Resorbing Bone Stimulates Tumor Cell Growth

A Role for the Host Microenvironment in Bone Metastasis

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Demineralized extracts of bone matrix and conditioned media from cultured fetal rat calvaria have been reported to contain growth stimulatory activity for bone cells. To investigate the potential role of these local bone growth factors in the development of bone metastases, we chose the Walker 256 carcinosarcoma, a rat mammary tumor which causes osteolytic bone metastases and hypercalcemia. ^{45}Ca-labeled, 19-day fetal Sprague–Dawley rat calvaria were cultured for 96 hours in BGJ$_b$ medium. Walker cells from ascites tumors or cultures were grown in unconditioned media or in conditioned media harvested from the bone cultures, in the presence of 10% fetal calf serum. Media were changed every 2 days, cells were counted daily for 5 days, and ^{3}H-thymidine uptake into acid insoluble residues was measured. The growth of tumor cells was 5–6-fold greater in conditioned media than in unconditioned media and the effect was dose dependent. Cells cultured in conditioned media demonstrated a ~3-fold enhancement of ^{3}H-thymidine incorporation. Generation of growth stimulatory activity correlated with the extent of bone resorption, measured by release of ^{45}Ca from the fetal parietal bones ($r = 0.85$; $P < 0.001$). Conditioned media from bones cultured with 10^{-7} M prostaglandin E_2 (PGE_2) contained greater amounts of growth stimulatory activity than untreated conditioned media, but PGE_2 itself did not stimulate tumor cell growth. Addition of 3.5 mM PO_4 to bone cultures blocked bone resorption and the generation of growth factors. Growth stimulatory activity was stable to heat (56 C for 30 minutes) and trypsin digestion, with an apparent molecular weight of less than 17,000 daltons by high-performance liquid chromatography. Conditioned medium also stimulated the growth of 13762 rat mammary adenocarcinoma cells, MB-MDA-231 human breast carcinoma cells, TE-85 osteosarcoma cells, a murine fibrosarcoma and rat embryonic fibroblasts, with the most potent effects noted for Walker tumor cells, the TE-85 osteosarcoma, and human breast carcinoma lines. These results suggest a mechanism by which bone resorption could promote the development of skeletal metastasis. (Am J Pathol 1986, 123:39–45)

IT HAS LONG BEEN recognized that the skeleton is one of the preferential sites for metastases, especially in patients with multiple myeloma and carcinomas of the breast, prostate, thyroid, and bronchus.[1] The pathophysiology of the associated bone destruction has been investigated[2,3] and is thought to be mediated either directly by the tumor[4–6] or through stimulation of osteoclasts by prostaglandin E_2 (PGE_2),[7–10] osteoclast activating factor,[11] parathyroid hormone-like substances,[12] or alpha transforming growth factors[13] produced by the tumor. However, the mechanisms responsible for the site specificity of metastasis are less well defined. Paget hypothesized that metastasis is dependent upon provision of a fertile environment (the soil) in which compatible tumor cells (the seed) could proliferate.[14] This view has received support from observations in mice wherein fragments of lung, maintained in ectopic sites *in vivo*, metastatic tumor deposits developed when the animals were given lung colonizing tumors by various routes.[15]

With regard to skeletal metastasis, several studies suggest that bone resorption may help to provide an environment favorable for the development of a metastatic focus. Exposure of Walker 256 carcinosarcoma cells to separate products of bone resorption causes chemotactic movement, adhesion, and aggregation of these rat mammary tumor cells.[16,17] Resorbing bone has also

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been noted to release stimulators and inhibitors of bone cell growth.[18,19] These substances are thought to represent local regulators of skeletal remodeling, maintaining bone volume in steady state by balancing osteoclastic and osteoblastic activity. Although osseous remodeling has been noted in over 90% of bone biopsies with metastasis[20] and is frequently observed radiologically around osteolytic metastases,[21] the possibility that products of bone resorption may influence tumor cell growth has not been studied. We chose to examine the relationship between bone resorption and tumor cell growth using the Walker 256 carcinosarcoma, a spontaneous rat mammary tumor which, like human breast cancer, tends to cause osteolytic bone deposits with hypercalcemia.[22] We report here that conditioned media harvested from resorbing rat calvaria contain growth-stimulating activity for tumor cells. We postulate that metastatic cells capable of inducing bone resorption could cause release of such substances, thereby creating an environment favorable to their own growth in bone.

