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J Clin Invest. 1985;76(5):2016-2019. https://doi.org/10.1172/JCI112202.

Research Article

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Human Transforming Growth Factor-alpha Stimulates Bone Resorption In Vitro

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Abstract

Tumor-derived transforming growth factors (TGF) have been proposed as possible mediators of hypercalcemia in malignancy. We have studied the action of recombinant human TGF-α in cultured bone cells and in bone explant cultures. In clonal UMR-106 rat osteosarcoma cells, TGF-α and epidermal growth factor (EGF) were equipotent in binding to the EGF receptor. TGF-α and EGF both stimulated resorption of neonatal mouse calvaria, and maximal responses were obtained with 10 ng/ml of TGF-α after 72 h in culture. The effects of both TGF-α and EGF in calvaria, but not those of parathyroid hormone, were inhibited by 5×10^{-7} M indomethacin. Fetal rat limb bone cultures were less sensitive to TGF-α than neonatal mouse calvaria, with a concentration of 30 ng/ml being required to stimulate resorption in this system. The bone-resorbing activity of TGF-α in fetal rat bones was inhibited by 10 ng/ml calcitonin but not by 5×10^{-7} M indomethacin. EGF at concentrations up to 300 ng/ml did not stimulate resorption of the limb bones at time periods up to 66 h. The results indicate that human TGF-α is a potent bone-resorbing agent, and support the concept that this growth factor exhibits some effects distinct from those of EGF. TGF-α could play an etiologic role in the hypercalcemia of malignancy.

Introduction

The etiology of hypercalcemia in malignancy is uncertain. Prostaglandins (1), a parathyroid hormone (PTH)[1]-like protein (2–4), and tumor-derived transforming growth factors (TGFs) are among the proposed mediators of the increased bone resorption and resultant hypercalcemia that typify some neoplasms. TGFs are polypeptide mitogens that are secreted by virus-transformed cells or neoplastic cells and confer the transformed phenotype, including anchorage-independent growth, on target cells (5, 6). TGFs of the α-subtype (TGF-α) bind to the receptor for epidermal growth factor (EGF) (7), a known stimulator of bone resorption in vitro (8, 9). Hence, TGF-α could be a good candidate to mediate the hypercalcemic effect of neoplasms, and has been implicated in the bone-resorbing effects of animal tumor cells. The bone-resorbing substance secreted by rat Leydig tumor cells partially copurifies with a component(s) that has activity in assays of mitogenesis, anchorage-independent growth, and EGF receptor binding (10); and the bone-resorbing effect of medium conditioned by Leydig tumor cells is blocked by an antiserum to the EGF receptor (11). The present studies were carried out to determine directly whether purified recombinant human TGF-α (12) is indeed a potent bone-resorbing substance.

Methods

Preparation of TGF-α. Human TGF-α was prepared by expression of a properly engineered cloned complementary DNA (cDNA) in *Escherichia coli.* The product was isolated as described (12), and the preparations used herein were purified by high performance liquid chromatography (HPLC). The multiple peaks of recombinant TGF-α from HPLC were identical by amino acid analysis, with the predicted amino acid composition, but had different tryptic peptides; the HPLC peak with the greatest EGF-equivalent activity was used. The specific activity of this material varied from 10 to 44% of that of EGF in competition for EGF binding, possibly because of variable folding and disulfide bond formation (12). TGF-α used in this work was assigned an EGF-equivalent activity (in nanograms/milliliter), based on competition for ^{125}I-EGF binding to CCL64 mink lung cells (12). When activities were expressed in this way, all TGF-α preparations used were equipotent in competing with ^{125}I-EGF for receptors in osteosarcoma cells.

