Zinc Sulfate-Failure as an Accelerator of Collagen Biosynthesis and Fibroblast Proliferation (35900)

MICHAEL D. WATERS, ROBERT D. MOORE, JOSEPH J. AMATO, AND JOHN C. HOUCK

Biomedical Department, Biophysics Laboratory, Research Laboratories, Edgewood Arsenal, Maryland 21010; Biochemical Research Laboratory, Children's Hospital, Washington, D.C. 20009; and Department of Pediatrics, George Washington University, School of Medicine, Washington, D. C. 20005

Although its precise role in tissue repair is undefined, current evidence suggests that zinc, an essential trace metal, is active locally at the site of injury. Pories *et al.* (1, 2) have reported zinc to accelerate wound healing in excised pilonidal sinus tracts. Savlov and co-workers (3) have demonstrated experimentally that radioactive zinc-65 is preferentially concentrated within the healing wound. According to Henzel and associates (4–6), zinc-deficient patients fail to concentrate zinc in wound granulation tissue and exhibit problems with wound healing.

The fact that the activities of several zinc-dependent enzymes are observed to increase during tissue disruption and repair (7, 8) suggests that the acceleratory role of zinc in wound healing may relate to enhanced biosynthetic activity. Collagen is the most important structural protein to be synthesized in the process of connective tissue repair and is principally responsible for gains in wound tensile strength.

The question arises, therefore, as to whether zinc may accelerate wound healing by promoting increased collagen production at the wound site. This question is answerable with difficulty *in vivo*. Green and Goldberg (9) and Shimizu *et al.* (10) have demonstrated in tissue culture, however, that human skin fibroblasts produce significant amounts of collagen if ascorbate is added to the culture medium. Thus, the tissue culture system provides an operable model in which to determine the direct effect of zinc on the biosynthesis of collagen.

Three parameters are to be evaluated in making this assessment: (a) cellular proliferation—measured by changes in total culture protein; (b) rate of collagen biosynthesis—measured by rate of conversion of radioactively labeled proline into hydroxyproline; and, (c) changes in culture collagen content—measured by total accumulation of hydroxyproline.

Materials and Methods. Diploid human fetal fibroblasts derived from skin and muscle (Microbiological Assoc., Bethesda, Maryland), were utilized in the 19th passage. Diploid human foreskin fibroblasts (courtesy of Mr. V. K. Sharma, Children's Hospital, Washington, D.C.) were subcultured five times prior to experimentation. All cultures were free of microbial contamination.

Monolayer cultures were maintained and harvested as previously described (11). Cells were pooled in Eagle's basal medium with Hanks' balanced salt solution (HBME); and aliquots of 20 ml (0.6 to 1.4 $\times$ 10^6 cells) were transferred to plastic culture flasks (Falcon Plastics, Los Angeles, California; growth surface 75 cm^2). Cultures were incubated at 36° for 48 hr at which time HBME was replaced by Eagle's basal medium with Earle's balanced salt solution (EBME). Both HBME and EBME were supplemented as previously described (11) except that amphotericin B was not added.

Zinc sulfate septahydrate (J. T. Baker Chemical Co., Phillipsburg, N.J.) was added to experimental media at 10^{-4} to 10^{-8} *M*. Sodium ascorbate (Nutritional Biochemicals Corp., Cleveland, Ohio) was added to both experimental and control media at 50 μg/ml.

In the experiment employing low density cultures, zinc sulfate and sodium ascorbate

were added with change of media after 48 hr and every 3 days thereafter. The rate of collagen biosynthesis was monitored using uniformly labeled L-proline-^{14}C (New England Nuclear Corp., Boston, MA; 205 μCi/μmole). Two μCi (0.2 ml) of L-proline-^{14}C were added 6 hr after medium change and cultures were harvested 18 hr later.

In the high density cultures, zinc sulfate and sodium ascorbate were added on the sixth day and every 3 days thereafter with change of media. L-Proline-3,5-^{3}H (New England Nuclear Corp.; 5 mCi/μmole) was continually present in the system permitting an independent determination of collagen content. Five microcuries (0.5 ml) were added to each culture immediately following change of medium.

Harvesting was carried out every 3 days at the time of media change. The cell monolayer was washed twice with warm (37°) 0.9% saline solution. Cells were scraped into warm saline, centrifuged at 500*g* for 10 min and stored frozen at —20°. Pellets were subsequently resuspended in distilled water (3.0 ml) and sonicated.

For determination of total culture protein, aliquots (0.3 ml) of cell sonicate were lysed with an equal volume of 2% sodium deoxycholate (Mann Research Lab., New York, N.Y.). Lysates (0.15 ml) were assayed by the method of Lowry *et al.* (12), using twice recrystallized bovine albumin (Nutritional Biochemicals, Cleveland, Ohio) as a standard.