Materials and Methods

Bone Cultures

On the 19th day of gestation, pregnant Sprague-Dawley rats (Charles River, Montreal) were given 40 μCi of ^{45}Ca by subcutaneous injection. Twenty-four hours later, the calvaria of the fetal rats were removed aseptically, and each parietal bone was placed into a culture well containing 1.0 ml BGJ_b medium (Fitton-Jackson Modification, GIBCO, Grand Island, NY). The bones were preincubated at 37 C in a humidified atmosphere with 5.0% CO_2 and air for 24 hours to allow exchange of loosely bound ^{45}Ca with the culture medium. The culture medium was then replaced with fresh medium. In some experiments various stimulators or inhibitors of bone resorption were added to the media, including 0.5 mg/ml bovine serum albumin,[23] 5% dextran-charcoal-treated, heat-inactivated fetal calf serum depleted of complement,[24] 10% complement-sufficient fetal calf serum, PGE_2,[16] and 3.5 mM phosphate.[25] The calvaria were cultured for 4 additional days and bone resorption was measured by calculating the percentage of ^{45}Ca released from each bone into the culture medium.[16,26]

Cell Lines

The Walker 256 carcinosarcoma was maintained by alternate passage in culture and as an ascites tumor.[17] Rat embryonic fibroblasts were prepared by a method modified from that of Duff and Rapp[27] as described previously.[28] The 13762 MAT tumor (a rat mammary adenocarcinoma), the MB-MDA-231 human breast carcinoma, a murine fibrosarcoma (FS/L10), and the TE-85 osteosarcoma cells were maintained as frozen stocks in a liquid nitrogen freezer and reestablished in tissue culture before the experiment.

Assays of Cell Growth

The kinetics of cell growth in BGJ_b medium with 5% and 10% fetal calf serum were determined for each of the cell lines to be tested. In experiments in which we were looking for growth-promoting factors in conditioned media, culture dishes containing various dilutions of conditioned media from the bone cultures were seeded with tumor cells at a concentration consistent with the lag phase of cell growth. Thereafter, daily counts of cell number were made by hemacytometer or by an electronic cell counter (Model ZBI, Coulter Electronics, Inc., Hialeah, Fla). The effects of fetal calf serum alone on cell replication were controlled by ensuring that all media tested for growth stimulatory activity contained the same concentration of serum prior to commencement of the assay. In some experiments DNA synthesis was determined by counting ^{3}H-thymidine incorporation into the trichloroacetic acid insoluble precipitates. After 48 hours' exposure to test media, cells were pulsed for 3 hours with ^{3}H-thymidine (6.7 Ci/mM) at a concentration of 0.5 μCi/ml, removed by centrifugation, and treated with three washes of 10% trichloroacetic acid and one wash of ethanol and counted in a liquid scintillation counter.

Characterization of Growth-Promoting Activity

Freshly harvested conditioned media from 96-hour cultures of resorbing bone were subjected to heating at 56 C for 30 minutes or to three cycles of freeze-thawing in liquid nitrogen. Conditioned media was also treated with agarose-bound trypsin beads (Sigma Chemical Co., St. Louis, Mo) at concentrations between 1 and 5 U/ml for 1 hour at 37 C.[29] Trypsin was removed by centrifugation, and the media were assayed for growth stimulatory activity.

Conditioned media from resorbing bone cultured with bovine serum albumin was concentrated 10-fold by lyophylization, reconstituted with sterile water, and analyzed by high performance liquid chromatography on a 600 × 7.5-mm Bio-Sil TSK-250 column equilibrated and eluted with a 10 mM Tris HCl and 0.15 M NaCl, pH 7.0, solution. Fractions of 2.0 ml were collected, sterilized by filtration through a 0.22-μ nitrocellulose filter (Millipore), supplemented with 10% fetal calf serum, and added to media for cell culture.

