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Tumor-Derived Growth Factor Increases Bone Resorption in a Tumor Associated with Humoral Hypercalcemia of Malignancy

Abstract. *Evidence is presented that a tumor-derived transforming growth factor is responsible for stimulating bone resorption and causing hypercalcemia in an animal tumor model of the hypercalcemia of malignancy. Both conditioned medium harvested from cultured tumor cells and tumor extracts of the transplantable rat Leydig cell tumor associated with hypercalcemia contained a macromolecular bone resorbing factor with the chemical characteristics of a tumor-derived transforming growth factor.*

The mechanisms underlying tumor-associated hypercalcemia have not been clearly defined. Some tumors cause hypercalcemia without invading bone, presumably by releasing a systemic factor that stimulates bone resorption (*1, 2*). A family of polypeptide growth factors called "transforming growth factors" (TGF's) are secreted by tumors and display some of the biological properties of epidermal growth factor (EGF) (*3–6*). Some (but not all) TGF's compete with EGF for binding to its receptor (*6*). Since EGF and other growth factors stimulate bone resorption (*7–9*), it is possible that a tumor-derived TGF with properties similar to those of EGF may be one of the factors responsible for increased bone resorption in the humoral hypercalcemia of malignancy. We report that both extracts from an animal tumor associated with hypercalcemia and conditioned medium from cultures of the tumor cells contain a bone resorbing factor, and that purified fractions of this factor contain biological activities associated with TGF's.

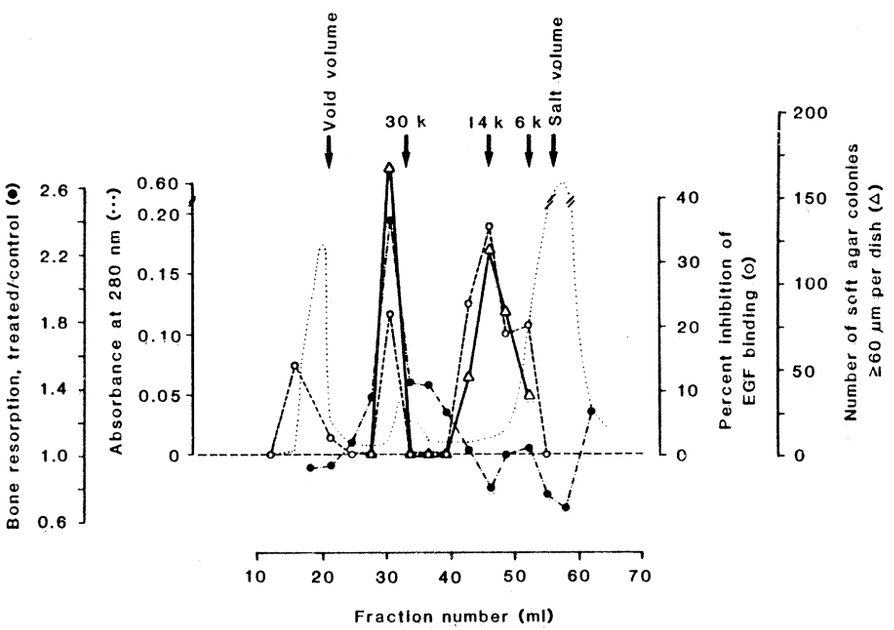

Fig. 1. Biological activities in extracts of Leydig tumors from hypercalcemic rats. Tumor (10 g) was extracted in acid and ethanol and chromatographed on a Bio-Gel P-100 column (1.5 by 40 cm) equilibrated with 1*M* acetic acid. Fractions (1 ml) were pooled in batches of three, divided into portions (2 ml for bone resorbing activity and 0.5 ml each for EGF competition and soft agar growth stimulating activity), and lyophilized. The portions were resuspended in the appropriate medium and tested for bone resorbing activity (bones incubated in control medium alone released 12.3 ± 2.4 percent of total ^{45}Ca), EGF competing activity, shown as the percentage of [^{125}I]EGF specifically bound, and soft agar growth stimulating activity, as described in the legend to Table 1. Values for soft agar colonies were corrected for colonies in control plates (26.6 ± 1.4). Molecular weight markers are blue dextran for the void volume, carbonic anhydrase (29,500), ribonuclease (13,500), insulin (6000), and [^{3}H]proline for the salt volume.