Aliquots of sonicate (2.7 ml) for determination of hydroxyproline-^{14}C or -^{3}H and total hydroxyproline were evaporated to dryness under a stream of warm air and hydrolyzed in 1.0 ml of 4 *N* NCl for 24 hr at 100°. Hydrolyzates were neutralized with approximately 1 ml of 4 *N* KOH prior to assay by the method of Switzer and Summer (13). In the radioassay, 9.0 ml of scintillation medium (14) were added to 1.0 ml aliquots of toluene-extracted pyrrole. Counting efficiency in a Nuclear Chicago model 725 liquid scintillation spectrometer was approximately 85% for ^{14}C and approximately 35% for tritium.

Results. Human skin fibroblasts synthesize significant amounts of collagen hydroxyproline when sodium ascorbate (50 μg/ml) is added every 3 days with change of medium. However, in newly established (low density) cultures collagen hydroxyproline content increases only after culture confluency is

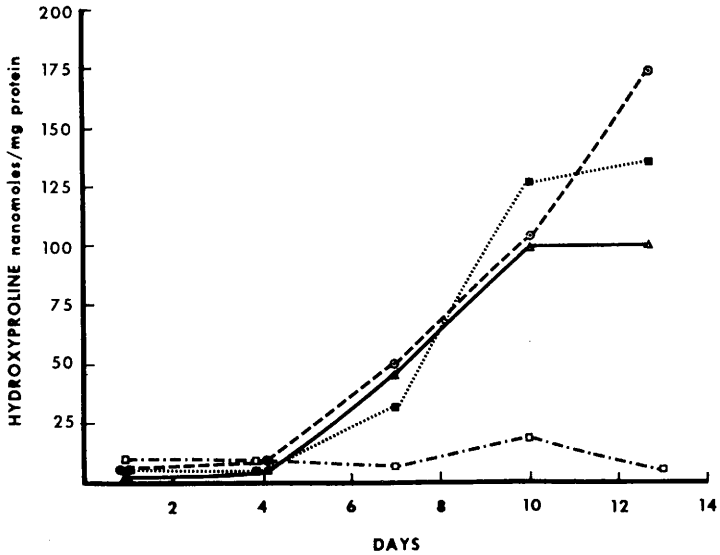

FIG. 1. Effect of zinc sulfate on collagen hydroxyproline content in newly established (low density) diploid human fibroblasts from skin and muscle: (⊙ ——) control; (□ - — -) 10^{-4} *M* $ZnSO_4$; (Δ—) 10^{-6} *M* $ZnSO_4$; (■- - -) 10^{-8} *M* $ZnSO_4$. Experimental day 0 was 2 days following planting. Media was changed on days 0, 3, 6, 9, and 12. All media contained sodium ascorbate (50 μg/ml).

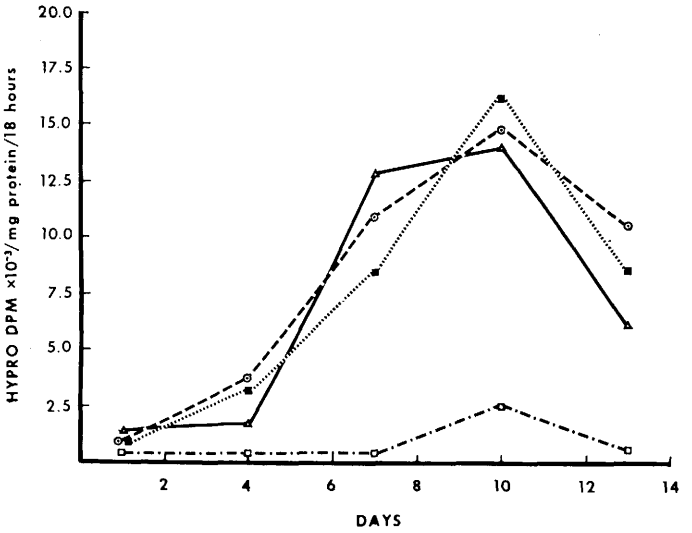

FIG. 2. Rate of collagen hydroxyproline biosynthesis in cultures treated with zinc sulfate. Labeled precursor L-proline-^{14}C (u.l.). See Fig. 1 for details.

reached about day 4 (Fig. 1). The increase in control cultures is linear with respect to time reaching a value of approximately 175 nmoles/mg of protein by day 13. In all cultures receiving zinc sulfate, the hydroxyproline content is less than in control cultures by day 13 as shown in Fig. 1.

The rate of collagen hydroxyproline production in low density cultures treated with 10^{-8} and 10^{-6} *M* zinc sulfate is approximately the same as in control cultures for the first 10 days (Fig. 2). By day 13, there is an apparent zinc sulfate concentration dependent decrease in both hydroxyproline content and rate of synthesis (Figs. 1 and 2). Throughout this period, the rate of collagen synthesis in cultures treated with 10^{-4} *M* zinc sulfate is minimal (Fig. 2).