Table 1—Effect of Conditioned Bone Culture Media on the Growth of Walker 256 Carcinosarcoma Cells

Medium	Cell growth* (Cell Number ± SE × 10^5) Experiment 1‡	Cell growth* (Cell Number ± SE × 10^5) Experiment 2‡	^{3}H-Thymidine incorporation† (CPM ± SE × 10^5) Experiment 2
BGJ_b unconditioned	6.3 ± 0.2	8.2 ± 0.1	2.2 ± 0.1
BGJ_b conditioned			
Undiluted	30.5 ± 0.5§	–	–
Diluted 1:2	–	23.5 ± 0.3§	6.3 ± 0.6‖
Diluted 1:5	11.3 ± 0.8§	–	–
Diluted 1:10	7.8 ± 0.7	–	–

* Cells counted 4 days after plating at 5 × 10^5/ml in media supplemented with 10% fetal calf serum. Media changed on Day 2.
† Radioactivity in trichloroacetic acid insoluble residues after 3-hour pulse of ^{3}H-thymidine, 0.5 μCi/ml.
‡ Bone resorption (percent ^{45}Ca release ± SE): Experiment 1, 43.4 ± 4.9; Experiment 2, 26.5 ± 4.2.
§ $P < 0.001$.
‖ $P < 0.05$.

Significance of differences was established with the use of the Student t test.

Results

Mitogenic Activity of Conditioned Media Harvested From Cultured Calvaria

Conditioned media, when applied to fresh cultures of Walker tumor cells, caused a dose-dependent increase in cell growth after 4 days of culture (Table 1). When growth activity was analyzed by cell counting, a 5–6-fold increase was observed in cultures containing conditioned media as compared with controls (Figure 1). A near 3-fold stimulation of ^{3}H-thymidine incorporation was also noted. The growth stimulatory effects of conditioned media on tumor cells were maintained for dilutions up to 1:10.

Relationships Between Bone Resorption and Generation of Growth Activity

We detected a direct relationship between the extent of bone resorption, measured by the percentage of calcium released from cultured bones, and growth stimulatory activity in the medium ($r = 0.85$; $P < 0.001$; Figure 2). Addition of various stimulators of resorption altered the generation of growth activity but did not themselves influence the growth of cells in unconditioned media (Table 2). As noted by Lorenzo and Raisz,[24] maximal bone resorption was obtained with 10% fetal calf serum, probably due to complement-mediated stimulation of prostaglandin synthesis by bone cells. Although elevated levels of PGE_2 have been reported in conditioned media from resorbing bones,[30] no significant growth stimulatory activity was noted when PGE_2 was added exogenously to unconditioned media. Phosphate blocked both bone resorption and release of growth activity into conditioned media. To rule out calcium as a mitogen, the calcium in fresh medium was chelated by 0.2 M EDTA and then replaced with known amounts of $CaCl_2$ in concentrations ranging from 0 to 5 mM. These far exceeded the range observed in conditioned media when the calcium concentration was analyzed by atomic absorption spectrophotometry (2.4–2.6 mM). No significant growth stimulation was noted after calcium was added to fresh media (data not shown).

Physical Properties of the Bone-Derived Growth Factor

The growth-promoting activity of conditioned media from resorbing bones was temperature-stable be-

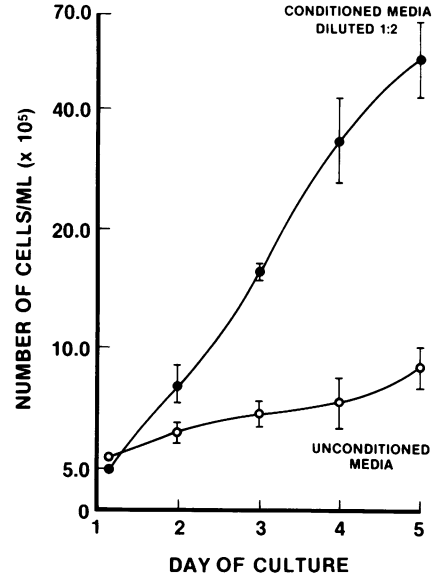

Figure 1—Effect of conditioned media from 96-hour cultures of resorbing rat calvaria on growth of Walker tumor cells. Mediator of resorption used in bone culture was 5% dextran-charcoal treated fetal calf serum. Percent ^{45}Ca release (± SE), 40.3 ± 3.9; 95% confidence intervals (± 2 SE) displayed on semi-log plot. n/group = 3.

