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The Effect of D-Penicillamine on Protein-Bound Homocyst(e)ine in Homocystinurics

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Summary

There is considerable evidence that homocystine has a direct damaging effect on vascular endothelium and other tissues. The demonstration of the existance of protein-bound homocyst(e)ine has strengthened this hypothesis. In an attempt to remove bound homocyst(e)ine, D-penicillamine was given to three patients with pyridoxine-nonresponsive homocystinuria. Before the clinical trial, it had been demonstrated that 0.1 μmole per ml concentration of D-penicillamine or cysteamine released approximately 50% of the homocyst(e)ine bound to plasma proteins *in vitro*. Oral D-penicillamine effectively reduced both free and plasma protein-bound homocyst(e)ine in homocystinurics from the second day of treatment. The homocystine excreted in the urine was mainly in the form of homocysteine-penicillamine disulfide. No mixed disulfide was detectable in the plasma, indicating an extremely high renal clearance. These observations suggested that oral D-penicillamine removed a considerable quantity of the bound homocyst(e)ine accumulated in the tissue proteins.

Speculation

D-Penicillamine treatment may be used on an experimental basis in pyridoxine-nonresponsive homocystinuric patients when dietary treatment is not practical. It may also be used in pyridoxine-responsive patients when control with pyridoxine is unsatisfactory. In addition, determination of protein-bound homocyst(e)ine should be used for the assessment of the effectiveness of therapy.

Homocystinuria may be due to a metabolic block in the synthesis of cystathionine from homocysteine and serine, or due to defects in the remethylation of homocysteine to methionine, resulting in an accumulation of homocystine in the plasma and urine (1, 3, 8, 13–15, 17, 20); however, homocystine is not found in tissue fluids of normal subjects when conventional methods are used for detection.

Recently, using a new method, we demonstrated the presence of protein-bound homocyst(e)ine in the plasma of both normal and homocystinuric subjects (10). Furthermore, we were able to demonstrate a significantly elevated concentration of protein-bound homocyst(e)ine (7–10-fold of normal value) in pyridoxine responsive patients when free homocystine was undetectable in the plasma. A large amount of protein-bound homocyst(e)ine was also demonstrated in the brain, liver and kidney of homocystinuric patients. These observations might account for the findings that some clinical abnormalities such as ectopia lentis and abnormal morphology in the hepatocytes persisted or developed in pyridoxine responsive homocystinuric patients despite the absence of free homocystine in their tissue fluids (4, 5, 7). We postulated that the formation of protein-bound homocyst(e)ine might be responsible for the direct damaging effect of homocystine on the vascular endothelium and other tissues. We therefore attempted to dissociate and remove protein-bound homocyst(e)ine.

The studies in this paper provide evidence that oral D-penicillamine produces a significant reduction of free and plasma protein-bound homocyst(e)ine in patients with pyridoxine-nonresponsive homocystinuria.

MATERIALS AND METHODS

Subjects. Patient I was a 21-year-old female. Patient II was a 15-year-old male and Patient III was a 13-year-old brother of Patient II. Each of these patients had the clinical and biochemical characteristics of cystathionine synthase deficiency and was pyridoxine-nonresponsive. All tests were performed during a period of reasonably constant protein intake. The dosage of oral D-penicillamine was 75 mg per kg body weight per day for the first 3 days, and 50 mg per kg per day for the rest of the experimental period. An informed consent was obtained for all procedures.

Synthesis of homocysteine-penicillamine disulfide. The synthesis of L-homocysteine-D-penicillamine disulfide was carried out according to the method described by Crawhall *et al.* (2) for the synthesis of cysteine-penicillamine disulfide. L-homocysteine thiolactone hydrochloride (Sigma), 9.6 mmole, and D-penicillamine (Sigma), 1.9 mmole, were dissolved in 20 ml of 4 N ammonium hydroxide. After the addition of 0.2 ml of 5% ferric chloride, the reaction was allowed to proceed at room temperature for 18 h. The reaction mixture was saturated with nitrogen during this period. The soluble fraction was separated by centrifugation and was lyophilized by freeze-drying. The dry powder was redissolved in water and was applied to an ion exchange chromatographic column (Dowex 50W-X4 in the H^+ form; 1.2 × 25 cm). A gradient elution of increasing pH and salt concentration with ammonium hydroxide and ammonium bicarbonate was used. The ninhydrin reaction was used for the detection of homocystine, D-penicillamine and homocysteine-penicillamine disulfide in the eluent. D-penicillamine, homocysteine-penicillamine disulfide and homocystine were eluted in that sequence without overlapping. The ammonia and ammonium bicarbonate were removed by evaporation in a rotary evaporator at 35°C. The disulfide synthesized was contaminated with a small quantity of homocystine and penicillamine. The solubility of homocysteine-penicillamine disulfide was 112 mg/100 ml water at 22°C, whereas that of L-homocystine was 11.2 mg/100 ml of water.

