrast, chase experiments directly address the efflux. Since in Menkes disease fibroblasts the efflux of copper (and silver) is defective and not the uptake, diagnostic conclusions can be drawn best from the chase experiments.

The long half life of ^{110m}Ag and its common availability will potentially facilitate both diag-

Figure 2 Silver incorporation by 10 different control and 10 different Menkes fibroblast cultures. The incorporation values, obtained after 16 hours' "pulse" and after subsequent 24 hours' "chase" are reported for each cell strain.

nosis and research aiming at improved therapy of Menkes disease and its allelic variant occipital horn syndrome.² The finding that CopB (*Enterococcus hirae*) and ATP7A (human), both members of a family of copper transporting type ATPases, also transport monovalent silver (this paper) suggests that other members of this family might also transport silver. Among these are the transporters involved in Wilson disease and Indian childhood cirrhosis.¹² It is not known whether the transport of silver is merely a biochemical property of the substrate binding site of copper transporters or that silver transport has a physiological role. There are only sporadic reports suggesting a specific role for silver ions in the oxidative burst of polymorphonuclear leucocytes.¹³

A few Menkes disease patients respond to copper supplementation whereas others do not. It is hypothesised that in some patient with residual copper transport, early copper supplementation therapy can be beneficial.¹⁴ In one of the mouse models for Menkes disease, the brindled mouse, the activity of copper dependent lysyl oxidase dramatically increases after subcutaneous injection of reduced copper (CuI). This suggests that the effectiveness of copper supplementation depends on its oxidation state.¹⁵ Copper loading tests in nine different mottled mouse variants do not correlate genotype with phenotype.¹⁶ Our data support the hypothesis that copper is transported as monovalent cation by ATP7A and that copper reduction represents an essential step preceding transport. Thus, comparison of copper and silver transport kinetics in different mutants might help to elucidate whether, in some of these mutants responsive to CuI supplementation therapy, the transport defect is secondary to a defective reduction mechanism.

Table 1 Diagnosis of Menkes disease by silver loading of cell cultures

	Pulse (16 h)	Chase (24 h)	% Retained
Fibroblasts			
Controls (n=10)			
Mean (SEM)	141.2 (18.8)	47.5 (11.0)	30 ± 3% Range 14–48%
Menkes (n=10)			
Mean (SEM)	261.7 (23.8)	230.7 (23.0)	88 ± 2% Range 77–100%
Amniocytes			
Control 1	14.2	3.7	26%
Control 2	33.0	3.3	10%
Menkes	33.2	30.7	93%

Each cell line was seeded to confluency in four separate wells of six well plates (Costar, 9.6 cm²/well). The cells were cultured for three to four days in F10⁺ medium (+ 15% FCS + PS) in a 3% CO₂ incubator at 37°C. To start the experiment, the medium was replaced by 2 ml fresh medium containing 1.25 μmol/l $^{110m}\text{AgNO}_3$ (Amersham spec act 2.7 μCi/μg Ag). After 16 hours wells were rinsed twice with 4 ml PBS. Two wells were harvested by lysis with 500 μl 0.2% SDS in 0.2 mol/l NaOH. The other two wells were subsequently cultured for 24 hours in fresh medium without label before harvesting. The lysates were neutralised and samples were taken for protein determination and liquid scintillation counting.

All Menkes cell lines exhibited increased accumulation in previous ^{64}Cu loading tests (The John F Kennedy Institute, Copenhagen). Loading is expressed as ng ^{110m}Ag /mg protein (average of duplicate wells). % Retained = chase/pulse * 100%.

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