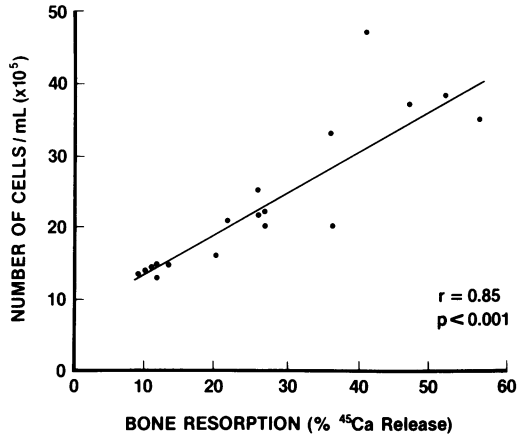

Figure 2—Relationship between bone resorption and the generation of growth stimulatory activity for tumor cells in conditioned media. Data represent resorption values of individual calvaria after 72 hours of organ culture and the effect of conditioned media from these bones on the growth of 5 × 10^5 Walker tumor cells after 3 days. As indicated in the text, various mediators of resorption were employed.

tween −20 and 37 C for storage periods of 1 week and was heat-stable at 56 C for 30 minutes. The mitogenic activity of conditioned medium was not decreased by trypsinization at concentrations between 5 and 1 U/ml (Table 3). After high-performance liquid chromatography, growth stimulatory activity was recoverable in a fraction which eluted after myoglobin, when assayed by both cell counts (Figure 3) and ^{3}H-thymidine incorporation (not shown). As a control, unconditioned medium was fractionated and tested for growth activity; no significant growth stimulation was noted in any of the column fractions. We were unable to detect any interleukin-1 activity in conditioned media using the thymocyte coactivator assay (data not shown[31]).

Cell Specificity of Skeletal Growth Factor

Table 4 demonstrates the effects of conditioned media on a variety of cell lines, when cultured under the same conditions as the Walker tumor cells. As previously reported by Canalis,[32] the actions of bone-derived growth factor were not specific for a single cell type. Growth stimulation was greatest for the Walker tumor but was also significant for the human breast carcinoma MB-MDA-231, the 13762 rat mammary adenocarcinoma, a methylcholanthrene-induced fibrosarcoma FS/L10, and in rat embryonic fibroblasts. Growth stimulation was also noted for the osteosarcoma line TE-85, a cell with osteoblastic properties previously reported to be sensitive to human skeletal growth factor.[33]

Table 3—Properties of Bone-Derived Growth Factor

Treatment	Cell Number ± SE × 10^5 Experiment 1*	Experiment 2†	% Inhibition of activity
Unconditioned medium (untreated)	18.8 ± 0.3	4.0 ± 0.6	–
Conditioned media			
Untreated	33.5 ± 2.8	10.4 ± 0.5	–
Trypsin‡ 5 U/ml	33.8 ± 1.3	–	0
Trypsin 1 U/ml	31.0 ± 1.0	–	0
Freeze-thaw × 3	–	12.6 ± 0.7	0
Heat 56 C for 30 min	–	15.6 ± 1.3	0

* Cells plated at 5 × 10^5/ml and counted on Day 3.
† Cells plated at 1 × 10^5/ml and counted on Day 3.
‡ Conditioned medium incubated with agarose-bound trypsin for 1 hour at 37 C.

