polysaccharides are superior to their uncharged counterparts (*10*).

Determination of the exact role of sialic acid in the organ-specific adherence of lymphocytes to HEV awaits the isolation and characterization of the HEV attachment sites from the various lymphoid organs.

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References and Notes

1. J. L. Gowans, *J. Physiol. (London)* **146**, 54 (1959).
2. J. L. Gowans and E. J. Knight, *Proc. R. Soc. London Ser. B* **159**, 257 (1964).
3. H. B. Stamper, Jr., and J. J. Woodruff, *J. Exp. Med.* **144**, 828 (1976).
4. ———, *J. Immunol.* **119**, 772 (1977).
5. E. C. Butcher, R. G. Scollay, I. L. Weissman, *ibid.* **123**, 1996 (1979).
6. E. C. Butcher *et al.*, *Eur. J. Immunol.* **10**, 556 (1980).
7. S. K. Stevens, I. L. Weissman, E. C. Butcher, *J. Immunol.* **128**, 8 (1982).
8. Y.-H. Chin, G. D. Carey, J. J. Woodruff, *ibid.* **129**, 1911 (1982); Y.-H. Chin *et al.*, *ibid.* **131**, 1368 (1983).
9. W. M. Gallatin, I. L. Weissman, E. C. Butcher, *Nature (London)* **304**, 30 (1983).
10. L. M. Stoolman and S. D. Rosen, *J. Cell Biol.* **96**, 722 (1983); L. M. Stoolman, T. Tenforde, S. D. Rosen, *ibid.* **99**, 1535 (1984). Although these experiments are reported for the rat, the pattern of inhibition by saccharides and polysaccharides is essentially the same in the mouse system (T. A. Yednock, in preparation).
11. S. D. Rosen and L. M. Stoolman, in *Vertebrate Lectins*, K. Olden, Ed. (Van Nostrand, New York, in press). Periodate experiments, which were first carried out in the rat system, were extended to the mouse as follows. Sections of mouse (female, BALB/c) peripheral nodes and Peyer's patches, frozen in a common block, were cut, mounted in 1.4-cm-diameter wells in epoxy-coated glass slides (Carlson Scientific) and fixed with paraformaldehyde as described in (*10*). Experimental sections in individual wells were exposed to 100 μl of 50 m*M* sodium metaperiodate in 50 m*M* sodium acetate, 100 m*M* NaCl, *p*H 5.0 for 1 hour at 4°C in the dark. The same treatment was used for control sections, except that periodate was omitted. The sections were then washed extensively, and binding of mesenteric node lymphocytes to the sections was carried out by the standard procedure (*10*) (legend to Fig. 2) in a physiological buffer, except that 10 m*M* sodium borohydride was included in the lymphocyte suspension during the binding step. The sodium borohydride prevented nonspecific binding of lymphocytes to aldehyde groups generated by periodate oxidation of the sections. The periodate treatment completely eliminated binding to both peripheral node HEV and Peyer's patch HEV. At least ten HEV segments were counted per section. Control binding to peripheral node HEV (eight independent sections) was 46.9 ± 5.1 lymphocytes per unit area of HEV (10^4 μm^2); binding to periodate-oxidized peripheral node sections (four sections) was 0.0 ± 0. Control binding to Peyer's patch HEV (eight sections) was 41.9 ± 4.1; binding to periodate-oxidized Peyer's patch sections (four sections) was 0.0 ± 0. See legend to Fig. 2 for a description of the counting procedure.
12. In an earlier study, J. J. Woodruff *et al.* [*J. Immunol.* **119**, 1603 (1977)] investigated the effect of sialidase treatment of lymphocytes on the ability of rat lymphocytes to bind to peripheral node HEV in frozen sections and found slightly augmented binding relative to untreated lymphocytes.
13. Standard assays [Hall *et al.*, *Methods Enzymol.* **50**, 439 (1978)] were carried out for α- and β-galactosidase, *N*-acetyl-β-glucosaminidase, *N*-acetyl-β-galactosaminidase, α-fucosidase, and α-mannosidase. Briefly, 0.1 unit of *Clostridium* sialidase (Sigma) was combined with 2.4 nmol of the appropriate *p*-nitrophenyl substrate (0.9 nmol in the case of *p*-nitrophenyl β-D-*N*-acetylgalactosamine) in a final volume of 5 ml of 500 m*M* sodium acetate buffer, *p*H 5.0. After a 90-minute incubation at 37°C, the reaction was stopped by the addition of 1 ml of 50 m*M* NaOH. Absorbance at 400 nm was measured against a substrate blank. No activity was detected with any of the substrates. The limit of detectability was the hydrolysis of 0.8 nmol of substrate per 90 minutes. Proteolytic activity was measured with azocasein as a substrate. Briefly, 0.1 unit of sialidase was mixed with 7.5 mg of azocasein (Sigma) in a total volume of 0.5 ml of 100 m*M* tris-HCl, 5 m*M* $CaCl_2$, *p*H 7.8. After 50 minutes at 37°C, the reaction was ended by adding 2.5 ml of 3 percent trichloroacetic acid to each tube. The solutions were filtered and read at 366 nm in comparison with a substrate blank. With a limit of detectability of 6 ng of trypsin, no activity was detected in the sialidase.
14. A role for sialic acid is not ruled out in Peyer's patch attachment sites, since certain *O*-acetylated forms of sialic acid are resistant to the bacterial sialidases used in this study [R. Schauer, *Methods Enzymol.* **50**, 64 (1978)]. Also certain sialylated structures (for example, ganglioside G_{M1}) are sialidase-resistant (*17*).
15. At 25 m*M*, sialic acid has no effect on lymphocyte binding to peripheral node HEV in the frozen section assay (legend to Fig. 2). At higher concentrations, sialic acid is inhibitory, but these effects are attributable to the elevated ionic strength of the sugar solution. The activity of sialic acid never exceeds that of an equimolar solution of NaCl [(*10*) and unpublished observations].
16. M. A. Markwell *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5406 (1981).
17. Branched oligosaccharides containing neighboring terminal L-fucose and sialic acid have been described [J. L. Magnani *et al.*, *J. Biol. Chem.* **257**, 14365 (1982); Y. Fukushi, E. Nudelman, S. B. Levery, S.-I. Hakomori, *ibid.* **259**, 10511 (1984).
18. S.D.R. was supported by NSF grant PCM-821-5581 and by NIH grant GM235472 and L.M.S. was supported by NIH grant 1K08CA00959.

