Electron Microscopic Changes in Hepatocytes of Patients with Homocystinuria

GERALD E. GAULL AND FENTON SCHAFFNER [36]

Departments of Pediatrics, Ophthalmology, and Pathology and The Clinical Research Center, Mount Sinai School of Medicine of The City University of New York, New York, 10029, and The Institute for Basic Research in Mental Retardation, Staten Island, New York, USA

Extract

Liver biopsy specimens from four patients with homocystinuria were examined by electron microscopy before and after treatment with massive doses of pyridoxine. The chief abnormalities found were as follows: unusually shaped mitochondria, hypertrophy of the smooth endoplasmic reticulum, and numerous pericanalicular lysosomes.

Several of the mitochondria in each cell in the various zones of the liver lobule had narrow projections and breaks in the outer mitochondrial membrane. Mitochondria were normal in size, but the mitochondrial dark granules appeared larger than normal.

Two patients responded biochemically to treatment by disappearance of hypermethioninemia and homocystinemia. No recognizable differences in hepatocellular structure resulted from treatment in any of the patients.

Speculation

Patients with homocystinuria who respond to pyridoxine treatment by disappearance of hypermethioninemia and homocystinemia exhibit no change in ultrastructural aberrations following treatment. This suggests that "toxic" accumulation of metabolites proximal to an enzymatic block may not account for all the phenotypical manifestations of inborn errors of the metabolism of amino acids.

Introduction

Homocystinuria is an inborn error of the metabolism of methionine associated with a deficiency of cystathionine synthase (EC. 4.2.1.13) in the liver and brain and inherited in an autosomal recessive pattern [3, 4, 8, 24]. The consistent phenotype includes: mental retardation in most cases, ectopia lentis, malar flush, thromboembolic and cardiovascular disease, skeletal deformities, and fatty liver [6, 13, 27]. Clinical and biochemical aspects have been reviewed recently [9, 12]. The fatty liver, unaccompanied by fibrosis, has been a fairly consistent finding at autopsy. Results of the various hepatic function tests, however, are nor-

mal [6, 27]. There is a twofold increase in liver lipids, the result of accumulation of triglyceride (neutral fraction) [6]. Examination of the fatty acids by gas chromatography reveals an increase in oleic acid, and decreases in palmitic, stearic, linoleic, and arachidonic acids [6]. The significance of these changes and their pathogenetic relation to the deficiency of cystathionine synthase are not clear.

Treatment of affected patients with massive doses of vitamin B₆, the putative cofactor of cystathionine synthase, has resulted in salutary changes in the concentrations of free amino acids in the plasma in some cases: hypermethioninemia and homocystinemia disap-

pear and concentrations of cystine in the plasma may return to normal in some, but not all cases [1, 2, 5, 7, 14, 15, 18, 19, 21, 30, 31]. Vitamin B₆, however, does not appear to exert its effect on cystathionine synthase activity [15], in some cases.

Biopsies of liver from four patients with homocystinuria taken before and after treatment with massive doses of pyridoxine have been studied by electron microscopy to define further the nature of the functional and structural changes in the liver, and to search for means of evaluating response to therapy. In this way, each patient serves as his own control, and difficulties in the assessment of treatment found in other rare inborn errors of the metabolism of amino acids are partially obviated.

Subjects and Methods

Patient 1

RC was an apparently fit, asymptomatic Puerto Rican male, 11 months at the time of first biopsy, and fed an apparently adequate diet *ad libitum*, without medication. After receiving 1,200 mg pyridoxine·HCl/24 hr and a normal diet limited to approximately 75 mg/kg/24 hr of methionine, hypermethioninemia and homocystinemia disappeared, but cystine concentrations in the plasma remained virtually unmeasurable. At the second biopsy, he was 14 months old and had been on this regimen for 3 months without other medication. Activities of three enzymes of the transsulfuration pathway in the liver were measured in parallel in the same biopsy specimens. The activity of cystathionine synthase was absent before and after treatment; activity of methionine-activating enzyme (EC. 2.5.1.6) was present at normal levels before and after treatment; and the activity of cystathionase (EC. 4.2.1.15) was normal before treatment, rising sixfold at the time of repeat biopsy 3 months later [15]. Although he subsequently developed some subluxation of the optic lenses while under treatment, his growth and development have otherwise been considered normal.

