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Mechanisms involved in the metastasis of cancer to bone

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Summary

The metastasis of cancer to bone is a frequent outcome of common malignancies and is often associated with significant morbidity due to osteolysis. Bone metastasis is also selective in that a disproportionately small number of malignancies account for the majority of tumors which spread to bone. While the mechanisms of bone destruction have been studied, those responsible for the site-specific nature of bone metastasis are poorly understood. As a metastatic target, bone is unique in that it is continuously being remodelled under the influence of local and systemic growth factors, many of which are embedded in the bone matrix. This review summarizes evidence for the hypothesis that the formation of metastatic tumors in bone is the consequence of a unique microenvironment where metastatic cells can alter the metabolism of bone, thereby regulating the release of soluble bone-derived growth factors as a consequence of bone resorption. These, in turn, can modulate the malignant phenotypic properties of receptive cells. Transforming growth factor-β is one factor which can promote the growth and motility of Walker 256 cells, a rat cell line with a propensity to metastasize spontaneously to bone.

Introduction

The lungs, liver, and bone are the most common sites for the growth of metastases from human primary malignancies. In bone, metastatic tumors account for the greatest number of neoplastic lesions, although accurate statistics on the relative frequencies of metastatic and primary tumors are difficult to obtain. Bone metastases cause significant clinical disease due to pain, pathological fractures, hypercalcemia, and bone marrow replacement [1-3]. Although the pathophysiology of bone metastasis is poorly understood, the spread of particular tumor types to bone (organ specific metastasis) and bone destruction are dominant aspects of the disease and afford clues to its mechanisms.

Bone metastasis is a *selective* process in that a limited number of primary neoplasms account for more than 80% of the tumors that cause clinically significant bone disease [4]. These include carcinomas of the breast, prostate, thyroid, bronchus, and kidney, and multiple myeloma. Approximately two-thirds of patients with metastatic

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breast cancer have bone involvement. While only a few tumor types account for the majority of clinically detectable metastases, studies of marrow aspirates indicate that bone marrow is often occupied by immunochemically-detectable metastatic cells at the time of initial cancer surgery. Moreover, the frequency of seeding by tumors that do not tend to cause clinically detectable metastatic bone tumors is similar to the frequency of early seeding by tumors that subsequently cause clinically significant disease (Table 1).

Factors which regulate the development of bone metastases

Observations on the selective nature of bone metastasis are not new. Over 100 years ago, Stephen Paget, an English surgeon, suggested that in cancer of the breast, the bones "suffer in a special way which cannot be explained by any theory of embolism alone". He noted that "some bones suffer more than others" and that the disease has its "seats of election" [5]. His suggestion that there is "dependence of the seed upon the soil" is a hypothesis which has considerable support from recent experimental data.

In 1942, Oscar Batson, in studying prostatic cancer metastasis, argued convincingly that anastomoses between the venous drainage of the prostate gland and paravertebral veins accounts for the frequent involvement of the spine by cancer of the prostate [6]. There is also experimental evidence to support his theory of mechanical selection of bone as a secondary target [7,8]. Thus, in some instances, bone may be the site of metastatic tumor growth because it is the first organ encountered by cells leaving a primary tumor or circulating in the blood stream [9].

Table 1. Median frequency of metastatic cells in bone marrow aspirates of cancer patients and frequency of metastatic tumors at death.

Primary tumor site	% positive marrows at diagnosis[a]	% with metastases at death[b]
Breast	27 (16-35)	70 (47-85)
Lung	34 (20-62)	40 (32-50)
Colon	27	9 (8-13)
Stomach	35	5 (3-11)

[a] Immunocytochemically-detectable tumor cells in bone marrow aspirates at the time of diagnosis. From references [98-106].

[b] From reference [4].

The blood supply of bone

Most cancers spread to bone via the blood stream. However, the relative frequency and extent of metastatic involvement of the skeleton is greater than would be predicted if the proportion of blood supplied to bone (5-10% of the cardiac output) [10] were the sole determinant. Bone metastases are more frequent at sites of red marrow where there are vascular sinusoids lined by endothelial cells that lack a basement membrane and display 60 Å fenestra [11]. Stromal or vascular adhesion molecules have been implicated in the homing of some avian hematopoietic neoplasms to bone [12].