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Stimulation of Bone Resorption in Vitro by Synthetic Transforming Growth Factor–Alpha

Abstract. *Experiments were conducted to test the hypothesis that tumor-derived transforming growth factor–alpha (TGF-α) is responsible for the increased bone resorption and hypercalcemia seen in some malignant diseases. Homogeneous synthetic TGF-α prepared by the solid-phase synthesis method stimulated bone resorption directly in vitro in a concentration-dependent manner. Incubation times of 72 hours or more were required to stimulate resorption, which is similar to the time course of bone resorption by epidermal growth factor.*

Transformed cells produce endogenous transforming growth factors (TGF's) that can reversibly induce the transformed phenotype in indicator cells in vitro. These polypeptide stimulators of cell growth and replication may represent examples of autocrine or paracrine secretion by tumor cells (*1*). One class of these factors, called TGF-α, competes for binding to the epidermal growth factor (EGF) receptor. Rat TGF-α has been sequenced and synthesized (*2*). We showed earlier that TGF-α activity is present in the partially purified material responsible for the bone-resorbing activity in tumors associated with hypercalcemia (*3*). We now report that synthetic TGF-α stimulates bone resorption directly in vitro. Increased bone resorption may be an endocrine effect of TGF's secreted by tumor cells and may be responsible for the bone destruction frequently associated with malignancy.