Bone cultures. ^{45}Ca-prelabeled 19-d fetal rat limb bones were cultured for 66 h in Dulbecco's modified Eagle's medium (DME) supplemented with 15% heat-inactivated (30 min, 56°C) horse serum. Details of the method have been described previously (13, 14). Resorption was quantified on the basis of release of ^{45}Ca, confirmed by morphologic assessment of resorption at a magnification of 16. Neonatal (3–5 d) mouse calvaria were cultured in roller tubes containing 2 ml of DME, plus 15% heat-inactivated horse serum. Details of the calvaria culture technique have been published previously (14). Other materials used include bovine (b)PTH(1–34) (Bachem, Inc., Torrance, CA), EGF (Collaborative Research, Inc., Waltham, MA; and Bethesda Research Laboratories, Gaithersburg, MD), salmon calcitonin (a gift of Dr. J. Bastian, Armour Pharmaceutical Co., Tarrytown, NY) and indomethacin (Sigma Chemical Co., St. Louis, MO).

PTH bioassay. The clonal rat osteosarcoma cell line UMR-106 (15) was kindly provided by Dr. T. J. Martin (Repatriation General Hospital, West Heidelberg, Australia). Osteosarcoma cells were cultured in DME containing 10% fetal bovine serum, 2 mM glutamine, 2% nonessential amino acids, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 2.5 μg/ml fungizone. Bioassays for PTH used confluent cultures in 24-well plates (Costar, Cambridge, MA) as previously described (16). Briefly, test substances were assayed for 10 min in 0.15 ml of DME containing 20 mM

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Received for publication 7 June 1985.

1. *Abbreviations used in this paper:* cAMP, cyclic AMP; DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; HPLC, high performance liquid chromatography; PTH, parathyroid hormone; TGF(s), transforming growth factor(s); TGF-α, TGFs of the α-subtype.

J. Clin. Invest.

0021-9738/85/11/2016/04 $1.00
Volume 76, November 1985, 2016–2019

Hepes, pH 7.4, and 1 mM isobutylmethylxanthine. Incubations were terminated by aspirating medium, washing the cells with 1 ml phosphate-buffered saline, and extracting cyclic AMP (cAMP) with three 1-ml washes of absolute ethanol. Ethanol was evaporated in air and the extracts were dissolved in 1.0 ml 50 mM sodium acetate, pH 4.0, for cAMP assay (17).

EGF binding assay. Confluent cultures of UMR-106 cells in 24-well plates were transferred to serum-free DME containing 0.1% bovine serum albumin. Cells were incubated at room temperature with ~10,000 cpm of ^{125}I-EGF (18) and unlabeled EGF or TGF-α. After 60 min, medium was removed and the cells were washed three times with 1 ml of phosphate-buffered saline. The cells were removed with 1 ml of 0.5 N NaOH, and counted for ^{125}I-radioactivity.

Statistics. Results were tested for significant differences by analysis of variance (19).

Results

TGF-α, at concentrations of 30–300 ng/ml, stimulated resorption of fetal rat limb bones (Fig. 1). Under these conditions (66-h incubation) EGF at similar concentrations had no bone-resorbing activity. This result was obtained using several preparations of EGF, whose biological activity was verified in assays of EGF receptor binding. The stimulatory effects of TGF-α were unaffected by indomethacin (Table I) but were inhibited by salmon calcitonin (percent ^{45}Ca released, mean±SE (n = 4): no treatment, 17.2±1.1; TGF-α, 300 ng/ml, 91.6±3.2; salmon calcitonin, 10 ng/ml, 14.8±0.4; TGF-α + salmon calcitonin, 16.4±1.0). In contrast, neonatal mouse calvaria were maximally stimulated by 10 ng/ml TGF-α (Fig. 2) and the effects of TGF-α, like those of EGF, were completely inhibited by 5×10^{-7} M indomethacin (Table I). As previously reported (8, 9), indomethacin did not block the bone-resorbing effect of PTH in either assay (data not shown). As shown in Fig. 3, EGF and

Table I. Effect of Indomethacin on TGF α-Stimulated Resorption in Fetal Rat Limb Bones and Neonatal Mouse Calvaria

A. Fetal rat limb bones	n	Percent bone ^{45}Ca released
No treatment	4	19.1±1.4
TGF-α, 300 ng/ml	4	45.5±7.8*
Indomethacin, 5×10^{-7} M	4	20.3±0.8
TGF-α + indomethacin	4	48.9±5.0*
B. Calvaria		**Medium ^{40}Ca at 72 h**
		(mM)
No treatment	5	2.23±0.16
TGF-α, 30 ng/ml	4	3.45±0.41*
Indomethacin, 5×10^{-7} M	4	1.99±0.17
TGF-α + indomethacin	4	2.21±0.10
EGF, 50 ng/ml	4	3.46±0.39*
EGF + indomethacin	4	2.44±0.18

* $P < 0.001$ vs. control.