Patient 2

MC was the 3-year-old sibling of RC. She presented with moderate mental retardation, severe ectopia lentis, and some *genu valgum*. Her regimen differed from that of RC only in that she required 800 mg pyridoxine·HCl/24 hr to attain the same biochemical response. Activities of the enzymes of transsulfuration in the liver before and after treatment for 3 months were similar to those of her brother [15]. She was given no other medication at the time of the biopsies, although

subsequently she has been started on 125 mg phenobarbital/24 hr for minor motor seizures, with apparent control.

Patient 3

LM was a 21-year-old, moderately retarded Puerto Rican male with severe ectopia lentis and thromboembolic disease. He showed persistent hypermethioninemia and homocystinemia after receiving as much as 1,000 mg pyridoxine·HCl/24 hr, and a dietary intake as low as 19 mg methionine/kg/24 hr with 13 mg cystine/kg/24 hr. The first biopsy was performed while the patient was receiving 35 mg methionine/kg/24 hr and 21 mg cystine/kg/24 hr, without supplementary pyridoxine; the second biopsy was performed 3 months later at the time the patient was receiving a diet containing 19 mg methionine/kg/24 hr, 13 mg cystine/kg/24 hr, and 1,000 mg pyridoxine·HCl/kg/24 hr. At the times of both biopsies, he was also taking folic acid, 15 mg three times a day, but no other medication. Assay of the activities of the enzymes of transsulfuration in the liver gave results similar to those for RC and MC [15]. Although hypermethioninemia and homocystinemia persisted despite pyridoxine therapy, during a subsequent 5-day period when this medication was withdrawn by the patient, there was a striking change in behavior characterized by uncontrollable bursts of inappropriate anger that apparently remitted when he was placed on pyridoxine again.

Patient 4

MS was a 19-year-old Jewish male, with normal mentation, severe ectopia lentis, and skeletal changes. He showed no response to a regimen consisting of as much as 750 mg pyridoxine·HCl/kg/24 hr, administered intramuscularly, or 1,500 mg/kg/24 hr administered orally, with as little as 15 mg methionine/kg/24 hr and 13 mg cystine/kg/24 hr obtained from the diet. The first biopsy was performed while the patient was on a regimen on which the diet contributed 32 mg methionine/kg/24 hr and 21 mg cystine/kg/24 hr, without supplementary pyridoxine; the second biopsy was performed one month later while the patient was receiving a diet consisting of 15 mg methionine/kg/24 hr, 13 mg cystine/kg/24 hr, and 1,500 mg pyridoxine·HCl/kg/24 hr. He received no other medications. Assay of cystathionine synthase in the first biopsy established the diagnosis unequivocally. Assay of methionine-activating enzyme and cystathionase were omitted in favor of *in vitro* studies of the metabolism of ³⁵S-methionine, to be reported elsewhere.

Liver Tissue

Liver biopsies were performed with a 70-mm by 1.4-mm Menghini needle. A sample, approximately 5 mm long, was placed in formalin for routine light microscopy. Two pieces, about 1 mm long, were placed in 4.0% glutaraldehyde buffered with cacodylate at pH 7.4 for 2 hours, and one was then placed in cold 1.0% osmium tetroxide, also buffered with cacodylate at the same pH. The osmium-fixed material was washed and dehydrated in graded alcohol and embedded in Epon 812 [23]. Sections were stained with periodic acid-Schiff and toluidine blue for lobular localization [29], and then thin sections were cut with a diamond knife on an ultramicrotome [32]. These were stained with lead citrate and examined with an electron microscope [33]. Results were compared with those obtained in the examination of over 100 biopsies of children and young adults with various metabolic disorders of the liver, including several with hepatic steatosis.

Fig. 2. Small portal tract from same liver as in Figure 1, with neither inflammation nor fibrosis. The bile ductules (arrows) are normal. $\times 630$.