Synthetic rat-type TGF-α was prepared as described by Tam *et al.* (*2*). Purity of the protein was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, amino acid analysis, and reversed-phase high-performance liquid chromatography (*2*). The biological activity of TGF-α preparations and synthetic TGF-α was monitored by

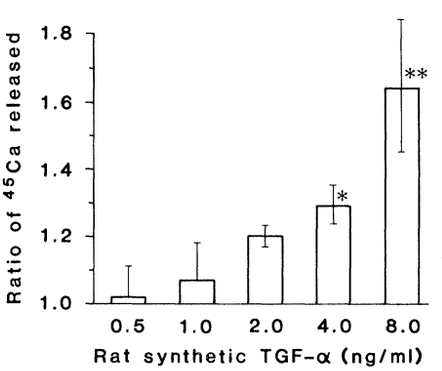


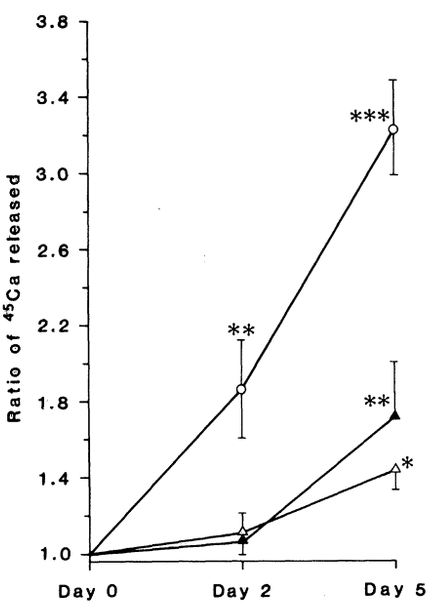
Fig. 1. Stimulation of bone resorption by synthetic TGF-α. Synthetic TGF-α was dissolved in 100 μl of 10 m*M* acetic acid containing bovine serum albumin (BSA) (fraction V; 5 mg/ml) (Sigma) and serially diluted into BGJb plus 5 percent fetal bovine serum. Doses are expressed as nanogram equivalents of EGF per milliliter of culture medium. Controls were treated with equivalent concentrations of 10 m*M* acetic acid containing BSA. Bone resorption was assessed by measuring the release of ^{45}Ca from previously labeled fetal rat long bones (*5*). Bones were cultured for 96 hours. Bone-resorbing activity is expressed as the treated-to-control ratio of ^{45}Ca release (± standard error of the mean of four bones per group). Statistical differences were calculated with Student's *t*-test for unpaired data (*$P < 0.05$; **$P < 0.025$).

means of an EGF radioreceptor assay (*4*). Bone resorption was assessed by measuring the release of ^{45}Ca from previously labeled fetal rat long bones. Pregnant rats at the 18th day of gestation were injected with 200 μCi of ^{45}Ca (*5*). The mothers were killed on the 19th day of gestation, and the fetuses were removed. The mineralized shafts of the radii and ulnae were dissected free of surrounding tissue and cartilage and placed in organ culture. The bones were incubated in BGJb medium (Irvine Scientific) for 24 hours at 37°C in a humidified atmosphere of 5 percent CO_2 and 95 percent air to allow for the exchange of loosely complexed ^{45}Ca. The bones were then cultured for 48 to 120 hours in BGJb medium supplemented with 5 percent fetal bovine serum (KC Biologicals) containing control or test substances. Bone-resorbing activity was measured as the percentage of total ^{45}Ca released into the medium and was expressed as a treated-to-control ratio. Statistical significance was determined with Student's *t*-test for unpaired data.