Determination of [^{35}S]-homocystine bound to protein. Aliquots of 0.1 ml fresh human plasma or 20 mg/ml of human serum albumin were incubated in the presence of 5 or 100 μM homocystine in a total volume of 0.5 ml mixture at 37°C for 2 h. The homocystine contained 4×10^3 cpm of L-[^{35}S]-homocystine (Amersham, specific activity, 40 mCi/mmole). The chemical being tested for its effect on homocystine binding was added in a concentration of 100 nmole/ml. The mixtures were reincubated for another 2 h. The reactions were terminated by the addition of buffered sulfosalicylic acid. Protein precipitates were separated by centrifugation and washed twice with 5 ml of buffered sulfosalicylic acid. The radioactivity in the washed precipitates was determined and the quantity of homocyst(e)ine bound to the protein was calculated.

Determination of free and protein-bound homocyst(e)ine. Hepa-

rinized venous blood was immediately centrifuged and the plasma sample was immediately precipitated with four volumes of 3.75% sulfosalicylic acid in 0.3 M lithium citrate buffer, pH 2.0. The acid precipitates of the plasma were washed twice with 3.75% sulfosalicylic acid, resuspended in water to the original volume of plasma, mixed with 1/10 volume of 2-mercaptoethanol, neutralized to pH 7, and incubated at 37°C for 120 min. Immediately after incubation, the mixture was treated with iodoacetic acid for 10 min and the protein was then reprecipitated with sulfosalicylic acid. The supernatant was used for the determination of protein-bound homocyst(e)ine. S-Carboxymethylation of protein-bound sulfhydryl compounds was described elsewhere (11). Urine samples were obtained from the pooled specimens of 24 h collections. The amount of homocystine and homocysteine-penicillamine disulfide was determined, using a Beckman Model 121M Amino Acid Analyzer, with a single column.

RESULTS

Effect of sulfhydryl compounds on homocyst(e)ine bound to plasma proteins in vitro. It was previously observed that in normal plasma incubated with 0.02–0.1 mM homocystine at 37°C for 2 h, more than 40% of exogenous homocystine was recovered as protein-bound homocyst(e)ine from the acid precipitate (10). Hence, a similar system was used to determine the effect of various compounds on [^{35}S]-homocyst(e)ine bound to proteins *in vitro*. After exogenous radioactive homocystine was allowed to bind to the protein molecules, the homocystine-protein mixtures were reincubated with the chemicals being tested (Table 1 and 2). It was found that ascorbic acid and cystine had no effect on the dissociation of homocyst(e)ine bound to plasma proteins. D-Penicillamine and cysteamine, on the other hand, released approximately 50% of the homocyst(e)ine from plasma proteins under the conditions of the experiments (Table 1). The results obtained by using human serum albumin showed an essentially similar pattern (Table 2).

Effect of oral D-penicillamine on free and protein-bound homocyst(e)ine in homocystinurics. D-Penicillamine has been used as a therapeutic agent in a number of human diseases. Its mode of action as well as side effects have been relatively well documented (9, 12, 16, 19). We, therefore, have selected D-penicillamine in the clinical trial for the release of protein-bound homocyst(e)ine in homocystinuric patients. It was found that the amount of plasma free homocystine progressively decreased from day 0 to the 73rd day of treatment (Table 3). Patient III, who had relatively low free homocystine initially, had only a small amount on the 73rd day. Plasma protein-bound homocyst(e)ine also decreased significantly from the 2nd day of oral D-penicillamine treatment (Table 3). Patient I and Patient II had very high plasma methionine concentrations (260–380 nmole/ml), whereas Patient III had moderate plasma methionine concentrations (40–110 nmole/ml); however, no significant change in methionine levels was observed during D-penicillamine therapy. Plasma free cystine concentrations were at low levels both before and during treatment. In contrast, plasma protein-bound cyst(e)ine levels decreased to approximately 40–60% of pretreatment values after 73 days of treatment (Table 3). No significant quantity of homocysteine-penicillamine disulfide was detected in the plasma from the patients throughout the period of treatment. In contrast, the major portion of homocystine was excreted as homocysteine-penicillamine disulfide in the urine (Fig. 1). Total homocystine excretion (*i.e.*, free homocystine plus homocysteine-penicillamine disulfide) increased significantly as compared with homocystine excretion before treatment; however, the total daily excretion of homocystine decreased consistantly with the progression of treatment (Fig. 1).