Measurements of the activities of the enzymes of transsulfuration and of the concentrations of amino acids in the plasma were as previously reported [15]. All liver biopsies were obtained while patients were in the fasting state because controls for the assays of the enzymes of transsulfuration were obtained under these conditions.

Results

Light Microscopy

The biopsy specimens of liver all appeared normal before and after treatment, except for modest accumulation of fat in the centrilobular hepatocytes (Fig. 1). Glycogen deposition in hepatocellular nuclei in the periportal zone, usually seen in biopsy specimens from children near puberty, was not noted, and portal inflammation and fibrosis were absent (Fig. 2).

Electron Microscopy

The over-all appearance of the hepatocytes was normal in all four patients. The borders between adjacent

Fig. 1. Photomicrograph of centrilobular portion of liver from patient 3 after treatment. Specimen embedded in epoxy resin and stained with periodic acid-Schiff and toluidine blue. Note the numerous small fat droplets (arrows), and the numerous dense pigment granules in the cells around the central vein (CV). $\times 400$.

cells were normal, as were the bile canaliculi. The sinusoidal borders of every cell had some blebs or so-called edematous microvilli projecting into the space of Disse (Fig. 3). The distribution of chromatin in the nuclei and the nucleolar detail appeared normal in the glutaraldehyde-fixed material where these are best vis-

ualized. The cytoplasm of some hepatocytes was depleted of glycogen presumably because all biopsies were obtained while patients were in a fasting state. Both glycogen-containing and glycogen-depleted cells near the central vein had one to three small fat droplets, seldom larger than $5\ \mu$ in diameter, usually near

Fig. 3. Low power electron micrograph of the cells in Figure 1 showing small fat droplets and pigment granules (arrow). Several irregular microvilli are in the space of Disse (D). The smooth endoplasmic reticulum is hypertrophied and is in the form of small vesicles. $\times 8,000$.

the sinusoidal border. Larger fat droplets occupying more than one-fourth of the area of the section of the cell were rare. The rough endoplasmic reticulum appeared normal in amount in about half the cells and reduced in the others. The smooth endoplasmic reticulum was increased in all cells, often in the form of small vesicles rather than tubules (Figs. 3, 4, 5). The number of mitochondria per section of cell was normal, and the organelles were normal in size although one or two very elongated forms over $5\ \mu$ in length were noted in each block. Megamitochondria or crystalloid inclusions in mitochondria were not found.

The shape of many mitochondria in each cell was unusual. There were finger-like projections up to $1\ \mu$ in length and about $0.1\ \mu$ in diameter. Other mitochondria had ends tapered to a point instead of rounded ends. Occasionally, adjacent mitochondria were linked by narrow projections from each one (Figs. 6, 7, 8). The mitochondrial matrix was of normal density except in the narrowed part, where it was more dense, and the dark granules were larger than usual (Figs. 6, 7). Mitochondrial inner membranes and cristae were intact, but breaks were common in outer membranes (Fig. 8). Microbodies were normal in size and number,

Fig. 4. Hypertrophy of the hepatocellular smooth endoplasmic reticulum in the form of vesicles in noncentrolobular cells (*patient 4* before treatment). The fiber bundle in the space of Disse (arrow) is larger than normal. $\times 8,000$.

Fig. 5. Numerous dark pigment granules (lysosomes) in centrolobular hepatocytes (*patient 3* before treatment). Note irregular shapes of mitochondria. $\times 10,000$.

and the Golgi zones also appeared normal. Pericanalicular lysosomes were large and numerous in the centrolobular zone, more so than would be expected in normal young people. Some clearly were of autophagic origin, since they still contained recognizable remnants of organelles, and most contained residual pigment (Figs. 3, 5, 7).

The fiber bundles in the spaces of Disse were more numerous than in normal persons of comparable age (Fig. 4), but the bundles were not enlarged.

No effect of pyridoxine therapy on the ultrastructure of the hepatocytes could be recognized in specimens from the same patient before and after treatment, even in *patients 1* and *2*, who showed a partial salutary effect.