The transplantable Leydig cell tumor that occurs in the aged Fischer rat causes hypercalcemia in these animals (*2*). The mechanism of hypercalcemia in this tumor model is increased osteoclastic bone resorption stimulated by a factor produced by the tumor cells. Hypercalcemic rats bearing the tumor have increased osteoclast numbers and activity in bones at sites distant from the tumor, increased urinary hydroxyproline, and reversal of hypercalcemia and the histological changes in bone after treatment with dichloromethylene diphosphonate, a drug that inhibits osteoclastic bone resorption (*10*). Hypercalcemia is due to a systemic mediator because excision of the tumor leads to reversal of the hypercalcemia (*2, 11*). To identify the mediator of bone resorption responsible for hypercalcemia, we examined tumor extracts and conditioned medium from cultures of tumor cells for bone resorbing activity by using a bioassay for bone resorption that is based on the release of previously incorporated ^{45}Ca from fetal rat long bones in organ culture (*12, 13*).

Pregnant rats in the 18th day of gestation were injected with 0.2 mCi of ^{45}Ca. The following day the fetuses were removed and the mineralized shafts of the radii were excised and placed in organ culture. Some bones were cultured with tumor products to be tested for bone resorbing activity and other bones were cultured with control medium. Bone resorbing activity was found both in conditioned medium and in tumor extracts (Table 1 and Fig. 1). The activity in the conditioned medium was consistently lost over a fourfold dilution [test/control ratio (mean ± standard error) at one-half dilution, 2.51 ± 0.12; at one-fourth dilution, 1.53 ± 0.11; and at one-eighth dilution, 0.97 ± 0.03).

Freshly excised tumors were extracted with acid and ethanol, precipitated with ethanol and ether, and lyophilized (*5*). The lyophilized samples were chro-

matographed on a Bio-Gel P-100 column (1.5 by 40 cm) and equilibrated in 1*M* acetic acid at flow rates of 6 ml/hour. Portions of fractions eluted from this column were lyophilized, resuspended in distilled water, relyophilized, suspended in culture medium, and assessed for bone resorbing activity. The activity was eluted from this column with proteins having an apparent molecular weight of approximately 30,000 (Fig. 1). Conditioned medium harvested from tumor cell cultures was lyophilized and extracted with 1*M* acetic acid. This extract was centrifuged at 100,000*g* for 60 minutes, and the supernate was chromatographed on a Bio-Gel P-60 column (2.5 by 95 cm) equilibrated in 1*M* acetic acid. Bone resorbing activity in the conditioned medium of cultured tumor cells coeluted with the molecular weight marker carbonic anhydrase (Table 1). These results indicate that the bone resorbing factor produced by the Leydig tumor cells is a macromolecular factor of about 30,000 daltons.

Tumor extracts and tumor cell–conditioned medium were then examined for bone resorbing activity and the biological activities associated with tumor-derived TGF's: competition with EGF for binding to cultured cells and promotion of anchorage-independent growth and mitogenesis (*5*). Competition with EGF for binding to its receptors was assessed by adding conditioned medium or tumor extracts to subconfluent monolayers of UMR-106 osteosarcoma cells (*14, 15*). Identical results were obtained when the human breast cancer cell line MCF-7 was used as the source of EGF receptors (*15*). The mitogenic activity was assessed by its ability to stimulate incorporation of [^{3}H]thymidine into cultured UMR-106 cells (*14*). Promotion of anchorage-independent growth was determined by adding the test medium to 3T3 mouse fibroblasts cultured in soft agar medium. It should be noted that, at 2 ng/ml, pure EGF causes 40 percent inhibition of [^{125}I]EGF binding to receptors on UMR-106 cells and stimulates the formation of 40 to 50 colonies per plate in the soft agar colony assay but has no bone resorbing activity. Maximum bone resorbing doses of pure EGF (10 ng/ml) cause 80 percent inhibition of binding. Thus the bone resorbing assay is less sensitive to pure EGF than the other two bioassays.

When media were assayed for immunoreactive EGF by measuring their cross-reactivity with rat EGF, no immunoreactive EGF (< 1 ng/ml) was detectable in starting material or control medium. The EGF radioimmunoassay was performed by using rabbit antibodies to purified mouse EGF, with ^{125}I-labeled mouse EGF as a tracer (*14*). Hence, authentic EGF could not account for the activity that competed with labeled EGF for binding to receptors on cultured cells.

The physicochemical properties of the bone resorbing factor produced by cultured tumor cells and tumor extracts were compared with those ascribed to other TGF's. The TGF's characterized to date have a number of properties, including acid stability, sensitivity to dithiothreitol, and sensitivity to trypsin (*4–6*). The bone resorbing activity was stable in 1*M* acetic acid (Fig. 1) and was lost when exposed to dithiothreitol and trypsin.