Synthetic rat-type TGF-α (molecular weight, 5600) in concentrations greater than 2 ng of EGF equivalents per milliliter stimulated bone resorption in a concentration-dependent manner in three separate experiments. The data from one experiment are shown in Fig. 1. Synthetic TGF-α caused no significant bone resorption during the first 48 hours of bone culture but clearly stimulated resorption over the following 3 days (Fig. 2). In this respect the synthetic form of TGF-α appears to be similar to EGF, which resorbs bone over a similar time course in this bioassay (*6*). The effect of TGF-α on bone resorption appeared to be independent of prostaglandin synthesis because bone-resorbing activity was not inhibited by $10^{-5}M$ or $10^{-6}M$ indomethacin (data not shown).

Since rat TGF-α resorbed bone in vitro, we also tested human preparations containing TGF-α activity for their effects on bone. Partially purified preparations of high and low molecular weight TGF-α were prepared from a human melanoma cell line (*7*). These preparations were partially purified by acid extraction and gel filtration chromatography (*7*). Both high (13,000) and low (6,000) molecular weight forms stimulated bone resorption (Fig. 3). The high molecular weight form stimulated resorption within 48 hours, whereas the low molecular weight form required 72 to 96 hours to stimulate resorption—a time course similar to that of synthetic rat-type TGF-α and EGF (*6*). The reason for the difference in time courses of the high and low molecular weight forms of TGF-α is not clear. The high molecular weight form may represent an incomplete processing of the precursor form (*8*) of TGF-α having slightly different properties. An alternative possibility is that the high molecular weight form contains other biologically active components that contribute to the bone-resorbing activity of the preparation.

We showed earlier that in a rat model of the humoral hypercalcemia of malignancy, TGF-α activity and bone-resorbing activity produced by the tumor cells eluted together from gel filtration columns (*3*). That observation led to the hypothesis that TGF-α, like EGF, could resorb bone (*6*). The bone-resorbing activity had an apparent molecular weight of 30,000 and eluted with TGF-α activity, as assessed by stimulation of soft agar growth of kidney fibroblasts and inhibition of EGF binding to its receptor (*3*). We also found that antiserums to the EGF receptor that inhibit the action of the low molecular weight TGF sarcoma growth factor (*9*) inhibited the bone-resorbing activity produced by the Leydig tumor cells (*10*). Our data in this report together with earlier observations suggest that the TGF's could be responsible for the increased bone destruction associated with some neoplastic diseases. These factors probably circulate since increased amounts of TGF activity have been found in the urine of patients with cancer (*11*), and extracts of urine from hypercalcemic cancer patients contain the bone-resorbing activity (*12*). All of these observations indicate that transforming growth factors may be humoral mediators of hypercalcemia in some neoplastic disease states.