Discussion

Two organelles, the smooth endoplasmic reticulum and the mitochondria, were altered in the hepatocytes of patients with homocystinuria. Although the significance of these changes should be expressed in terms of altered function, present knowledge of correlations of ultrastructure with function render discussion somewhat speculative. Although none of the changes described was specific, the constellation of structural changes seen in these four patients was consistent and unique. That they occurred in association with a specific enzymatic deficiency in patients receiving no medication renders them of unusual interest.

Each of the mitochondrial abnormalities may have a different significance. Breaks in the outer membranes

Fig. 6. Region around nucleus showing irregular shaped mitochondria with finger-like projections in places apparently linking segments of elongated mitochondria (*patient 2* before treatment). Note the large dense mitochondria (arrows). $\times 24,000$.

have been encountered in alcoholic cirrhosis in man, although usually in megamitochondria with inclusions of crystalloid, and following administration of toxins such as dieldrin or butter-yellow to rats, under which conditions mitochondria are swollen [22]. Neither megamitochondria nor swelling of mitochondria was noted in the livers studied here. The outer membrane of the mitochondria contains both structural and enzymatic proteins [17, 25], including enzymes of fatty acid elongation and oxidation. In light of the changes in the fatty acid profile [6], further study of the metabolism of fatty acids in this disease seems warranted. Mitochondria with peculiar finger-like or pointed projections were reminiscent of mitochondrial changes

that have been described in the mucopolysaccharidoses [20], but no relation to homocystinuria was apparent. Increases in the size of the intramitochondrial dense bodies have been related to the amount of calcium in the organelle [26], although this has been disputed.

Increase in the smooth endoplasmic reticulum is most often associated with inductive adaptation to exogenous toxins and drugs, but the initial biopsy in each of our cases showed these changes without any known exogenous inducers. It would be of interest to know whether or not this hypertrophy of smooth endoplasmic reticulum was accompanied by hyperactivity of enzymes characteristic of this organelle, *e.g.*, hydroxylases, nitroreductases, demethylases, and hemoprotein

Fig. 7. Zone near bile canaliculus (C) showing numerous lysosomes (L) and mitochondria with projections (arrows) (*patient 2* before treatment). $\times 30,000$.

P₄₅₀, or whether it represents a hypertrophic, hypoactive smooth endoplasmic reticulum [22].

Whether or not the increase in smooth endoplasmic reticulum represents a response to an endogenous inducer or toxin cannot be determined. One possibility is that methionine, which was found to accumulate intracellularly in a biopsy from a patient with homo-

cystinuria and was accompanied by an even larger accumulation of methionine sulfoxides [28], induces the proliferation of the smooth endoplasmic reticulum. Analogously, chlorpromazine, which is enzymatically converted to a sulfoxide as the first step in detoxification [16], induces proliferation of the smooth endoplasmic reticulum. Although the concentrations of

Fig. 8. Mitochondria with segments of their outer membranes missing (*patient 1* before treatment). Some have long, finger-like projections and the mitochondrial dense granules are large. $\times 36,000$.

methionine and homocystine in the plasma were reduced in *patients RC* and *MC*, it is not known to what extent the intracellular concentrations were affected. This point is now under investigation.

The numerous pigment-containing lysosomes in the pericanalicular zone, including some autophagic vacuoles, suggested that organelle turnover had been increased, possibly as a result of injury. The breaks in the outer membranes of the mitochondria supported this suggestion, as did the increase in the number of collagen bundles in the space of Disse. The injury and fibrosis, however, were so mild that clinically apparent liver injury would not be expected to occur, and, in fact, results of routine hepatic tests were normal in all four patients.

The fact that the structural abnormalities persisted even when massive doses of pyridoxine resulted in a disappearance of the hypermethioninemia and homo-

cystinemia in *patients 1* and *2* supports the hypothesis that accumulations of metabolites proximal to an enzymatic block do not alone result in all of the phenotypic manifestations of the disease [7, 10, 11]. Some patients with homocystinuria treated with massive doses of pyridoxine also show a return to normal of the low concentrations of cystine in the plasma; *i.e.*, metabolism of compounds distal to the block is favorably affected. Two such patients have been studied enzymatically but not ultrastructurally [15]. The role of abnormal metabolism of cysteine in homocystinuria and its possible role in the pathogenesis of the ultrastructural changes appear worthy of further study. These findings reinforce the contention that examination of blood is not a total body biopsy and that the concentrations in the plasma of metabolites proximal to the enzymatic block may be an incomplete index of a salutary therapeutic response [10, 11].

