Zinc and Zinc-dependent Enzymes in Penicillamine-Treated Patients with Generalized Scleroderma

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In 7 penicillamine-treated patients with generalized scleroderma zinc in serum, erythrocytes and granulocytes, alkaline phosphatase activity in serum and granulocytes, carbonic anhydrase activity in erythrocytes were examined. No significant difference was found between patient and control values, but granulocyte zinc strongly tended to be decreased ($0.1 > p > 0.05$). It is our hypothesis that penicillamine may produce a zinc depletion at a

cellular level. *Key words: Granulocyte-zinc; Erythrocyte-zinc; Zinc-depletion.* (Received February 21, 1984.)

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In generalized scleroderma (GS) dermal fibroblasts have been found to synthetize abnormally large quantities of both glycosaminoglycans and collagen (1, 2). Penicillamine (PS) has been used as a therapeutic agent because of its influence on the collagen metabolism (3). The biological actions of PS predominantly depend on reaction with carbonyl groups, thiol-disulphide reactions and chelation of metals (4). The last mentioned effect might degenerate into a side effect as metals are often acting as co-factors of enzymes. Over 70 metallo-enzymes are known to require zinc for their function. Some of these enzymes are participating in the synthesis of RNA and DNA (5).

In rheumatoid arthritis the zinc concentration in granulocytes decreased during 6 months of penicillamine therapy (6), indicating a tissue depletion. As the zinc metabolism in GS has only been examined by means of urine zinc and serum zinc (S–Zn) (7), we found it relevant also to examine what happens to zinc at a cellular level (granulocytes and erythrocytes) and to the zinc dependent enzymes (alkaline phosphatase EC 3.1.3.1. and carbonic anhydrase EC 4.2.1.1.).

MATERIAL AND METHODS

Eight patients (3 males and 5 females) with GS, age 35–66 years (mean 51.8 years), from an out-patient clinic were included in the study. Treatment with penicillamine had been given for a period of 2.5–7.5 years (mean 5 years). The maintenance dose ranged from 300–750 mg/24 hours. One patient received glutamin 300 mg daily, and one patient diuretics. One patient was excluded from the study because she had taken oestrogens which are known to influence the zinc metabolism (8).

The control group comprised voluntary healthy hospital staff members.

The two groups were matched according to age and sex. Fasting blood samples (4 ml serum and 20 ml heparinized venous blood) were taken between 8 and 10 a.m. Zinc was determined in serum, erythrocytes (E-Zn) and granulocytes (Gr-Zn), alkaline phosphatase in serum (S-AP) and granulocytes (Gr-AP) and carbonic anhydrase in erythrocytes (E-HCA).

Equipment

Zinc was determined by atomic absorption spectrophotometry (Perkin Elmer 372). Alkaline phosphatase was assayed by means of a Gilford photometer. Carbonic anhydrase activity was measured in a specially constructed glass-chamber, supplied with a water envelope of a constant temperature (1°C). An air stream (1 800 ml/min) passed through the floor, made of a porous plate.

Separation

The separation of whole blood in granulocytes and erythrocytes was achieved by a Percoll® density gradient in the following way, described in a previous paper (9): An isosmotic stock solution was prepared from 63 ml Percoll® (density 1.131 kg/l) and 7 ml NaCl 1.5 mol/l. By further dilution with NaCl 0.15 mol/l solution I (density 1.075 kg/l) and solution II (density 1.096–1.098 kg/l) were made. The heparinized venous blood was diluted 1:2 with NaCl 0.15 mol/l and 4 ml distributed in each test-tube. Then 3 ml of solution I were placed below the blood and after that 3 ml of solution II below solution I. The test-tubes were now centrifuged at 200 G for 25 min at room temperature (20–22°C). Corresponding to the interphases two separate bands (the upper (which was discharged) made of mononuclear cells and thrombocytes, the lower of granulocytes) were seen, and at the bottom the erythrocytes. After separation the granulocytes were transferred to 0°C NaCl 0.15 mol/l (centrifugated at 4°C and 800 G for 10 min). Additionally, two washes were done in 5 ml 0°C NaCl 0.15 mol/l (centrifugation at 4°C and 200 G for 10 min). Contaminating erythrocytes were lysed in between by re-suspending the granulocytes in 1 ml 0°C NaCl 0.15 mol/l, adding 3 ml 0°C redistilled water (giving a hypotonic solution of NaCl 0.0325 mol/l) and after 90 sec making the solution isoton by adding 1 ml 0°C NaCl 0.616 mol/l (centrifugation at 4°C and 800 G for 10 min). Finally, the granulocytes were resuspended in NaCl 0.15 mol/l, counted on a Coulter Counter (in duplicate) and 1 ml stored at −18°C. A minimum of 25 000 granulocytes per microliter are necessary.