Table 1. *In vitro [^{35}S]-homocystine binding in plasma proteins*

Concentration of homocystine in incubation mixture	5 μM		100 μM	
Protein-bound homocyst(e)ine[1]	nmole/ml plasma	%	nmole/ml plasma	%
Chemical added				
None	4.8	100	60.1	100
Ascorbic acid	4.9	102	79.4	132
Cystine	4.6	95	59.7	99
Cysteine	2.6	62	50.8	85
D-penicillamine	2.1	44	39.1	65
Cysteamine	2.4	50	37.0	62

[1] Mean of triplicate determinations.

Table 2. *In Vitro [^{35}S]-homocystine binding in serum albumin*[1]

Chemical added	Protein-bound homocyst(e)ine[2] nmole/g albumin	%
None	44.0	100
Ascorbic acid	40.5	92
Cysteine	36.5	83
D-penicillamine	25.5	58
Cysteamine	23.9	48

[1] Incubation mixture contained 100 μM homocystine.
[2] Mean of triplicate determinations.

Table 3. *Free and protein-bound cyst(e)ine and homocyst(e)ine in the plasma (nmole/ml) of homocystinuric patients during treatment with D-penicillamine*

		Days of treatment				
	Patient	0	2	4	6	73
Free cystine	I	0.6	0.9	1.1	0.9	0.2
	II	0.6	0.4	0.2	1.0	0.5
	III	1.6	1.6	1.7	0.3	0.6
Protein-bound cyst(e)ine	I	18.8	10.5	12.5	11.5	7.9
	II	22.7	19.0	20.5	18.1	14.2
	III	29.9	27.4	24.2	22.0	18.7
Free homocystine	I	137	108	84	80	24
	II	76	70	64	42	30
	III	45	30	19	16	4
Protein-bound homocyst(e)ine	I	40	21	19	18	10
	II	27	20	16	16	13
	III	31	21	16	10	8

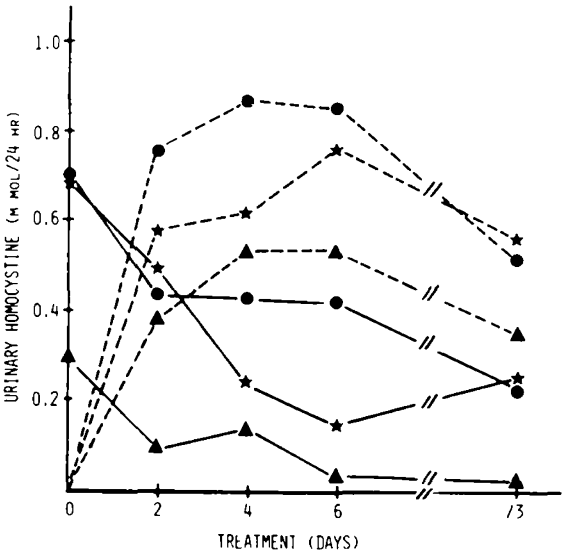

Fig. 1. Urinary excretion of homocystine and homocysteine-penicillamine disulfide during D-penicillamine therapy. The *solid lines* indicate homocystine and the *broken lines* indicate homocysteine-penicillamine disulfide. *Circles*, Patient I; *stars*, Patient II; and *triangles*, Patient III.

The toxic effects of D-penicillamine were monitored by physical examination and laboratory studies, including blood cell counts, urine analysis, and coagulation tests. No undesirable side effects of oral D-penicillamine were observed in these patients.

DISCUSSION

D-Penicillamine is an analogue of the naturally occurring amino acid, cysteine, and is metabolized by the body to a small extent. This compound has been widely used for the treatment of Wilson's disease (19), heavy metal poisoning (6, 9, 18), cystinuria (2, 12) and rheumatoid arthritis (16). The present studies have demonstrated that both cysteamine and D-penicillamine released homocyst(e)ine bound to protein molecules efficiently. The mechanism of the release with D-penicillamine probably was related to the formation of homocysteine-penicillamine disulfide, which increased markedly in the urine during treatment. Because the mixed disulfide was undetectable in the plasma, it was possible that the high renal clearance of this compound contributed to a facilitated excretion of homocystine from the tissue fluids as well as from tissue proteins.