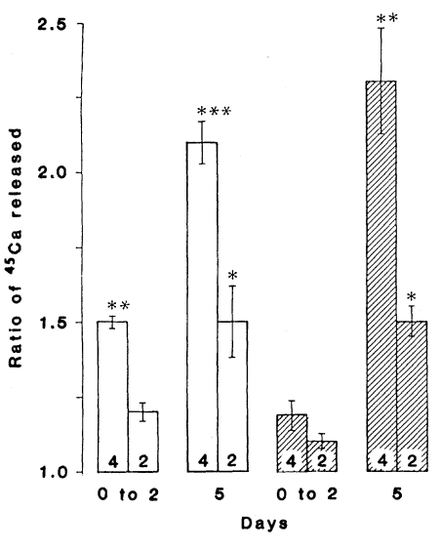


Fig. 2 (left). Time course of action of (○) parathyroid hormone (400 ng/ml); (▲) rat-type synthetic TGF-α (6 ng of EGF equivalents per milliliter; and (△) EGF (30 ng/ml). The bone resorption assay was performed as described in Fig. 1, except that the bones were first incubated for 48 hours, then transferred to wells containing fresh medium and parathyroid hormone, TGF, or EGF as appropriate and incubated for 72 hours longer. The release of ^{45}Ca over each incubation period was measured. Bone-resorbing activity is expressed as the treated-to-control ratio of ^{45}Ca release (± standard error of the mean of four bones per group) ($^*P < 0.05$; $^{**}P < 0.025$; $^{***}P < 0.005$). Fig. 3 (right). Bone resorbing activity of BioGel P10 column fraction of TGF-α from medium conditioned with human melanoma cells (*7*). Open bars represent the 13,000-dalton fraction and hatched bars the 6,000-dalton fraction. Lyophilized material was dissolved in 100 μl of 10 m*M* acetic acid containing BSA (5 mg/ml). The amount of TGF-α added was either 4-ng or 2-ng equivalents of EGF per milliliter. The bone resorption assay was performed as described in Fig. 1, except that the bones were first incubated for 48 hours, then transferred to wells containing fresh medium and TGF as appropriate and incubated for 72 hours longer. The release of ^{45}Ca over each incubation period was measured. Bone-resorbing activity is expressed as the treated-to-control ratio of ^{45}Ca release (± standard error of the mean of four bones per treatment) ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$).

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References and Notes

1. M. B. Sporn and G. J. Todaro, *N. Engl. J. Med.* **303**, 878 (1980).
2. J. P. Tam *et al.*, *Nature (London)* **309**, 376 (1984).
3. K. J. Ibbotson *et al.*, *Science* **221**, 1292 (1983).
4. G. J. Todaro, J. E. DeLarco, S. Cohen, *Nature (London)* **264**, 26 (1976).
5. L. G. Raisz, *J. Clin. Invest.* 44, 103 (1975).
6. ——— *et al.*, *Endocrinology* **107**, 270 (1980); A. H. Tashjian and L. Levine, *Biochem. Biophys. Res. Commun.* **85**, 966 (1978).
7. H. Marquardt and G. J. Todaro, *J. Biol. Chem.* **257**, 5220 (1982).
8. R. Derynck *et al.*, *Cell* **38**, 287 (1984).
9. G. Carpenter *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5627 (1983).
10. K. J. Ibbotson *et al.*, *Endocrinology* **116**, 469 (1985).
11. D. R. Twardzik *et al.*, *J. Natl. Cancer Inst.* **69**, 793 (1982); S. A. Sherwin *et al.*, *Cancer Res.* **43**, 403 (1983).
12. K. J. Ibbotson *et al.*, *Calcif. Tissue Int.* **35**, 605 (1983).

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Selection for Increased Safety Factors of Biological Structures as Environmental Unpredictability Increases

Abstract. *Theory predicts that selection should increase the ratio of the performance of a biological structure or system to the requirements placed upon it (that is, its safety factor) as conditions become increasingly unpredictable. Although conventional safety factors are rarely measurable, an alternative, truncation safety factor (the ratio of mean strength to maximum possible load), can be measured quantitatively for certain load-bearing structures. For intertidal limpet shells subject to prying forces, truncation safety factor was found to increase with increased variability in shell strength, thus providing direct support for the theory.*

Most biological structures, from the cellular to organ system level, have evolved in environments that are, to a greater or lesser degree, unpredictable. In particular, using load-bearing structures as an example, one would expect lifetime maximum loads on such structures to vary in an unpredictable fashion. Through environmental effects on development and aging, the strength of these structures should also vary unpredictably. Therefore, selection for the strength of a structure, relative to the maximum load it must bear, should be sensitive not only to the average maximum load likely to be encountered, but also to variability in the strength of the structure or the maximum load on the structure, or both (*1*). One measure of relative strength is "safety factor"—that is, the ratio of mean strength to mean lifetime maximum load (*2*).

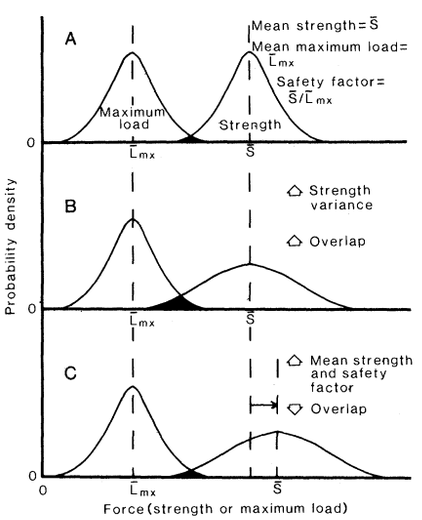

Fig. 1. Potential effect of increasing variance (unpredictability) on safety factor. The curves illustrate, for a given load-bearing, biological structure, the distribution of structural strengths and lifetime maximum loads for individuals within a single population as strength variance is increased. Increasing maximum load variance would have an analogous effect.