Conditioned medium from the tumor cell cultures was chromatographed on a Bio-Gel P-60 column, and portions of the elution fractions were examined for mitogenic activity and EGF competing activity as well as bone resorbing activity. The conditioned medium contained activity (molecular weight, approximately 30,000) that was mitogenic, prevented binding of EGF to its receptors, and coeluted with the bone resorbing activity (Table 1). A peak of soft agar colony stimulating activity coeluted with these other activities. Tumor extracts were chromatographed on a Bio-Gel P-100 column and portions of eluted fractions were tested for bone resorbing activity,

Table 1. Bone resorbing activity, EGF competing activity, mitogenic activity, and soft agar colony stimulating activity in conditioned medium harvested from cultures of Leydig tumor cells. Conditioned medium (60 ml) harvested from tumor cells (10 ml of medium per 10^7 cells) was extracted into 1*M* acetate and the supernate was chromatographed on a Bio-Gel P-60 column. Pooled column fractions were divided into equal portions and assessed for biological activity. Blue dextran eluted in fraction A, carbonic anhydrase (29,500 daltons) in fraction B, and insulin (6000 daltons) in fraction D. Protein concentration in the starting material was 1.4 mg/ml; in fraction A, 1.4 mg/ml; and in fraction B, 0.05 mg/ml. Bone resorbing activity was assessed by measuring the release of previously incorporated ^{45}Ca from fetal long bones. Results shown are test/control ratios of ^{45}Ca released during 48 hours in culture (N = 4 bones). Bones incubated in control medium alone release 21.2 ± 2.1 percent of total ^{45}Ca. EGF competing activity was assessed by adding test or control materials in the presence of [^{125}I]EGF (150 pg per well; Collaborative Research) to subconfluent UMR-106 cells in 12-well plates. After 2 hours at 37°C the medium was removed, the cells were washed three times with ice-cold phosphate-buffered saline and solubilized in 0.5*M* NaOH, and the quantity of [^{125}I]EGF specifically bound was determined. Nonspecific binding in the presence of unlabeled EGF (1 μg/ml) was 5 percent of total binding; EGF at 3 ng/ml resulted in 50 percent competition. Results are counts per minute × 10^{-3} per 10^6 cells (N = 3 wells). Mitogenesis was assessed by incubating UMR-106 cells with test substances for 24 hours and then with [^{3}H]thymidine (0.5 μCi/ml; specific activity 20 to 30 Ci/mmole) for 2 hours. Trichloroacetic acid–precipitable material was collected on Millipore filters that were dried, dissolved in tetrahydrofuran (2 ml; Waters), and counted in 5 ml of Aquasol (New England Nuclear). Values shown are counts per minute × 10^{-3} per well (N = 3 wells). Soft agar growth stimulating activity was measured by mixing test or control substances with 3T3 cells (3 × 10^3 cells per plate) in 0.3 percent (weight to volume) agar in Dulbecco's minimum essential medium plus 10 percent fetal calf serum and plating 1 ml of the mixture over a 1-ml base layer of 0.5 percent (weight to volume) agar into each 35-mm petri dishes. Colonies ≥ 60 μm in diameter were counted 12 days later by using a Bausch & Lomb FAS II image analyzer. N.T., not tested. Statistical comparisons for bone resorbing activity were made with modified t-tests; for other bioassays the formula for the comparison of means of small samples with unknown variances not assumed to be equal was used. The probability of a significant difference between two means was then determined from Student's t tables.

Biological activity	Starting material	Column fraction			
		A	B	C	D
Bone resorbing activity (test/control ratios)	1.79 ± 0.20*	1.32 ± 0.11†	1.50 ± 0.06*	1.03 ± 0.08	0.89 ± 0.09
EGF competing activity					
Test	2.50 ± 0.10‡	9.00 ± 0.20	6.90 ± 0.40†	10.70 ± 0.40	9.60 ± 0.30
Control	4.80 ± 0.10	11.10 ± 0.50	11.80 ± 0.50	11.80 ± 0.60	10.50 ± 0.30
[^{3}H]thymidine incorporation					
Test	20.90 ± 0.30‡	18.00 ± 0.60†	20.90 ± 0.30‡	15.30 ± 0.50	13.60 ± 0.50
Control	13.00 ± 0.30	11.70 ± 0.70	12.70 ± 0.30	12.70 ± 0.40	10.50 ± 0.30
Soft agar colony stimulating activity					
Test	49.00 ± 3.00†	N.T.	N.T.	N.T.	N.T.
Control	12.00 ± 1.00	N.T.	N.T.	N.T.	N.T.

