Copper Deficiency in Patients with Cystinosis with Cysteamine Toxicity

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Objectives To assess whether copper deficiency plays a role in the recently described cysteamine toxicity in patients with cystinosis, and to examine whether polymorphisms in copper transporters, lysyl oxidase, and/or type I procollagen genes could be responsible for the occurrence of cysteamine toxicity in a small subset of patients with cystinosis.

Study design Thirty-six patients with cystinosis were included: 22 with Fanconi syndrome (including 7 with cysteamine toxicity), 12 after renal transplantation, 1 receiving hemodialysis, and 1 with ocular cystinosis. Serum copper and ceruloplasmin levels and urinary copper/creatinine ratio were measured. Genes *ATP7A* and *CTR1* (encoding copper transporters), *LOX* (encoding lysyl oxidase), and *COL1A1* and *COL1A2* (encoding type I procollagen) were analyzed in patients with (n = 6) and without (n = 5) toxicity. Fibroblast (pro)collagen synthesis was compared in patients with (n = 3) and those without (n = 2) cysteamine toxicity.

Results All 22 patients with Fanconi syndrome had increased urinary copper excretion. Serum copper and ceruloplasmin levels were decreased in 9 patients, including all 7 patients with cysteamine toxicity. No specific sequence variations were associated with toxicity. All fibroblasts exhibited normal (pro)collagen synthesis.

Conclusion Patients with cystinosis with cysteamine toxicity demonstrate copper deficiency. This can cause decreased activity of lysyl oxidase, the enzyme that generates the aldehydes required for collagen cross-linking. Thus, copper supplementation might prevent cysteamine toxicity. *(J Pediatr 2013;163:754-60)*.

Cystinosis is an autosomal recessive disorder marked by intralysosomal cystine accumulation in various tissues. It causes generalized proximal tubular dysfunction, termed renal Fanconi syndrome (FS), which usually leads to end-stage renal disease in the second decade of life.[1] The amino thiol cysteamine, currently the only available treatment for cystinosis, depletes lysosomal cystine and postpones renal and extrarenal organ damage.[2-5]

Recent reports of bruise-like skin lesions, striae, bone abnormalities, and muscle weakness in patients with cystinosis treated with cysteamine has led to concerns regarding the use of high doses (above the recommended maximum of 1.95 g/m^2/day), although only one-half of the reported patients received such high doses. Histological analysis of skin biopsy specimens obtained from the bruise-like lesions revealed proliferation of microvascular endothelial cells (reactive angioendotheliomatosis) and irregular collagen fiber calibers, resembling those found in classical Ehlers-Danlos syndrome; irregular elastin fibers were found in some patients. The etiology of these lesions remained unclear; however, the causative role of cysteamine administration was supported by the fact that symptoms ameliorated in all patients after cysteamine doses were decreased. There was no clear dose-dependent effect, however.[6]

Given the structural similarity between cysteamine and D-penicillamine, we suggested that cysteamine can interfere with collagen cross-linking.[6] Aldehydes required for collagen and elastin cross-linking are synthetized in the presence of copper by an enzyme, lysyl oxidase.[7,8] It has been demonstrated that D-penicillamine can block aldehydes, making them unavailable for cross-linking.[7] Interestingly, the bone problems related to cysteamine toxicity clinically resemble osteolathyrism, which is caused by β-aminopropionitrile–induced inhibition of lysyl oxidase.[8] Previous reports of osteolathyrism have described bone pains and skeletal deformities, including lack of ossification centers in the iliac creasts, ischial tuberosities, and vertebrae, as well as bowing with thickening of the femoral shaft.[9-12] Because copper is a cofactor of lysyl oxidase,[13] symptoms of copper deficiency also overlap with those of osteolathyrism.[8]

We hypothesized that copper deficiency might have contributed to the adverse effect of cysteamine on collagen cross-linking in patients with cystinosis. We

FS Fanconi syndrome

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examined whether polymorphisms in copper transporters, lysyl oxidase, and/or type I procollagen genes could be responsible for the occurrence of cysteamine toxicity in only a small subset of patients with cystinosis.