The isolated erythrocytes were transferred to 0°C NaCl 0.15 mol/l, subsequently washed thrice (centrifugation at 4°C, 1000 g for 5 min), diluted with redistilled water (1 : 10) and stored at −18°C (the concentration of zinc in this hemolysate equals that of serum).

Zinc determination

All samples were deproteinized with trichloroacetic acid (TCA), serum and erythrocytes with TCA 5%, granulocytes with TCA 10% containing Zn 2.5 µmol/l (granulocytes were, prior to deproteinizing digested with papain for 24 hours at 60°C). Zinc was determined by aspirating the supernatant, in case of granulocytes via a Teflon sampling cup (which reduced the aspirated volume to 100 µl). The method has been described in details in another paper (10). Samples belonging together were analysed in the same run.

Determination of enzymes

AP in plasma was assayed by the Scandinavian recommended method (11), which was used for alkaline phosphatase in granulocytes (Gr–AP) too. Three ×20 µl of isolated granulocytes were transferred to 3×2 ml diethanolamine solution (0.01 ml/l + $MgCl_2$ 0.505 mmol/l), sonicated (5 microns) for 4 min, stored at 4°C and analysed the following day.

The carbonic anhydrase in isolated erythrocytes was measured by the dehydration reaction according to Hansen & Magid (12). The hemolysate for the assay contained $2.4–2.6 \times 10^{-5}$ mol hemoglobin/l. It was stored at 4°C and analysed within 10 days (6).

Statistics

Student's test for unpaired data.

RESULTS AND DISCUSSION

The results appear from Table I. Mean S-Zn, S-AP and Gr-AP in GS were not different from control values. E-Zn and E-HCA were not significantly changed, but tended to be decreased. Gr-Zn was decreased though not significantly. No difference was observed between the activity of Gr-AP in GS and controls.

The finding of unchanged S-Zn and S-AP is in agreement with the findings of Knudsen & Weisman (7), who reported unchanged S-Zn values during the first 16 weeks of PS treatment in GS. In the present study actually one S-Zn value was significantly decreased and one increased. E-Zn and E-HCA have not previously been examined in PS treated GS.

In PS treated patients with rheumatoid arthritis E-Zn increased and E-HCA decreased during the first 6 months of treatment (6). The apparent discrepancy between the behaviour of E-Zn in the two investigations may be due to the dissimilarity of the patients or it may reflect the different periods of treatment. The activity of E-HCA was decreased in both studies. Though E-HCA is not inhibited in vitro by PS (unpublished data) an in vivo inhibition of the enzyme cannot be excluded, but it is made less likely. A decreased

Table I. *Zinc and zinc dependent enzymes in GS and controls*

Values: mean ± SD. Number of patients in parentheses. AP=alkaline phosphatase, HCA=carbonic anhydrase, E=erythrocytes, Gr=granulocytes

	S-Zn (µmol/l)	S-AP (units/l)	E-Zn (mmol/ mol Hgb)	E-HCA (units × 10^{10}/ mol Hgb)	Gr-Zn (µmol/ 10^{10} gran.)	Gr-AP (units/ 10^{10} gran.)
GS (7)	14.4±3.04	164±43	10.0±1.06	0.94±0.11	0.94±0.13	118±113
Controls (13)	14.0±1.0	149±41	10.6±1.05	1.02±0.14	1.07±0.18	160±94
t-test (unpaired data)	t=0.547	t=0.768	t=1.154 $0.15 > p > 0.1$	t=1.305 $0.15 > p > 0.1$	t=1.684 $0.1 > p > 0.05$	t=0.889

activity may else be due to a decreased concentration of one or both isoenzymes in the red cells (13).