Cancer cell properties

Tumor malignancy has been correlated with autonomous growth, the production of proteinases [13,14] and angiogenesis factors [15], motility [16], and adhesion [17,18]. Properties specifically associated with the propensity to colonize bone include estrogen receptor status, histologic grade, and expression of plasminogen activator (reviewed in reference [19]). In addition to genetic regulation, effected by the activation or inhibition of "metastasis" and "antimetastasis" genes [20-22], the expression of these properties, generally termed "the metastatic phenotype", is also under the control of environmental influences such as drugs, radiation [23], and growth factors [23-25]. The evidence that growth factors can regulate

metastasis has been summarized recently [26]. This includes clinical observations of metastatic organ-preference, identification of growth factors in target organs, the presence of growth factor receptors on malignant cells, the production of autocrine growth factors by malignant cells, and evidence that growth factors can selectively promote the growth of malignant subpopulations within heterogeneous tumors.

The bone microenvironment

Bone is unique among metastatic target tissues because it is continuously being remodelled. Bone is constantly formed by osteoblasts and degraded by osteoclasts. These two processes are balanced by local growth factors which are generated and/or released as part of the bone remodelling process [27]. Evidence from anecdotal clinical observations and experiments *in vivo* suggest that skeletal metabolism and the bone microenvironment can influence the formation of metastatic lesions in bone. For example, patients with malignant tumors and active Paget's disease have been reported to develop their first hematogenous metastases in the pagetic bones where there is active bone remodelling [28,29]. Following intra-arterial injection of Walker 256 tumor cells, rats treated with 1,25-vitamin D_3, a stimulator of bone turnover, had significantly more skeletal metastases than untreated controls [30]. In contrast, inhibitors of prostaglandin synthesis (aspirin and indomethacin) and bisphosphonates (agents which inhibit bone resorption) have been reported to reduce the incidence of skeletal metastases in rats injected with Walker tumor [31-33] and in some clinical trials [34,35].

The organic phase of bone matrix contains a milieu of osteoblast-derived growth factors which regulate the differentiation and proliferation of cells indigenous to bone [36] and which are potentially mitogenic to metastatic cells. Transforming growth factor-β (TGF-β) is produced by osteoblasts [37], and is present in higher quantities in bone than any other tissue [38]. Transforming growth factor β has profound effects on many cell types as a growth and differentiation factor. Insulin-like growth factors I and II are also abundant bone-derived growth factors [39] which can modulate the growth of colorectal [40] and breast [41] carcinoma cell lines. Basic fibroblast growth factor can stimulate human prostate cancer cell growth *in vivo* [42], is produced by cultured bone cells, and is stored in their extracellular matrix [43]. Platelet-derived growth factor is mitogenic for a variety of cell lines [44-46]. The cytokine interleukin-1 is produced by osteoblasts, and influences bone cell replication [47]. Interleukin-6 is also produced by osteoblasts [48,49], and human bone-metastasizing PC-3 prostate carcinoma cells have been reported to express receptors for this cytokine [50]. Interleukin-6 is an autocrine growth stimulator for invasive, late stage melanoma cells, but is an inhibitor of early stage melanoma cell growth [51].

In addition to regulating tumor cell growth, extracellular matrix components can regulate the synthesis, secretion, and activity of matrix metalloproteinases in cancer cells. Interleukin-1, basic fibroblast growth factor, and platelet-derived growth factor can up-regulate the gene expression of interstitial collagenases in fibroblast, fibrosarcoma, and osteoblast-like osteosarcoma cell lines [52,53]. Growth factors can induce expression of urokinase type plasminogen activator which can subsequently activate latent procollagenase I [54].