TGF-α (whose activity was calibrated in CCL64 cells) were approximately equipotent in competing for binding of ^{125}I-EGF to UMR-106 osteosarcoma cells. In other experiments, the concentration of EGF required for half-maximal inhibition of ^{125}I-EGF binding ranged from 10 to 25 ng/ml, in agreement with previous results in this cell line (20). However, TGF-α displayed no PTH-like bioactivity in UMR-106 osteosarcoma cells. At saturating concentrations up to 1 μg/ml, TGF-α did not elicit an increase in osteosarcoma cell cAMP levels (basal, 8 pmol/well; TGF-α, 1 μg/ml, 8 pmol/well), while in the same experiments, PTH (20 ng/ml) stimulated an increase in cellular cAMP to 465 pmol/well.

Discussion

The results indicate that human TGF-α is a potent bone-resorbing agent in two culture systems in vitro; fetal rat limb bones

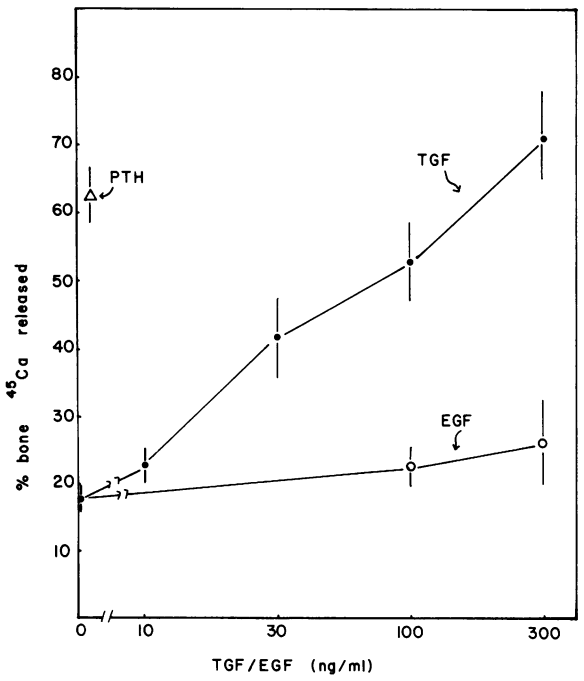

Figure 1. Effects of TGF-α and EGF on resorption of fetal rat limb bones in vitro. Bones were cultured for 60 h with the indicated concentrations of TGF-α and EGF or with 1 nM PTH. Values are the means and standard errors of responses from six bones per point.

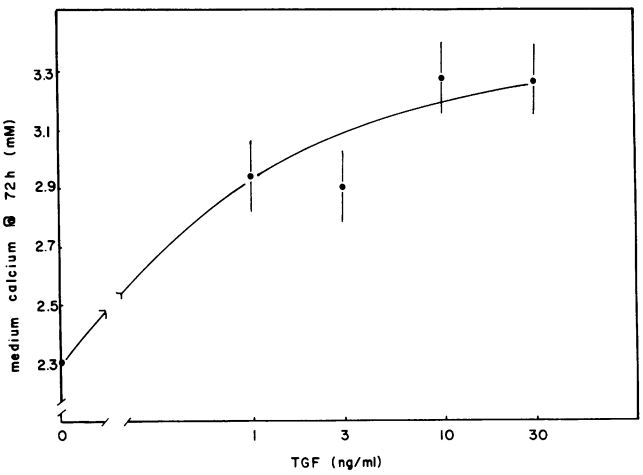

Figure 2. Effect of TGF-α on resorption of neonatal mouse calvaria in vitro. Bones were cultured for 72 h with the indicated concentrations of TGF-α. Values are the means and standard errors of responses from four bones per point.