A marked decrease of both plasma free and protein-bound homocyst(e)ine during D-penicillamine treatment in patients with homocystinuria has been demonstrated in this study. Thus, D-penicillamine may be used on an experimental basis in pyridoxine-nonresponsive homocystinuric patients when dietary treatment is not practical. It may also be considered as an adjunct therapeutic agent for pyridoxine responsive homocystinurics when pyridoxine alone is inadequate to produce a satisfactory chemical response. Inasmuch as there was a significant decrease in protein-bound cyst(e)ine as well as homocyst(e)ine, dietary supplementation of cystine appeared to be important during D-penicillamine treatment.

The observations in these experiments suggest that the determination of plasma free and protein-bound homocyst(e)ine or total homocystine is more valuable and informative than the traditional determination of free homocystine for the assessment of treatment in homocystinurics. Because the method is simple, it should be used routinely by all laboratories dealing with homocystinuric patients.

REFERENCES AND NOTES

1. Carson, N. A., Dent, C. E., Field, C. M. B., and Gaull, G. E.: Homocystinuria: clinical and pathological review of ten cases. J. Pediatr., *66:* 565 (1965).
2. Crawhall, J. C., Scowen, E. F., and Watts, R. W. E.: Further observations on the use of D-penicillamine in cystinuria. Brit. Med. J., *1:* 1411 (1964).
3. Freeman, J. M., Finkelstein, J. D., and Mudd, S. H.: Folate responsive homocystinuria and schizophrenia: a defect in methylation due to deficient 5, 10-methylenetetrahydrofolate reductase activity. N. Engl. J. Med., *292:* 401 (1975).
4. Gaull, G. E. and Schaffner, F.: Electronmicroscopic change in hepatocytes of patients with homocystinuria. Pediatr. Res., *5:* 23 (1971).
5. Gaull, G. E., Sturman, J. A., and Schaffner, F.: Homocystinuria due to cystathionine synthase deficiency: enzymatic and ultrastructural studies. J. Pediatr., *84:* 381 (1974).
6. Goldberg, A., Smith, J. A., and Lochhead, A. C.: Treatment of lead poisoning with oral penicillamine. Brit. Med. J., *1:* 1270 (1963).
7. Hagberg, B., Hambraeus, L., and Bensch, K.: A case of homocystinuria with a dystonic neurological syndrome. Neuropediatric, *1:* 337 (1970).
8. Hollowell, J. G. Jr., Hall, W. K., Coryell, M. E., McPherson, J. Jr., and Hahn, D. A.: Homocystinuria and organic aciduria in a patient with vitamin B_{12} deficiency. Lancet, *2:* 1428 (1969).
9. Harris, C. E. C.: A comparison of intravenous calcium disodium versenate and oral penicillamine in promoting elimination of lead. Can. Med. Ass. J., *79:* 664 (1958).
10. Kang, S., Wong, P. W. K., and Becker, N.: Protein-bound homocystine in normal subjects and in patients with homocystinuria. Pediatr. Res., *13:* 1141 (1979).
11. Kang, S., Wong, P. W. K., and Milanez, S.: Determination of protein-bound sulfhydryl amino acids. Submitted to Anal. Biochem.
12. McDonald, W. B. and Fellers, F. X.: Penicillamine in the treatment of patients with cystinuria. J. Amer. Med. Ass., *197:* 396 (1966).
13. Mudd, S. H., Finkelstein, J. D., Irreverre, F., and Lancaster, L.: Homocystinuria: an enzymatic defect. Science, *143:* 1443 (1964).
14. Mudd, S. H., Levy, H. L., and Abeles, R. H.: A derangement in B_{12} metabolism leading to homocystinuria, cystathioninemia and methyl malonic aciduria. Biochem. Biophysi. Res. Commun., *35:* 121 (1969).
15. Mudd, S. H., Uhlendorf, B. W., Freeman, J. M., Finkelstein, J. D., and Shih, V. E.: Homocystinuria associated with decreased methyltetrahydrofolate reductase activity. Biochem. Biophys. Res. Commun., *46:* 905 (1972).
16. Multicentre Trial Group. Controlled trial of D(-)penicillamine in severe rheumatoid arthritis. Lancet, *1:* 275 (1973).
17. 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).
18. Selander, S.: Treatment of lead poisoning: effects of sodium calcium edetate and penicillamine administered orally and intravenously. Brit. J. Int. Med., *24:* 272 (1967).
19. Sternlieb, I. and Scheinber, I. H.: Penicillamine therapy for hepatolenticular degeneration. J. Am. Med. Ass., *189:* 784 (1964).
20. Wong, P. W. K., Justice, P., Hruby, M., Weiss, E. B., and Diamond, E.: Folic acid nonresponsive homocystinuria due to methylenetetrahydrofolate reductase deficiency. Pediatrics, *59:* 749 (1977).
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