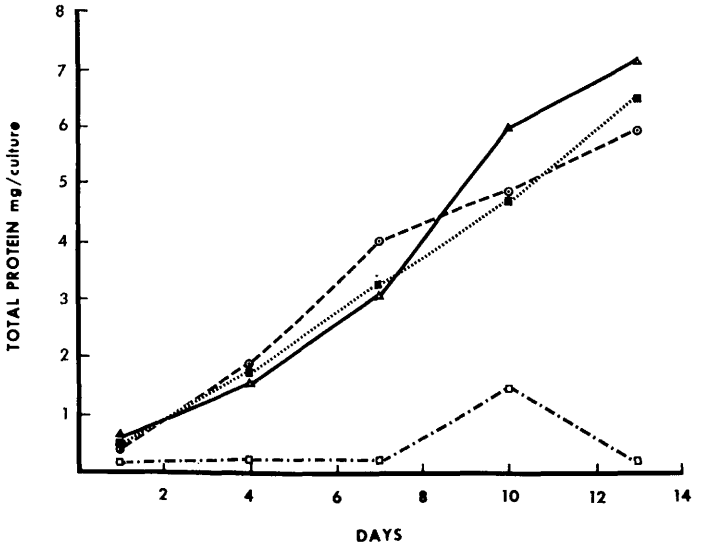

FIG. 3. Cellular proliferation as measured by total culture protein in cultures treated with zinc sulfate. See Fig. 1 for details.

Total culture protein in low density cultures treated with 10^{-6} and 10^{-8} *M* zinc sulfate is approximately the same as in control cultures (Fig. 3). Cell rounding and detachment observed microscopically in low density cultures receiving 10^{-4} *M* zinc sulfate suggest that this concentration is cytotoxic.

Similar results are obtained when zinc sulfate is added to completely confluent (high density) cultures beginning on day 6 (Table I). Both colorimetric determination and radioassay of hydroxyproline indicate that zinc sulfate at 10^{-4} *M* reduces accumulation of collagen by nearly 50% compared to controls. Lower concentrations of zinc sulfate (10^{-5} to 10^{-7} *M*) do not influence collagen hydroxyproline content.

The increase in total protein content with time is approximately the same in all high density cultures (Table I). The inhibition of cellular proliferation observed in Fig. 1 when 10^{-4} *M* zinc sulfate is added to low density cultures is not seen in the case of confluent cultures.

Discussion. The present experiments attempted to further elucidate the role of zinc in the normal healing process by considering the role of this element in fibroblast proliferation and collagen biosynthesis. Low density, rapidly growing cultures were employed to evaluate principally the effect of zinc sulfate on fibroblast proliferation. Confluent, stationary cultures, on the other hand, were utilized to study specifically the influence of zinc on the production of collagen.

Collagen hydroxyproline content in low density cultures treated with zinc sulfate was less after day 10 than in control cultures at every concentration employed (10^{-4} to 10^{-8} *M*). The addition of zinc sulfate (10^{-4} to 10^{-7} *M*) to high density cultures also failed to increase collagen content significantly.

The cell rounding and detachment observed microscopically in low density cultures treated with 10^{-4} *M* zinc sulfate suggests that the decreased collagen hydroxyproline production is the result of cytotoxicity. Toxicity at this concentration (10^{-4} *M* or 28.75 μg/ml) is surprising since the average zinc concentration in human skin is reported by separate laboratories at means of 16.01 (4) and 16.63 μg/g (15), but see Raff and Houck (16).

In the experiment with high density cultures, microscopic observations, as well as total protein determinations, did not indicate that 10^{-4} *M* zinc sulfate was cytotoxic. The fact that in confluent cultures 10^{-4} *M* zinc sulfate inhibits collagen hydroxyproline synthesis and deposition in the absence of significant effect of cellular proliferations suggests that under these conditions collagen biosynthesis may be selectively inhibited. Concentrations of zinc sulfate below 10^{-4} *M* do not appear to exert an inhibitory effect on collagen biosynthesis in high density cultures.

While these studies in tissue culture do not rule out indirect acceleratory effects of zinc sulfate on collagen biosynthesis *in vivo* they do suggest that zinc does not have a direct

TABLE I. Effect of Zinc Sulfate on Mean[a] Collagen Hydroxyproline Content in Confluent (high density) Diploid Human Fibroblasts from Foreskin.