Not only bone but also marrow is an important source of growth factors and chemoattractant molecules which are normally involved in the regulation of hematopoiesis. Some of the growth factors released from marrow and marrow stromal cells or fibroblasts have been shown to be capable of stimulating the growth of cancer cells with the potential to form bone metastases [55,56] (Figure 1). Recently, Arguello *et al* have demonstrated that injection of B16 melanoma cells into mutant mice deficient in stem cell growth factor resulted

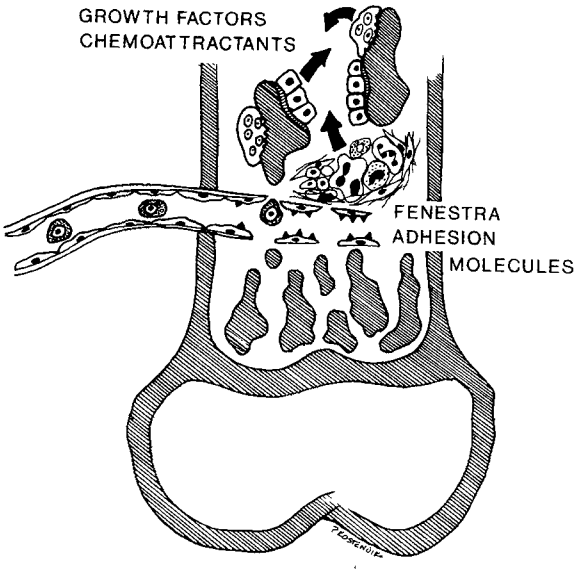

Figure 1. Properties of bone which have been identified as responsible for organ site specificity of metastasis. These include unique adhesion molecules in the vascular supply of bone and the microvascular anatomy as well as growth factors and chemoattractants involved in the regulation of bone remodelling and hemopoiesis.

in fewer bone metastases than injection of the same cell population into a control group deficient in stem cell growth factor receptor [57].

Mechanisms of cancer-induced osteolysis

The growth of metastatic cells in bone often alters both bone metabolism and structure. Metastases frequently present as osteolytic lesions with pathological fractures [58-60]. Osteoclasts, cancer cells, and tumor-associated macrophages have been identified as mediators of metastasis-associated osteolysis [61]. Osteoclast-mediated mechanisms have been most extensively examined, especially in regard to hypercalcemic syndromes [62]. Some cancer cells stimulate osteoclastic activity by secreting interleukin-6, interleukin-1β, prostaglandins, transforming growth factors [58,63], or parathyroid hormone-related peptide [64]. Recent evidence suggests that tumor-associated macrophages can also mediate osteolysis. Macrophages from human lung and murine mammary carcinoma specimens produce resorption pits on bone surfaces [65] and may be synergistically stimulated by paracrine factors derived from marrow stromal cells [66]. There is some evidence that cancer cells can directly degrade bone matrix by generating active matrix metalloproteinases and other enzymes [67], but this mechanism requires evaluation with modern techniques. Based on *in vivo* observations with the VX2 squamous cell carcinoma model in rabbits, Galasko proposed two phases of metastasis-associated osteolysis, the first predominantly osteoclastic followed by a second phase in which cancer cell-mediated degradation occurs [68].

An animal model for spontaneous bone metastasis

Several animal species and tumor models have been used to study bone metastasis experimentally. These have involved direct invasion of bone from contiguous intramuscular tumor [32], or intraosseous injection [69]. Intracardiac injection has been used to obtain bone colonization by several established non-human tumor lines, including the B16 melanoma or human cancer cell lines (in immunodeficient animals) [70,71]. The studies of Shevrin *et al* with a human prostate cancer cell line [7] and of Geldof with a rat prostate cancer cell line [72] provide evidence in support of Batson's original concept that the vertebral column is at particular risk from mechanical seeding of cancer cells via the vertebral venous plexus.

We have recently reported the development of a model of spontaneous bone metastasis which allows for the simultaneous quantitation of metastatic tumor burden, cancer cell growth rate, and progressive changes in bone morphology. This model employs the Walker 256 cell line, a highly malignant allogenic rat tumor which expresses

monocytoid differentiation markers [73] and which had been shown to form bone metastases after intraarterial [31] or intraosseous injection [69]. When Walker 256 (W256) cells or vehicle were injected into the muscle of male Fischer rats, metastases appeared after 7 days in distal femurs, liver, kidneys, and lungs. At day 14, femoral metastases were associated with a 53±10% decrease in trabecular bone (Figure 2), a 61±15% increase in osteoclasts, and a 95±10% decrease in osteoblasts as compared to non-tumor-bearing controls (Figure 3). W256 cells adjacent to trabecular bone surfaces had a 33±7% greater growth rate than W256 cells >50 μm from bone surfaces ($p<0.05$), suggesting a mitogenic effect of bone [74].