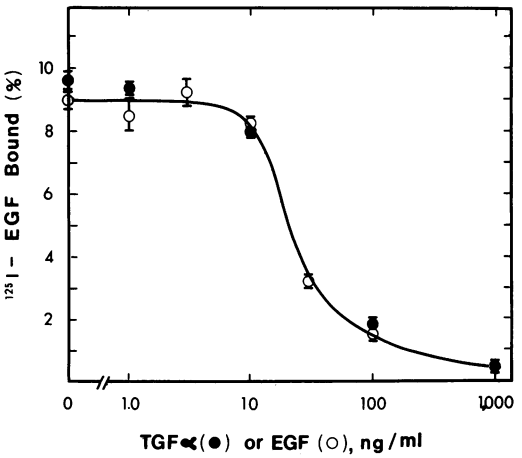

Figure 3. Inhibition of ^{125}I-EGF binding to UMR-106 osteosarcoma cells by EGF (○) or TGF-α (●). Values are the means and standard errors of triplicate determinations.

and neonatal mouse calvaria. In limb bones, TGF-α concentrations as low as 30 ng/ml stimulated resorption after 66 h. The calvaria were more sensitive to TGF-α, with maximal responses obtained with 10 ng/ml. The response of the two systems to TGF-α also differed, in that the effect in limb bones was insensitive to indomethacin, whereas that in the calvaria was completely blocked by the prostaglandin synthesis inhibitor. The two systems also differed in their relative responses to TGF-α and EGF. Neonatal mouse calvaria appeared to be equally sensitive to TGF-α and EGF. Previous studies with EGF in this same calvarial culture system indicated that 10 ng/ml was a maximally effective concentration of EGF (8) consistent with the effects of TGF-α seen here. Like the effect of TGF-α, the effect of EGF was inhibited by indomethacin in calvaria. In contrast, EGF failed to stimulate resorption in the rat limb bone cultures. The poor responses to EGF were not unexpected in view of the previous observations that long exposures (3–6 d) were required to produce resorption with EGF in fetal rat limb bones (9). Similar disparities between the responses of fetal rat limb bones and neonatal mouse calvaria have been described previously, not only with respect to EGF (8, 9), but with other factors that can produce release of prostaglandins. For example, we have shown that KCl stimulates resorption of neonatal mouse calvaria by a pathway that is significantly inhibited by indomethacin, where KCl does not stimulate resorption in fetal rat or mouse limb bones (21).

Certain of the data are consistent with the possibility that TGF-α stimulates resorption by interaction with a receptor for EGF. Since UMR-106 cells have osteoblast-like characteristics (16) and are known to possess surface receptors for EGF (20), we used them to determine the relative affinities of EGF and recombinant human TGF-α for binding to the EGF receptor in rat bone cells. The finding that human TGF-α and EGF bind with similar affinity to the EGF receptor in osteosarcoma cells is consistent with results in fibroblast cell lines (12) and in the neonatal mouse calvaria. The observation that TGF-α was considerably more effective than EGF in stimulating bone resorption in fetal rat limb explants is more difficult to reconcile with the concept that they are acting through the same receptor. It has been suggested that in addition to the EGF receptor, TGF-α binds to a unique receptor in NRK and A431 cells (22). Such a unique receptor may also be present in bone cells. However, in preliminary studies, specific ^{125}I-TGF-α binding to UMR-106 cells can be displaced completely by EGF (Nissenson, R. A., and G. J. Strewler, unpublished observation). It is also possible that in contrast to its binding affinity in osteosarcoma cells, TGF-α has a markedly higher affinity than EGF itself for a common receptor in fetal rat bone. However, the concentration of TGF-α required to effect bone resorption is similar to the concentration necessary for binding to osteosarcoma cells. It remains possible that TGF-α and EGF bind similarly to the EGF receptor in rat limb bone cultures, but receptors occupied by TGF-α more effectively activate bone resorption.