	Hydroxyproline (nmoles/mg of protein)		Hydroxyproline (dpm × 10^{-3}/mg of protein)		Total protein (mg/culture)	
Days	Control and 10^{-5}–10^{-7} *M* $ZnSO_4$	10^{-4} *M* $ZnSO_4$	Control and 10^{-5}–10^{-7} *M* $ZnSO_4$	10^{-4} *M* $ZnSO_4$	Control and 10^{-5}–10^{-7} *M* $ZnSO_4$	10^{-4} *M* $ZnSO_4$
6	7.17	7.17	0.00	0.00	0.68	0.68
9	9.34–13.00	6.13	5.35– 6.25	3.20	1.76–2.19	1.96
12	18.97–20.49	10.20	11.15–13.51	5.80	2.61–2.98	3.43
15	18.86–24.96	12.08	12.81–18.15	8.88	3.09–4.30	4.86

[a] Each value represents 3 cultures—duplicate assays on each culture. Low and high means are given under control and 10^{-5} to 10^{-7} *M*. Final concentrations of zinc sulfate in media were 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} *M*.

acceleratory effect on collagen biosynthesis. Other agents such as ascorbate, iron, α-ketoglutarate, and oxygen are known cofactors for collagen hydroxylation *in vivo* and can be shown to increase collagen biosynthesis by fibroblasts *in vitro* (9, 10, 17).

The explanation for accelerated wound healing observed by Pories and co-workers (1, 2) is probably related to increased zinc requirements following tissue trauma as suggested recently by Henzel and co-workers (4). It should be noted, however, that acceleration of wound healing, as determined by planimetry or wound volume measurements, need not involve increases in collagen production. Since wound tensile strengths have not been determined, there is no evidence from work *in vivo* to suggest that collagen production, indeed, has increased. According to Pories *et al.* (1, 2), the greatest acceleration of wound healing was observed during the phase of epithelialization. This is perhaps where the beneficial effect of zinc therapy is most profound.

Summary. The effect of zinc sulfate on collagen biosynthesis and fibroblast proliferation has been studied in a tissue culture model system using *human* skin fibroblasts. Addition of zinc sulfate to newly established (low density) cultures in concentrations of 10^{-4} to 10^{-8} *M* results in concentration-dependent cytotoxicity with inhibition of collagen biosynthesis, as well as cellular proliferation. Zinc sulfate at 10^{-4} to 10^{-7} *M* added to confluent (high density) cultures does not influence cellular proliferation, but results in selective inhibition of collagen biosynthesis at the 10^{-4} *M* concentration. Lower concentrations of zinc sulfate (10^{-5} to 10^{-7} *M*) do not affect collagen biosynthesis in high density cultures. The results show that zinc does not have a direct acceleratory effect on either cellular proliferation or collagen biosynthesis in human skin fibroblasts *in vitro*.

The authors thank Donald R. Sundeen and Daniel J. Kraft for excellent technical assistance, Larry M. Sturdivan for his assistance in statistical analysis and John J. Heieck, M.D., for valuable criticism of the manuscript.

1. Pories, W. J., Henzel, J. H., Rob, C. G., and Strain, W. H., Lancet **1**, 121 (1967).

2. Pories, W. J., Henzel, J. H., Rob. C. G., and Strain, W. H., Ann. Surg. **165**, 432 (1967).

3. Savlov, E. D., Strain, W. H., and Huegin, F., J. Surg. Res. **2**, 209 (1962).

4. Henzel, J. H., DeWeese, M. S., and Lichti, E. L., Arch. Surg. **100**, 349 (1970).

5. Henzel, J. H., DeWeese, M. S., and Holtmann, B., Presented: Fundamental Forum, St. Louis Surg. Soc., St. Louis, 1968.

6. Henzel, J. H., DeWeese, M. S., and Pories, W. J., Arch. Surg. **95**, 991 (1967).

7. Fell, H. B., and Danielli, J. F., Brit. J. Exp. Pathol. **24**, 196 (1943).

8. Sullivan, J. F., and Lankford, H. G., Amer. J. Clin. Nutr. **17**, 57 (1965).

9. Green, H., and Goldberg, B., Proc. Soc. Exp. Biol. Med. **117**, 258 (1964).

10. Shimizu, Y., McCann, D. S., and Keech, M. K., J. Lab. Clin. Med. **65**, 286 (1965).

11. Waters, M. D., and Summer, G. K., Proc. Soc. Exp. Biol. Med. **133**, 926 (1970).

12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

13. Switzer, B. R., and Summer, G. K., Anal. Biochem. **39**, 487 (1971).

14. Patterson, M. S., and Greene, R. C., Anal. Biochem. **37**, 854 (1965).

15. Smoot, W., Presented: Amer. Dermatol. Soc., Chicago, 1967.

16. Raff, E. C., and Houck, J. C., J. Cell. Physiol. **74**, 235 (1969).

17. Goldberg, B., and Green, H., Proc. Nat. Acad. Sci. U.S.A. **59**, 1110 (1968).

Received April 22, 1971. P.S.E.B.M., 1971, Vol. 138.