To test the hypothesis that the development of bone metastases is influenced by the rate of bone remodelling, we examined the effect of stimulating bone resorption on the growth of spontaneously metastatic W256 tumor cells *in vivo*. This was accomplished by subcutaneous transplantation of the non-metastatic Rice H-500 Leydig cell tumor which stimulates bone resorption with increased osteoclast number or activity, decreased bone formation, and hypercalcemia, attributed to the release of TGF-β and parathyroid hormone-related protein [74-76]. Enhanced bone resorption was confirmed quantitatively in a pilot study by evaluating parameters of bone morphometry after 4, 7, and 10 days of Leydig tumor injection (Table 2). To evaluate the growth response of W256 cells to Leydig tumor-induced bone resorption, 20 rats were injected intramuscularly with 2 x 10^7 W256 cells, and 20 rats were vehicle-injected. Two days later, 10 rats from each group were injected sc with Leydig tumor cells. Twelve days after W256/vehicle injection, rats were injected with ^{3}H-thymidine and killed 2 hrs later, and their femurs, liver, lungs, and kidneys were processed for histology. In rats injected with Leydig tumor cells only, enhanced bone resorption was indicated by a 40±4%increase in serum calcium concentration and by a 48±8% decrease in trabecular bone content, compared with non-tumor-bearing rats. In Leydig tumor-bearing rats, metastatic W256 cells adjacent to trabecular bone had a 56±18% greater relative ^{3}H-thymidine labeling index than did W256 cells in the bones of non-Leydig tumor-bearing rats (Table 3). The labeling indices of W256 cells in the liver, lungs, and kidneys were not affected by Leydig tumor burden. These results suggested that enhanced bone resorption is associated with the *selective* growth promotion of metastatic W256 cells in bone, and were consistent with the existence of a bone-derived factor which is mitogenic to W256 cells [77].

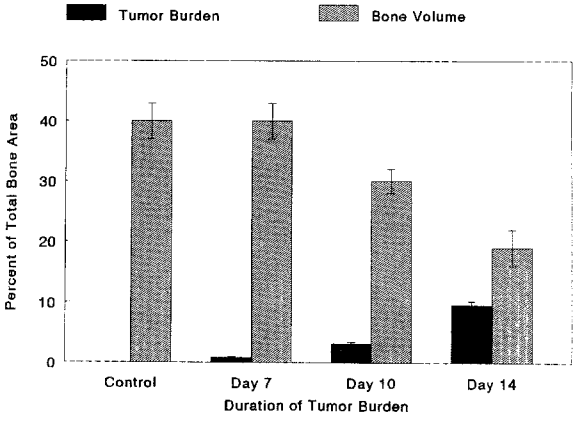

Figure 2. The kinetics of development of metastatic tumors in rat bone by Walker 256 cells released spontaneously from a solid intramuscular tumor transplant. See text and reference [74] for details.

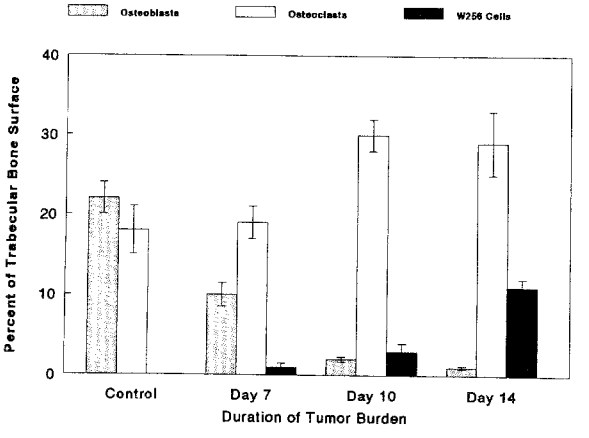

Figure 3. Effects of spontaneously metastatic Walker 256 tumor burden on the composition of rat trabecular bone surface cells. See text and reference [74] for details.