Considerable evidence supports the proposal that in many instances, hypercalcemia in malignancy is mediated by a tumor-derived protein that stimulates bone resorption via the PTH receptor, even though it is immunochemically distinct from PTH (2). This protein binds to the PTH receptor coupled to adenylate cyclase in renal plasma membranes (3, 4) and in osteosarcoma cells (16, 23). It appears that human TGF-α elicits increased bone resorption by a different pathway, since TGF-α does not stimulate cAMP production in osteosarcoma cells. It is of interest that the same renal carcinoma cell line from which messenger RNA (mRNA) for TGF-α was isolated (12) also secretes a PTH-like protein that binds to the PTH receptor and stimulates cAMP in osteosarcoma cells (16).

TGFs have also been proposed as mediators of hypercalcemia in malignancy, based on the following evidence: (*a*) rat Leydig tumor cells produce a bone-resorbing substance that copurifies with mitogenic activity, transforming activity (stimulation of anchorage-independent growth), and EGF receptor binding activity (10); (*b*) an antiserum to the EGF receptor blocks bone resorption by Leydig tumor cell medium (11); and (*c*) a hypercalcemic variant of Walker 256 carcinosarcoma produces a bone-resorbing substance with some similarities to TGF-α (e.g., acid stability, sensitivity to dithiothreitol), whereas a normocalcemic variant does not (24). The present results indicating that recombinant human TGF-α is a potent bone-resorbing substance also support this view. The effects of TGF-α are clearly distinguishable from those of a PTH-like protein that is strongly associated with hypercalcemia in malignancy (2–4). Considerably more work will be required to define the frequency with which either of these two putative mediators causes hypercalcemia. In this regard, the availability of specific antisera (25) could permit the correlation of blood levels of TGF-α with humoral hypercalcemia in patients and animal subjects.

Acknowledgments

We gratefully acknowledge the technical assistance of Thalia Mavreas, Shirley Snerling, and Steven C. Leung.

These studies were supported by National Institutes of Health research grant AM11262, grant PDT 229 from the American Cancer Society, and funds from the Medical Research Service of the Veterans Administration.

References

1. Seyberth, H. W., G. V. Segre, J. L. Morgan, B. J. Sweetman, J. T. Potts, Jr., and J. A. Oates. 1975. Prostaglandins as mediators of hypercalcemia associated with certain types of cancer. *N. Engl. J. Med.* 293: 1278–1283.

2. Stewart, A. F., R. Horst, L. J. Deftos, E. C. Cadman, R. Lang, and A. E. Broadus. 1980. Biochemical evaluation of patients with cancer-associated hypercalcemia: evidence for humoral and nonhumoral groups. *N. Engl. J. Med.* 303:1377–1383.

3. Strewler, G. J., R. D. Williams, and R. A. Nissenson. 1983. Human renal carcinoma cells produce hypercalcemia in the nude mouse and a novel protein recognized by parathyroid hormone receptors. *J. Clin. Invest.* 71:769–774.

4. Stewart, A. F., K. L. Insogna, P. Goltzman, and A. E. Broadus. 1983. Identification of adenylate cyclase-stimulating activity and cytochemical glucose-6-phosphate dehydrogenase-stimulating activity in extracts of tumors from patients with humoral hypercalcemia of malignancy. *Proc. Natl. Acad. Sci. USA.* 80:1454–1458.

5. DeLarco, J. E., and G. J. Todaro. 1978. Growth factors from murine sarcoma virus-transformed cells. *Proc. Natl. Acad. Sci. USA.* 75: 4001–4005.

6. Roberts, A. B., C. A. Frolik, M. A. Anzano, and M. B. Sporn. 1983. Transforming growth factors from neoplastic and nonneoplastic tissues. *Fed. Proc.* 42:2621–2625.

7. Todaro, G. J., C. Fryling, and J. E. DeLarco. 1980. Transforming growth factors produced by certain tumor cells: polypeptides that interact with epidermal growth factor receptors. *Proc. Natl. Acad. Sci. USA.* 77: 5258–5262.

8. Tashjian, A. H., Jr., and L. Levine. 1978. Epidermal growth factor stimulates prostaglandin production and bone resorption in cultured mouse calvaria. *Biochem. Biophys. Res. Commun.* 85:966–975.