Summary

Hepatocytes from four untreated patients with homocystinuria showed bizarrely shaped mitochondria and an increase in the smooth endoplasmic reticulum and lysosomes. Breaks in the outer membranes of the mitochondria and mitochondrial dark granules which were larger than normal were also seen. This picture remained unchanged in two patients who responded to massive doses of pyridoxine with disappearance of hypermethioninemia and homocystinemia, suggesting that accumulations of these metabolites were not responsible for the ultrastructural changes in the liver.

References and Notes

1. BARBER, G. W., AND SPAETH, G. L.: Pyridoxine therapy in homocystinuria. *Lancet*, **1**: 337 (1967).
2. BARBER, G. W., AND SPAETH, G. L.: The successful treatment of homocystinuria with pyridoxine. *J. Pediatr.*, **75**: 463 (1969).
3. BRENTON, D. P., CUSWORTH, D. C., AND GAULL, G. E.: Homocystinuria. Some biochemical studies. In: *Proceedings of the International Congress for the Scientific Study of Mental Retardation*, Copenhagen, p. 79 (1964).
4. BRENTON, D. P., CUSWORTH, D. C., AND GAULL, G. E.: Homocystinuria. Biochemical studies of tissues including a comparison with cystathioninuria. *Pediatrics*, **35**: 50 (1965).
5. CARSON, N. A. J., AND CARRÉ, I. J.: Treatment of homocystinuria with pyridoxine. *Arch. Dis. Childhood*, **44**: 387 (1969).
6. CARSON, N. A. J., DENT, C. E., FIELD, C. M. B., AND GAULL, G. E.: Homocystinuria: Clinical and pathological review of ten cases. *J. Pediatr.*, **66**: 565 (1965).
7. CUSWORTH, D. C., AND DENT, C. E.: Homocystinuria. *Brit. Med. Bull.*, **25**: 42 (1969).
8. FINKELSTEIN, J. C., MUDD, S. H., IRREVERRE, F., AND LASTER, L.: Homocystinuria due to cystathionine synthetase deficiency: The mode of inheritance. *Science*, **146**: 785 (1964).
9. GAULL, G.: Homocystinuria. *Advan. Teratol.*, **11**: 101 (1967).
10. GAULL, G. E.: The pathogenesis of homocystinuria. Implications for treatment. *Amer. J. Dis. Child.*, **113**: 103 (1967).
11. GAULL, G. E.: The pathogenesis of inborn errors of amino acid metabolism: Are classical concepts adequate? In: *E. van Praag: Brain Damage in Inborn Errors of Metabolism*, p. 67 (Bohn, Haarlem, 1968).
12. GAULL, G.: Abnormal metabolism of sulphur-containing amino acids associated with brain dysfunction. In: *A. Lajtha: Handbook of Neurochemistry*, vol. VII (Pergamon Press, New York, in press).
13. GAULL, G. E., CARSON, N. A. J., DENT, C. E., AND FIELD, C. M. B.: Homocystinuria: Clinical and pathological description of 10 cases. In: *Proceedings of the International Congress for the Scientific Study of Mental Retardation*, Copenhagen, p. 91 (1964).
14. GAULL, G. E., RASSIN, D. K., AND STURMAN, J. A.: Pyridoxine dependency in homocystinuria. *Lancet*, **2**: 1302 (1968).
15. GAULL, G. E., STURMAN, J. A., AND RASSIN, D. K.: Enzymatic and metabolic studies of homocystinuria: Effects of pyridoxine. *Neuropädiatrie*, **1**: 199 (1969).
16. GILLETTE, J. R., AND KAMM, J. J.: The enzymatic formation of sulfoxides: The oxidation of chlorpromazine and 4,4'-diaminophenyl sulfide by guinea pig liver microsomes. *J. Pharmacol. Exp. Ther.*, **130**: 262 (1960).