Table 2. Effects of the Rice-Leydig cell tumor on parameters of bone morphometry[a]

Duration of tumor burden	Trabecular bone area[b]	Surface osteoclasts[c]	Surface osteoblasts[c]	Serum calcium[d]
Control	42±2	19±2	20±2	2.7±0.1
Day 4	46±2	14±2	16±3	2.6±0.2
Day 7	36±4	20±2	12±1	2.8±0.1
Day 10	24±2	28±1	4±1	3.8±0.2

[a] Rats were injected subcutaneously with Leydig tumor cells and their femurs dissected 4, 7, or 10 days later. From reference [77].
[b] % total area of distal metaphysis (400 μm from the growth plate) occupied by trabecular bone.
[c] % of the trabecular bone surface occupied by these cells.
[d] mmol/l, determined at sacrifice.

Application of a bone organ culture system to study metastasis

To examine *in vitro* the hypothesis that products of bone can regulate the metastatic phenotype of cancer cells, we have used a bone organ culture system [78] to generate soluble products of bone resorption. On the 18th day of gestation, fetal rat bones were radiolabeled *in utero* by injecting 40 μCi ^{45}Ca subcutaneously into pregnant Sprague-Dawley rats. One day later, the fetal parietal bones were placed into tissue culture. After a 24 hr preculture period, to allow exchange of loosely bound ^{45}Ca, various mediators or inhibitors of bone resorption were added to the medium and the cultures were maintained for an additional 3 day period. The extent of bone resorption was measured by the release of ^{45}Ca. The conditioned bone culture media were then analyzed for their ability to alter the phenotypic properties of the Walker 256 cells.

Cancer cell motility

Cancer cell motility [16] and chemotaxis [79,80] can contribute to metastasis. Our initial experiments on cell motility demonstrated the ability of bone culture-derived conditioned medium to stimulate the directed migration (chemotaxis) of W256 cells. The magnitude of the chemotactic activity was directly proportional to the extent of bone resorption. Moreover, W256 cells generated soluble mediators of bone resorption which up-regulated the release of chemoattractants from the cultured bones [81,82]. Subsequent experiments have shown that products of bone resorption can stimulate the directed migration (chemotaxis) and adhesion of W256 and other cells [81-84], as can purified matrix constituents, including type I collagen (comprising 90% of the bone matrix) [85-87], α_2 HS glycoprotein, osteocalcin, and synthetic peptides containing amino acids found frequently in the collagen helix [87-89].

TGF-β has been identified in the media of bone organ cultures [90], and bone has levels of TGF-β in excess of many other tissues [38,91]. Since TGF-β is a potent chemoattractant and activator of fibroblasts [92] and macrophages

Table 3. Effects of stimulated bone resorption on the growth of metastatic Walker 256 cells *in vivo*.

Metastatic site	Growth in Leydig-bearing rats compared to growth in controls[a]
Bone	+56%[b]
Liver	+2%
Lung	+12%
Kidney	−5%

[a] The effect of Leydig tumor burden on ^{3}H-thymidine uptake by spontaneously metastatic W256 cells in Leydig tumor-bearing animals compared to ^{3}H-thymidine uptake by W256 cells in non-Leydig tumor-bearing animals 12 days after W256 cell injection. Data from reference [77].
[b] $P<0.05$.

Table 4. Correlations between bone resorption, TGF-β concentration, and activities for chemotaxis and growth in rat parietal bone organ culture media.