9. Raisz, L. G., H. A. Simmons, A. L. Sandberg, and E. Canalis. 1980. Direct stimulation of bone resorption by epidermal growth factor. *Endocrinology.* 107:270–273.

10. Ibbotson, K. J., S. M. D'Souza, K. W. Ng, C. K. Osborne, M. Niall, T. J. Martin, and G. R. Mundy. 1983. Tumor-derived growth factor increases bone resorption in a tumor associated with humoral hypercalcemia of malignancy. *Science (Wash. DC).* 221:1292–1294.

11. Ibbotson, K. J., S. M. D'Souza, D. D. Smith, G. Carpenter, and G. R. Mundy. 1985. EGF receptor antiserum inhibits bone resorbing activity produced by a rat Leydig cell tumor associated with the humoral hypercalcemia of malignancy. *Endocrinology.* 116:469–471.

12. Derynck, R., A. B. Roberts, M. E. Winkler, E. Y. Chen, and D. V. Goeddel. 1984. Human transforming growth factor-α precursor structure and expression in *E coli. Cell.* 38:287–297.

13. Stern, P. H., T. E. Phillips, and T. Mavreas. 1980. Bioassay of 1,25-dihydroxyvitamin D in human plasma purified by partition, alkaline extraction, and high-pressure chromatography. *Anal. Biochem.* 102:22–30.

14. Stern, P. H., and N. S. Krieger. 1983. Comparison of fetal rat limb long bones and neonatal mouse calvaria: effects of parathyroid hormone and 1,25-dihydroxyvitamin D. *Calcif. Tissue Int.* 35:172–176.

15. Partridge, N. C., D. Alcorn, V. P. Michelangeli, G. Ryan, and T. J. Martin. 1983. Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin. *Cancer Res.* 43: 4308–4314.

16. Nissenson, R. A., G. J. Strewler, R. D. Williams, and S. C. Leung. 1985. Activation of the parathyroid hormone receptor-adenylate system in osteosarcoma cells by a human renal carcinoma factor. *Cancer Res.* In press.

17. Gilman, A. G. 1970. A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. USA.* 67:305–312.

18. Vlodavsky, I., K. D. Brown, and D. Gospodarowicz. 1978. A comparison of the binding of epidermal growth factor to cultured granulosa and luteal cells. *J. Biol. Chem.* 253:3744–3750.

19. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State University Press, Ames, IA. 1-593.

20. Ng, K. W., N. C. Partridge, M. Niall, and T. J. Martin. 1983. Epidermal growth factor receptors in clonal lines of a rat osteogenic sarcoma and in osteoblast-rich rat bone cells. *Calcif. Tissue. Int.* 35:298–303.

21. Krieger, N. S., and P. H. Stern. 1983. Potassium effects on bone: comparison of two model systems. *Am. J. Physiol.* 245:E303–E307.

22. Massague, J., M. P. Czech, K. Iwata, J. E. DeLarco, and G. J. Todaro. 1982. Affinity labeling of a transforming growth factor receptor that does not interact with epidermal growth factor. *Proc. Natl. Acad. Sci. USA.* 79:6822–6826.

23. Rodan, S. B., K. L. Insogna, A. M.-C. Vignery, A. F. Stewart, A. E. Broadus, S. M. D'Souza, G. R. Mundy, and G. A. Rodan. 1983. Factors associated with humoral hypercalcemia of malignancy stimulate adenylate cyclase in osteoblastic cells. *J. Clin. Invest.* 72:1511–1515.

24. D'Souza, S. M., K. J. Ibbotson, D. D. Smith, and G. R. Mundy. 1984. Production of a macromolecular bone-resorbing factor by the hypercalcemic variant of the Walker rat carcinosarcoma. *Endocrinology.* 115:1746–1752.

25. Linsley, P. S., W. R. Hargreaves, D. R. Twardzik, and G. J. Todaro. 1985. Detection of larger polypeptides structurally and functionally related to type 1 transforming growth factor. *Proc. Natl. Acad. Sci. USA.* 82:356–360.