Figure 1A illustrates, for a hypothetical load-bearing structure, the mean force required to break the structure ($\bar{S}$) and the mean maximum force that structure experiences during its lifetime ($\bar{L}_{mx}$), where these means are calculated for a population of individuals; safety factor equals $\bar{S}/\bar{L}_{mx}$. Since individual structures in the population will not all break at the same force (S), there will be a variance in S. Also, since individual structures will not all experience the same lifetime maximum load (L_{mx}), there will be a variance in L_{mx}. If the distribution of L_{mx} is described by function f and the distribution of S is described by function g, then the probability (PF) that an individual structure, chosen at random from the population, will fail sometime during its lifetime is

$$PF = \int_0^\infty g(x) \int_x^\infty f(y)dydx$$

(relative magnitudes of PF are depicted as shaded overlap regions in Fig. 1). If such a failure is deleterious, selection should act to minimize PF by increasing $\bar{S}$ when it is not possible to reduce $\bar{L}_{mx}$ or either variance. However, if there is a cost to strengthening the structure, PF may not be reduced to zero. If the variance in S or L_{mx} is increased without changing the means, the overlap, and thus PF, will increase (Fig. 1B). Consequently, selection should act to increase the safety factor by increasing $\bar{S}$ to bring PF back down to a suitably low value (Fig. 1C). Therefore, more variable systems should experience selection for greater safety factors—that is, greater relative strengths (*3*).

Testing this prediction requires a consistent measure of relative strength—that is, a measure (with statistically fixed points such as $\bar{S}$ and $\bar{L}_{mx}$ used to define a safety factor) of the relative positions of the two curves illustrated in Fig. 1A. The mean and variance of strength are readily obtained for many structures by measuring the force required to break several test specimens from the population of interest. The mean and variance of lifetime maximum load, however, are usually difficult to measure under natural conditions because of the heterogeneous environment of forces that most load-bearing structures experience. For example, $\bar{L}_{mx}$ for a given segment of an adult zebra femur would be a complex function of several variables including the adult lifetime probabilities of a wide range of accelerations, decelerations, cruising speeds, falls, and collisions with predators and conspecifics. Biewener (*4*) has shown that a nonrigorous choice of $\bar{L}_{mx}$ can lead to the perhaps unreasonable conclusion that the limb bones of

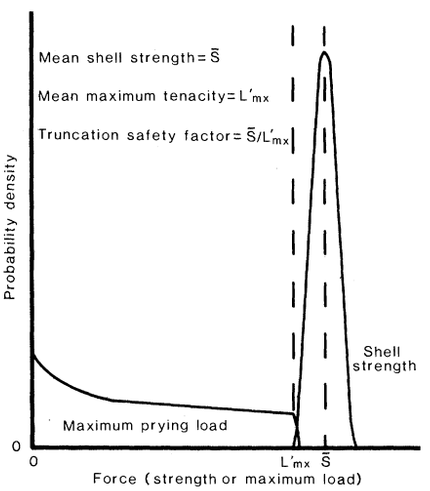

Fig. 2. Truncation safety factor for hypothetical limpet population subject to prying forces. The shapes of the maximum prying load and shell strength distributions are meant to suggest that (i) maximum load variance is likely to be greater than strength variance because of the heterogeneity of the intertidal environment, (ii) many limpets probably live to reproduce and then die without ever having experienced a prying load, and (iii) maximum tenacity determines the right-hand truncation of the maximum load curve.