The zinc concentration of the granulocytes was not significantly decreased. The finding is, however, in agreement with earlier findings of decreasing Gr-Zn during PS treatment of rheumatoid patients.

CONCLUSION

None of the parameters turned out to be significant, probably because of the small number of patients available in the department (the remaining GS received combined therapy and were not fit for the study). If the present results, however, are added to our previous results (6), it seems as if a relatively moderate dose of penicillamine may provoke a zinc depletion at a cellular level. The consequences are not clear, but as zinc, for instance, influences the function of granulocytes (14) some change might be produced in the inflammatory response.

REFERENCES

1. Uitto J, Bauer EA, Eisen AZ. Scleroderma. Increased biosynthesis of triple-helical type I and type III procollagens associated with unaltered expression of collagenase by skin fibroblasts in culture. J Clin Invest 1979; 64: 921–929.
2. Uitto J, Helin G, Helin P, Lorenzen I. Connective tissue in scleroderma. Acta Derm Vereneol (Stockh) 1971; 51: 401–406.
3. Rodnan GP. Progressive systemic sclerosis and penicillamine. J Rheumatol (Suppl. 7) 1981; 8: 116–120.
4. van de Stadt RJ, Muijsers AO, Henrichs AMA, van der Korst JK. D-penicillamine: Biochemical, metabolical and pharmacological aspects. Scand J Rheumatol 1979; Suppl. 28: 13–20.
5. Lindeman RD, Mills BJ. Zinc homeostasis in health and disease. Mineral Electrolyte Metab 1980; 3: 233–236.
6. Jepsen LV, Hørslev Pedrsen K. Changes in zinc and zinc-dependent enzymes in rheumatoid patients during penicillamine treatment. Scand J Rheumatol. Accepted for publication.
7. Knudsen L, Weismann K. Taste dysfunction and changes in zinc and copper metabolism during penicillamine therapy for generalized scleroderma. Acta Med Scand 1978; 204: 75–79.
8. Cumming F, Briggs MH, Fardy JJ. Some aspects of the effect of oral contraceptives on trace element metabolism. In: Trace elements metabolism in man and animal. Howell JMcC, Gawthorne J, eds. Berlin, Heidelberg, New York: Springer-Verlag, 1983: 265.
9. Jepsen LV, Skottun T. A rapid one-step method for the isolation of human granulocytes from whole blood. Scand J Clin Lab Invest 1982; 42: 235–238.
10. Jepsen LV. Determination of zinc in serum, granulocytes and erythrocytes by flame atomic absorption spectrophotometry. Scand J Clin Lab Invest. Accepted for publication.
11. Committee of Enzymes, the Scandinavian Society for Clinical Chemistry and Clinical Physiology. Recommended methods for the determination of four enzymes in blood. Scand J Clin Lab Invest 1974; 33: 291–303.
12. Hansen P, Magid E. Studies on a method of measuring carbonic anhydrase activity. Scand J Clin Lab Invest 1966; 18: 21–32.
13. Weatherall DJ, McIntyre PA. Developmental and acquired variations in erythrocyte carbonic anhydrase isoenzymes. Br J Hematol 1967; 13: 106–114.
14. Chvapil M, Stankova L, Weldy P, Bernard D, Campbell J, Carlson E, Cox T, Peacock J, Bartos Z, Zukoski C. The role of zinc in the function of some inflammatory cells. In: Zinc metabolism: Current aspects in health and disease. Brewer GJ, Prasad, AS, eds. New York: Alan R. Liss Inc., 1977: 103.