Discussion

The observation that products of bone resorption stimulate tumor cell growth supports Paget's hypothesis that the host organ may contribute to the development of metastases.[14] Although previous studies on site specificity of metastasis using organ extracts have reported variable effects on cell replication,[34] the organ culture of bone represents a unique opportunity to test Paget's hypothesis, because the normal physiology of bone remodeling can be recreated and modulated *in*

Table 2—Effect of Mediators of Bone Resorption on Release of Growth Activity for Walker Tumor Cells Into Conditioned Bone Culture Media

Mediator*	Bone resorption in conditioned media (% ⁴⁵Ca released)	Cell growth† (Cell number ± SE × 10^5) Conditioned media	Unconditioned media
None (BGJ_b alone)	15.0 ± 2.8	15.9 ± 1.6	15.5 ± 0.5
PO_4 3.5mM	9.8 ± 0.7	14.3 ± 0.4§	15.2 ± 1.1
Albumin 0.5 mg/ml	28.7 ± 2.5	20.8 ± 0.5	13.7 ± 0.2
PGE_2 (10^{-7} M)‡	36.7 ± 2.4	24.1 ± 0.7	13.7 ± 0.8
10% fetal calf serum	48.9 ± 3.8	39.1 ± 2.7	15.5 ± 0.5

* Mediators present during bone organ culture were also added to unconditioned media as controls prior to assay of cell growth.
† Cells plated at 7 × 10^5/ml in media supplemented with 10% fetal bovine serum and counted on Day 3. n/group = 4.
‡ Bone culture medium also contained bovine serum albumin, 0.5 mg/ml.
§ $P > 0.05$. $P < 0.05$ for remaining conditioned media growth and resorption values when compared with corresponding controls.

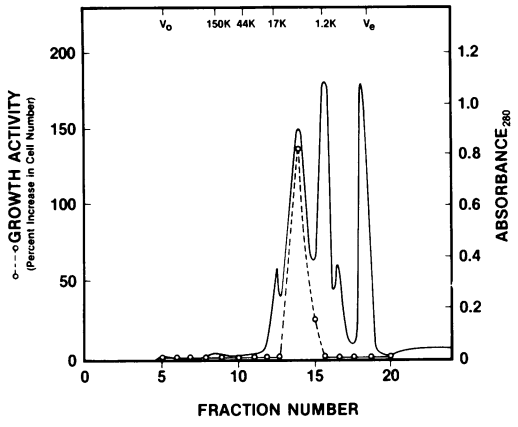

Figure 3—Growth of cells in fractions of conditioned bone culture media after high performance liquid chromatography on a Bio-Sil TSK-250 column. Molecular weight standards included thyroglobulin (V_o) 700,000 daltons, bovine gamma globulin 150,000 daltons, ovalbumen 44,000 daltons, myoglobin 17,500 daltons, cyanogen bromide 1200 daltons and phenol red (V_e) 350 daltons. Cells were plated at 6 × 10^5/ml and counted 3 days later. Results are expressed as percent increase in cell number above that observed for cells cultured in unconditioned media.

Table 4—Effects of Conditioned Bone Culture Media on Growth of Various Cell Lines

Cell line*	n/group	Number of cells† ± SE × 10^5 Unconditioned medium	Conditioned medium	Treatment/ control
Walker tumor	3	8.6 ± 0.6	53.5 ± 0.6‡	6.2
TE-85	3	2.4 ± 0.4	5.7 ± 0.4‡	2.4
MB-MDA 231	3	3.5 ± 0.3	6.5 ± 0.1‡	1.9
Fibroblasts	3	5.3 ± 0.2	7.7 ± 0.3‡	1.5
FS/L10	3	2.7 ± 0.2	3.6 ± 0.2‡	1.3
13762MAT	4	6.4 ± 0.1	8.2 ± 0.1‡	1.3

* Cell lines included the Walker 256 carcinosarcoma, TE-85 osteosarcoma, MB-MDA-231 human breast carcinoma, rat embryonic fibroblasts, FS/L10, a methylcholanthreneinduced murine fibrosarcoma, and 13762 rat adenocarcinoma.

† Cells plated at 1 × 10^5/ml and counted on Day 4, with media changed on the second day of culture.

‡ $P < 0.01$.

vitro. The capacities of certain tumors to induce bone resorption and then respond to bone-derived growth factors may represent an example of how neoplastic cells can subvert normal homeostatic mechanisms to aid their own growth.