Culture condition	Resorption (^{45}Ca release)	TGF-β assay[a] (ng TGF-β/ml)	Chemotactic activity[b] (cells/hpf)	Cell growth[c] (% of control)
Experiment 1				
Dead bones	9.3 ± 1.3	<0.05	0	111 ± 7
Medium only	14.8 ± 1.5	<0.05	16 ± 2	140 ± 6
10^{-12}M PGE_2	19.0 ± 1.5	0.10	19 ± 4	218 ± 6
2% serum	24.6 ± 2.8	0.15	30 ± 2	269 ± 6
Experiment 2				
Dead bones	15.4 ± 2.1	<0.05	0	121 ± 5
Medium only	17.8 ± 2.3	0.07	9 ± 2	154 ± 11
10^{-12}M PGE_2	29.0 ± 3.0	0.12	34 ± 3	292 ± 8
2% serum	31.7 ± 2.1	0.13	43 ± 3	323 ± 16

[a] The concentration of TGF-β was determined from a standard curve based upon NRK colony formation of soft agar culture with TGF-β ranging from 0.005 to 10 ng/ml as a control.

[b] Values for random migration in corresponding unconditioned media have been subtracted. (In Experiment 1 = 20 ± 1 cells/hpf; in Experiment 2 = 32 ± 2 cells/hpf). Data are from reference [94].

[c] Cell numbers were determined on day 3 of culture. Values represent mean ± standard deviation. Data are from reference [97].

[93], we questioned whether TGF-β might also stimulate W256 cell motility. We observed that purified platelet-derived TGF-β elicits dose dependent migration of W256 cells in the Boyden chamber assay with half-maximal responses (ED_{50}) elicited by 0.12±0.01 ng/ml TGF-β. Checkerboard analysis confirmed dependence of the response upon a concentration gradient. Conditioned media from organ cultures of bone contained TGF-β and chemotactic activity in proportion to the extent of bone resorption (Table 4). Further, the chemotactic activity in conditioned bone culture medium and that of the purified platelet-derived TGF-β were both inhibited after incubation with anti-TGF-β1. We have concluded that TGF-β, released from resorbing bone, can influence the migratory behavior of the osteotropic W256 cell line [94].

Tumor cell growth

The conditioned medium from resorbing rat calvarial cultures was also found to contain growth-stimulatory activity for Walker 256 cells as well as for cells from human osteosarcoma and breast carcinoma lines [95]. While TGF-β has generally been regarded as a growth inhibitor and differentiation factor for malignant cells, more recent data support the notion that this factor may selectively promote the growth of metastatic populations [51, 96]. In the presence of 20 ng/ml epidermal growth factor, purified platelet-derived TGF-β produced a dose-dependent growth response in Walker 256 cells with an ED_{50} equal to 0.5 ng/ml. Epidermal growth factor or platelet-derived growth factor, by themselves, had no significant effect on cell growth in concentrations from 1-100 ng/ml. Bone-derived TGF-β activity in conditioned media, measured by NRK fibroblast colony formation, correlated with resorption of bone organ cultures ($r>0.95$ in several experiments) and with growth promotion of the W256 cells ($r=0.98$, $p<0.01$, Table 4). Antibodies to TGF-β1 blocked the growth response normally induced by conditioned bone culture media [97].

Since growth factor effects may relate to the activation of proliferation-associated genes, we