Methods

Physicians treating patients with cystinosis diagnosed with cysteamine toxicity were invited to include their patients in this study. The physicians collected samples for serum copper and ceruloplasmin measurements and urinary copper excretion. These patients were treated in 5 different centers: Radboud University Nijmegen Medical Center (Nijmegen, The Netherlands), Bambino Gesù Children's Hospital (Rome, Italy), Copenhagen University Hospital (Copenhagen, Denmark), Besançon University Hospital (Besançon, France), and Leuven University Hospital (Leuven, Belgium). In 3 centers (The Netherlands, Italy, and Belgium), samples from patients with cystinosis but without cysteamine toxicity were collected as well; these patients served as a control group. A total of 36 patients with cystinosis were included, 7 of whom were diagnosed with cysteamine toxicity. DNA samples were obtained from 6 of the patients with cysteamine toxicity; fibroblasts, from 3 of these patients. DNA samples and fibroblasts from patients with cystinosis without cysteamine toxicity were obtained from local biobanks in Belgium and The Netherlands and served as controls.

The study protocol was approved by the Ethical Board of Leuven University Hospital, the center that coordinated the study. Informed consent was obtained from all patients included in the study. The subjects who provided the biobank specimens had provided consent for use of their material for scientific research at the time of sample collection. Informed consent forms were signed either by the patients (if aged ≥18 years) or their parents (if aged <18 years).

Copper and Ceruloplasmin Measurements

Blood samples for copper and ceruloplasmin measurement were collected in a vacutainer tube containing clot activator and gel for serum separation (BD, Erebodegem, Belgium). Serum was collected by allowing blood samples to clot for 30 minutes, followed by centrifugation at 1700 × *g* for 10 minutes at 4°C. Midstream urine samples were not centrifuged. Both serum and urine samples were immediately frozen and shipped to Leuven on dry ice, where they were stored at −80°C until analysis. Copper was measured by electrothermal atomic absorption spectrometry (Varian SpectrAA 220Z; Varian Medical Systems, Diegem, Belgium). Ceruloplasmin was measured by nephelomatic analysis (IMMAGE 800; Beckman Coulter, Suarlée, Belgium).

DNA Analysis

Genomic DNA was extracted from blood or cultured fibroblasts from 6 patients with cystinosis with cysteamine toxicity and compared with samples from 5 patients with cystinosis without cysteamine toxicity. The open reading frames of *ATP7A* (23 exons), *CTR1* (4 coding exons), and *LOX* (7 coding exons) were investigated by polymerase chain reaction, followed by bidirectional fluorescent DNA sequencing. The primer sequences of *ATP7A*[14] and *LOX*[15] have been described previously; primer sequences of *CTR1* are available on request. Then the open reading frames of *COL1A1* (52 exons) and *COL1A2* (52 exons) were investigated by polymerase chain reaction, followed by bidirectional fluorescent DNA sequencing, as described previously.[16]

(Pro)collagen Synthesis Studies

These experiments were performed on 3 fibroblast cell lines obtained from patients with cystinosis with cysteamine toxicity and 2 fibroblast cell lines from patients with cystinosis without cysteamine toxicity. Cells were cultured in culture medium supplemented with ascorbic acid, to ameliorate the production of type I and type III (pro)collagen. Each cell line was cultured both with and without the addition of 0.1 mM cysteamine, close to the peak cysteamine plasma levels measured in patients with cystinosis.[17] A patient with cystinosis with cysteamine toxicity was reported to have a similar plasma level.[6] Synthesis of (pro)collagens was examined as described previously.[18] In brief, fibroblasts were grown to confluency and then incubated with [^{14}C]proline at 37°C for 16 hours. Medium and cell layer fractions were harvested separately; both were supplemented with protein inhibitors and stored at −80°C until analysis. Both medium and cell layer procollagens were converted to the respective collagen molecules by pepsin digestion, lyophilized, and redissolved in sample buffers. Analyses of secreted pro-α-collagen chains and secreted and intracellular α-collagen chains were performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were fixed and processed for fluorography, dried, and exposed to an X-ray film.

Statistical Analyses

The Student *t* test and Pearson correlation were used for statistical analyses. Values were considered statistically significant at a *P* value <.05.

Results

Patients' characteristics are summarized in Table I. Copper and ceruloplasmin data are reported for 36 patients with cystinosis, including 22 patients with renal FS (including 7 with signs of cysteamine toxicity), 12 patients who underwent transplantation with a functional renal graft, 1 anuric patient who was treated with hemodialysis after renal graft failure, and 1 patient with ocular cystinosis without renal involvement. All patients with cysteamine toxicity exhibited skin abnormalities, including bruise-like lesions and/or red skin striae. In addition, patients 3 and 4 had severe musculoskeletal pain, limiting their ability to walk. Patient 3 also suffered from neurologic complications causing behavioral changes. Severe bone deformities were reported despite adequate phosphate and vitamin D supplementations in 3 of 6 patients with cysteamine toxicity, including severe genu valgum requiring surgical