*Significantly different from corresponding control value at $P < 0.005$. †$P < 0.01$. ‡$P < 0.01$

soft agar growth stimulating activity, and EGF competing activity (Fig. 1). Bone resorbing activity was again eluted from this column with a molecular weight of approximately 30,000. A peak of EGF competing activity and a peak of soft agar growth stimulating activity consistently (in four different experiments) coeluted with the bone resorbing activity. In addition, other peaks of soft agar growth stimulating activity and EGF competing activity were eluted from this column. None of these was associated with bone resorption. It is not surprising that several peaks of soft agar growth stimulating activity and EGF competing activity were found in tumor extracts, since others have shown that multiple peaks of TGF activity of similar molecular weight and bioactivity may be present in tissue extracts (*4, 16*).

The evidence that a TGF produced by the tumor cells is responsible for increased bone resorption is strong. The bone resorbing factor released by the tumor cells shares all the known chemical properties of the TGF released by the same cells: it is acid-stable, depends on disulfide bonds for activity, is trypsin-sensitive, coelutes from gel filtration columns with soft agar colony stimulating activity and mitogenic activity, and is able to prevent binding of EGF to its receptors. However, final proof that the bone resorbing factor is the same polypeptide as the TGF will not be possible until a pure homogeneous preparation of the bone resorbing factor is shown to contain all the relevant biological activities of the TGF (competing activity for EGF receptors, mitogenic activity, and promotion of anchorage-independent growth of nontransformed cells). It seems likely that only small amounts of protein are responsible for these activities, so purification to homogeneity will probably be difficult (*17*).

Rats carrying the Leydig cell tumor have other features that have been associated with the humoral hypercalcemia of malignancy seen in humans (*2, 18*). There is absence of bone metastasis, increased generation of nephrogenous adenosine 3′,5′-monophosphate (cyclic AMP), hypophosphatemia, and renal phosphate wasting. The relation of the production of a tumor-derived TGF to nephrogenous cyclic AMP generation and renal phosphate wasting is unknown. Recently, we found that medium conditioned by these tumor cells contains a factor that causes renal phosphate wasting (*19*). Clarification of the interrelations between the factor responsible for hypercalcemia and these other parameters associated with the humoral hypercalcemia of malignancy will require further study.

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Axonal Proteins of Presynaptic Neurons During Synaptogenesis

Abstract. *Changes occur in the synthesis and axonal transport of neuronal proteins in dorsal-root ganglia axons as a result of contact with cells from the spinal cord during synapse formation. Dorsal-root ganglia cells were cultured in a compartmental cell culture system that allows separate access to neuronal cell bodies and their axons. When cells from the ventral spinal cord were cultured with the dorsal-root ganglia axons, synapses were established within a few days. Metabolic labeling and two-dimensional electrophoresis revealed that four of more than 300 axonal proteins had changed in their expression by the time synapses were established. The highly selective nature of these changes suggests that the proteins involved may be important in the processes of axon growth and synapse formation and their regulation by the regional environment.*

During the formation of a synapse, ultrastructural changes occur in the postsynaptic neuron, in the intercellular cleft, and in the presynaptic neuron (*1*). The cytoarchitectural and functional conversion of the probing growth cone to a transmitting synapse is complex, and its molecular basis is far from being understood. The proteins involved in the implementation of the structural and functional specializations of the axon and its tip are synthesized in the neuronal soma and moved to their site of destination by axonal transport. Neurons can be grown in a three-compartment cell culture system that offers separate access to cell somas and axons (*2*). We have used this cell culture technique in conjunction with metabolic labeling of proteins and two-dimensional gel electrophoresis (*3*) to provide a selective and high-resolution analysis of newly synthesized axonal proteins. We now report that a few distinct axonal proteins change in their expression when axons from dorsal-root ganglion (DRG) neurons and ventral spinal cord (VSC) neurons are cultured together under conditions in which synapse formation occurs.

Dissociated DRG cells from 10-day-old chick embryo were plated in the center compartment of the three-compartment cell culture system (Fig. 1). The outgrowing axons crossed the barrier between center and side compartments through a thin film of medium, whereas the cell somas were retained in the center compartment. After 10 days, cells from the VSC of 6-day-old chick embryos were cultured as presumptive postsynaptic cells in the two side compartments (*4*). Four days after they were