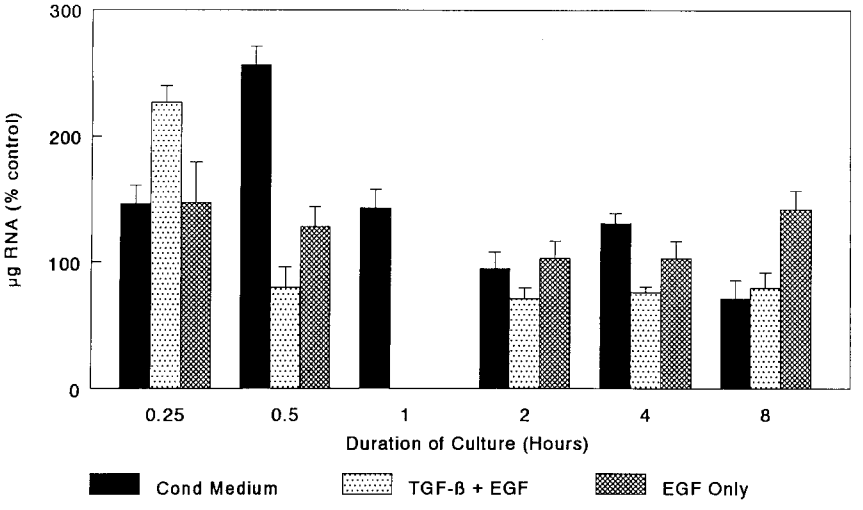

Figure 4. C-myc mRNA expression in W256 cells exposed for varying periods of time to conditioned medium from resorbing bone cultures, to TGF-β (0.1 ng/ml) + epidermal growth factor (20 ng/ml), or to epidermal growth factor only. Total cellular RNA was slot-blotted, probed with an α-^{32}P-dATP-labelled human c-myc DNA probe, and analyzed densitometrically. Values have been standardized to control (BGJ_b) medium and error bars are standard deviations.

have recently examined the expression of the oncogenes c-*myc* and c-*fos* in W256 cells after incubation with TGF-β and with bone-derived conditioned medium. While c-*fos* mRNA was not altered, c-*myc* mRNA was elevated after 15 and 30 min and returned to basal levels by 1 hr (Figure 4). Nuclear c-*myc* protein levels were enhanced 3-fold after 2 hr exposure and returned to control levels at 4 hr (unpublished). We concluded that the mitogenic response of W256 cells to bone-derived conditioned medium and to TGF-β is accompanied by an induction of c-*myc* mRNA which may have a role in mediating this growth response.

Conclusion

This brief review summarizes evidence that bone derived factors, including transforming growth factor β, can promote the growth and migration of rat Walker 256 cells. We postulate that the formation of a metastatic bone tumor depends upon a synergistic relationship between the cancer cell and the bone such that bone resorption is up-regulated by mediators released from cancer cells or host leukocytes in the metastatic focus. In turn, the growth of cancer cells is promoted at these sites by factors released during bone resorption. These growth factors can activate proliferation associated oncogenes leading to a preferential growth of cancer cells in bone (Figure 5). The hypothesis provides an example of the way in which an appropriate soil can facilitate the growth of a responsive seed, as suggested by Paget over 100 years ago.

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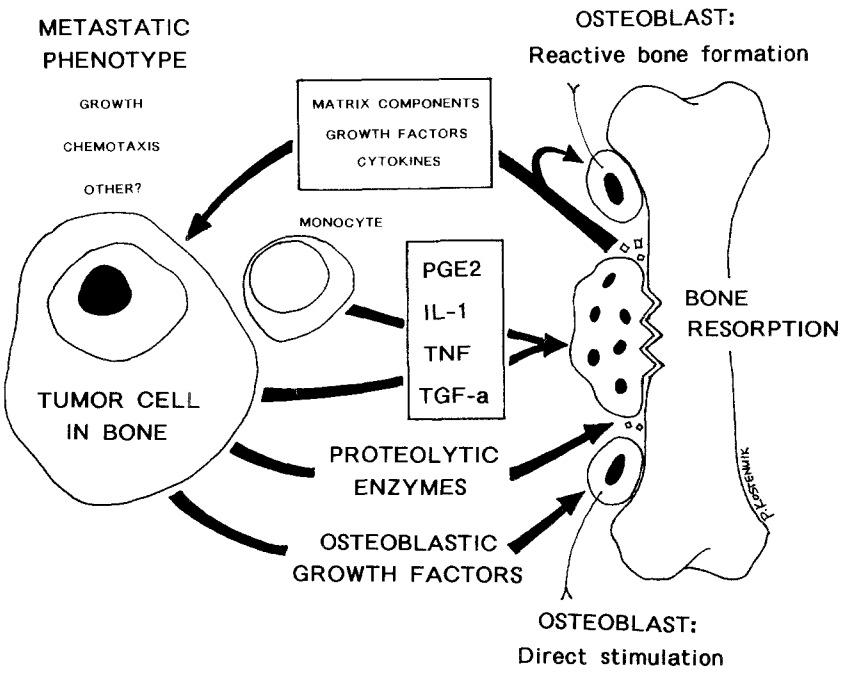

Figure 5. Summary of postulated relationships between metastatic tumor cells in bone and the bone microenvironment. See text for details. Modified from reference [95].

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