17. GREEN, D. E., AND PERDUE, J. F.: Correlation of mitochondrial structure and function. *Ann. N. Y. Acad. Sci.*, **137**: 684 (1966).
18. HALLOWELL, J. G., JR., CORYELL, M. E., HALL, W. K., FINDLEY, J. F., AND THEVAOS, T. G.: Homocystinuria as affected by pyridoxine, folic acid and vitamin B₁₂. *Proc. Soc. Exp. Biol. Med.*, **129**: 327 (1968).
19. HAMBRAEUS, L., WRANNE, L., AND LORENTSSON, R.: Biochemical and therapeutic studies in two cases of homocystinuria. *Clin. Sci.*, **35**: 457 (1968).
20. HAUST, M. D., ORIZAGA, M., BRYANS, A. M., AND FRANK, H. F.: The fine structure of liver in children with Hurler's syndrome. *Exp. Molec. Pathol.*, **10**: 141 (1969).
21. HOOFT, C., CARTON, D., AND SAMYN, W.: Pyridoxine treatment in homocystinuria. *Lancet*, **1**: 384 (1967).
22. HUTTERER, F., KLION, F. M., WENGRAF, A., SCHAFFNER, F., AND POPPER, H.: Hepatocellular adaptation and injury. Structural and biochemical changes following dieldrin and methyl butter yellow. *Lab. Invest.*, **20**: 455 (1969).
23. LUFT, J. H.: Improvements in epoxy resin embedding methods. *Biophys. Biochem. Cytol.*, **9**: 409 (1961).
24. MUDD, S. H., FINKELSTEIN, J. D., IRREVERRE, F., AND LASTER, L.: Homocystinuria: An enzymatic defect. *Science*, **143**: 1143 (1964).
25. PARSONS, D. F., WILLIAMS, G. R., AND CHANCE, B.: Characteristics of isolated and purified preparations of the outer and inner membranes of mitochondria. *Ann. N.Y. Acad. Sci.*, **137**: 643 (1966).
26. REYNOLDS, E. S.: Liver parenchymal cell injury. III. The nature of calcium-associated electron-opaque masses in rat liver mitochondria following poisoning with carbon tetrachloride. *J. Cell Biol.*, **25**: 53 (1965).
27. SCHIMKE, R. N., MCKUSICK, V. A., HUANG, T., AND POLLACK, A. D.: Homocystinuria. Studies of 20 families with 38 affected members. *J. Amer. Med. Ass.*, **193**: 711 (1965).
28. STURMAN, J. A., AND GAULL, G. E.: Unpublished observations.
29. TRUMP, B. F., SMUCKLER, E. A., AND BENDITT, E. P.: A method for staining epoxy sections for light microscopy. *J. Ultrastruct. Res.*, **5**: 343 (1961).
30. TURNER, B.: Pyridoxine treatment in homocystinuria. *Lancet*, **2**: 1151 (1967).
31. YOSHIDA, T., TADA, K., YOKOYAMA, Y., AND ARAKAWA, T.: Homocystinuria of the vitamin B₆ dependent type. *Tohoku J. Exp. Med.*, **96**: 235 (1968).
32. Ultratome III, LKB Instruments, Rockville, Md.
33. Hitachi HS 7S, Hitachi, Ltd., Tokyo, Japan.
34. We are grateful to Miss M. Kalin for the calculation and planning of special diets and to Miss K. Young and her staff for superb research nursing. Informed consent was obtained in accordance with the provisions set forth in the Declaration of Helsinki.
35. This work was supported by Public Health Service Research Grant no. AM 03846, by Clinical Research Center Grant no. FR-71 from the National Institutes of Health, and by the New York State Department of Mental Hygiene.
36. Requests for reprints should be addressed to: Fenton Schaffner, M.D., Department of Pathology, Mount Sinai School of Medicine of The City University of New York, New York, N. Y. 10029 (USA).
37. Accepted for publication May 12, 1970.