The observed correlation between bone resorption and the generation of low molecular weight, temperature-stable, trypsin-insensitive mitogens is consistent with data obtained by others using similar systems to study bone cell growth.[18,35] Although these substances have not been defined, reports of their extractability from demineralized bone matrix suggest that they may represent connective tissue elements common to bone.[33,36-38] When tested individually, certain purified matrix constituents appear to be capable of modifying tumor cell properties. Type I collagen, which comprises 90% of the bone matrix,[39] has been shown to promote the attachment and growth of various cells in culture.[40] Alpha$_2$ HS glycoprotein, osteocalcin, and synthetic peptides containing amino acids found frequently in the collagen helix have been shown to elicit chemotactic activity for a number or cells, including the Walker tumor.[41-43] In addition to these matrix constituents, monocytes, found frequently at the margins of skeletal metastases, may both resorb bone[44] and promote the growth of osteoblasts, through production of peptides with interleukin-1 activity.[45] Although we were unable to detect interleukin-1 activity in our system, it is possible that monocytes may play an important role in the host-tumor cell interaction *in vivo*.

Several observations suggest that skeletal metabolism may influence the development of bone metastases. Fol-

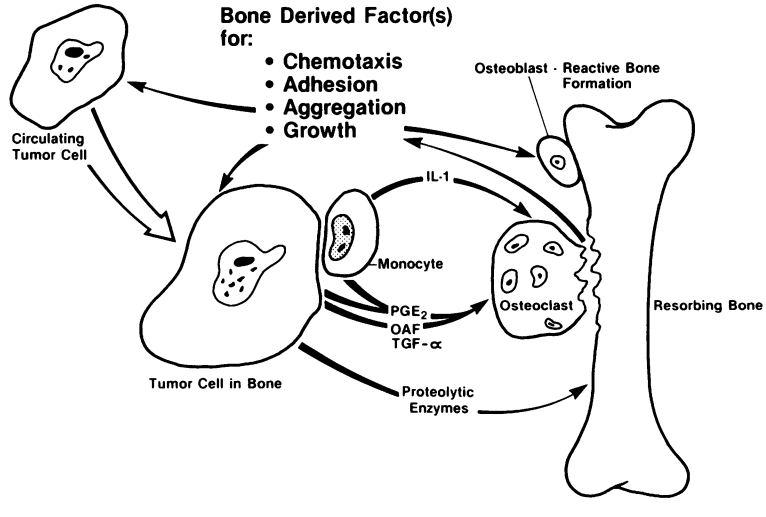

Figure 4—Postulated interrelationships between neoplastic cells, host elements, and bone resorption (see text). *TGF*, transforming growth factor, alpha. *OAF*, osteoclast activating factor. *IL-1*, interleukin-1.

lowing intraarterial injection of Walker tumor cells, rats treated with 1,25-vitamin D_3, a stimulator of bone turnover,[46] had significantly more skeletal metastases than untreated controls.[47] Diphosphonates, agents which inhibit osteoclastic bone resorption,[48] caused a reduced incidence of skeletal metastases in rats given the Walker tumor.[49] Further, patients with known malignancy and active Paget's disease have been reported to develop their first hematogenous metastasis in areas of the skeleton affected by Paget's disease.[50,51] Our understanding of metastatic bone disease may therefore require additional knowledge of the local factors responsible for the regulation of bone metabolism.

Based on these findings, a model of the potential host-tumor cell interactions occurring in bone metastasis is illustrated in Figure 4. Bone is continually undergoing remodeling, and humoral mediators of resorption released systemically by certain primary tumors might accelerate the process. Circulating tumor cells, when present in bone undergoing active resorption, could then aggregate, become lodged in capillaries, and migrate across blood vessels in response to chemotactic stimuli from bone. The metastatic cell and surrounding monocytes might then resorb bone either directly or through stimulation of local osteoclasts. This would result in release of growth factors from the bone matrix, thereby stimulating both tumor cell growth and reactive bone formation. Bone resorption may therefore help to create an environment favorable to the establishment of a metastatic focus in bone.

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