Ascites Sarcoma 180 Secretes a Soluble Factor (s) which Inhibits Mineralized Nodule Formation In Vitro

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Abstract: Ascites sarcoma 180 (S180A) is a transplantable tumor that induces hypercalcemia in tumor-bearing mice without producing parathyroid hormone-related protein (PTHrP) and stimulates bone resorption in cultured neonatal mouse calvaria. To investigate the effects of S180A on bone formation, bone marrow cells were cultured in the presence of ascorbic acid, dexamethasone and β-glycerophosphate and then cell proliferation and mineralized nodule formation were evaluated. Serum-free conditioned media of ascites cell cultures greatly stimulated the 3H-thymidine uptake (5.5-fold on day 10) throughout the experimental period up to 14 days. On the other hand, they limited the rise in alkaline phosphatase activity significantly compared to control (44.1% and 70.8% of control on day 10 and 14, respectively). After 14 days of culture, many mineralized nodules were observed in control and recombinant human TGF groups, whereas nodule formation was completely abolished by the addition of S180A CM. Thus the results of the present study indicate that tumor-produced factors cause hypercalcemia by inhibiting bone formation, which cooperates with the stimulation of bone resorption in S180A-bearing mice.

Key words: Ascites sarcoma 180, Hypercalcemia in malignancy, Bone formation

Introduction

Hypercalcemia frequently occurs in advanced malignancies and continues to be a major cause of cancer-associated morbidity and mortality. Humoral hypercalcemia in malignancy (HHM) is characterized by a decreased renal threshold for phosphate, leading to hypophosphatemia, and can be discriminated from other hypercalcemic syndromes by higher nephrogenous cAMP excretion compared to local osteolytic hypercalcemia and by lower plasma 1,25-dihydroxyvitamin D values compared to primary hyperparathyroidism. Parathyroid-hormone-related protein (PTHrP) that is secreted by tumor cells was first identified as the cause of hypercalcemia in malignancy. The specific tumors that characteristically produce humoral hypercalcemia by secreting PTHrP include squamous, renal and breast carcinoma. This peptide has similar amino acid sequence to PTH at the N-terminal end, interacts with a common cell surface receptor, PTH/PTHrP receptor, and mimics the biological effects of PTH both on bone resorption and on renal tubular function. As expected, infusion of PTHrP can reproduce most aspects of the clinical syndrome of hypercalcemia, serum levels of PTHrP are increased in hypercalcemia, and neutralizing antibodies to PTHrP can reverse hypercalcemia induced in animals by human tumor cells.

In the normal adult skeleton, coordinated resorption and formation, which is known as bone remodeling, is taken place to maintain the bone homeostasis. Bone remodeling is most likely regulated locally in the bone microenvironment by various local factors including growth factors, cytokines, and prostaglandins, as well as systemic hormones. However, in the pathological conditions like HHM, this coordination is largely disrupted by the tumor cells, which produce and secrete substantial amount of growth factors, and which in turn causes the imbalance between resorption and formation of bone. Parathyroid hormone is well known as a potent bone-resorbing factor that regulates the
osteoclast activity through its receptor on osteoblasts, which is believed to be the case also in PTHrP produced by tumors. Mundy proposes there may be a vicious cycle in the bone marrow microenvironment between the myeloma cells and osteoclastic bone destruction—the more aggressive the behavior of the myeloma cells, the greater the bone destruction, which in turn causes excess production of IL-6, which makes the myeloma cell growth even more aggressive5).

Thus, the tumor-induced hypercalcemia is thought to be essentially due to a marked stimulation of osteoclast-mediated bone resorption. On the other hand, there has been not much information about the bone formation in malignancy. We have been investigating the features of hypercalcemia in malignancy by using Ascites Sarcoma 180 (S180A)-bearing mice as an animal model and previously reported6-8) that 1) plasma Ca and Pi levels are elevated in S180A-bearing mice, whereas hypophosphatemia was observed in PTHrP-producing tumors; 2) The serum-free conditioned media of S180A cell cultures (S180A CM) exhibited dose-dependent bone-resorting activity, which coeluted with either TGFα activity or lymphocyte-activating factor activity in Bio-Gel P-100 column chromatography; 3) S180A CM failed to stimulate cAMP production in either UMR 106-01 cells or neonatal mouse calvaria at concentrations that stimulated bone resorption; and 4) S180A cells did not express mRNA for PTHrP based on Northern blot analysis. The purpose of our present study is to investigate whether S180A produces the factors which affect the bone formation, as assessed by the mineralized nodule formation in vitro.

Materials and Methods

1. Animals
S180A-bearing mice were generously provided by Dr Akio Hoshi (National Cancer Center Research Institute, Tokyo). This tumor was transplanted weekly in ddY mice by ip injections of ascites containing 4-5×10⁶ cells.

2. Preparation of S180A-conditioned medium
Cells obtained from the ascites of S180A-bearing mice, which were negative for non-specific esterase-staining (α-naphthyl esterase kit, Sigma Chemical), were suspended in serum-free Dulbecco’s modified Eagle’s minimal essential medium (10⁶ cells/mL) and incubated at 37°C for 48hr. Conditioned media were diafiltrated against 1% acetic acid and concentrated by ultrafiltration with an Amicon YM 10 membrane (MW cutoff at 10kDa) in a stirred cell (Amicon, Beverly, MA). The supernatant was lyophilized and dissolved in a small amount of 1M acetic acid, and the clear supernatant after centrifugation was lyophilized again (S180A CM) and stored at −20°C until use. The lyophilized S180A CM was reconstituted in a small volume of PBS and added into the cultures at a final concentration of 40μg/mL for the assay.

3. Mineralized nodule formation
Bone marrow cells were obtained from male 6-week-old Wistar rats. The femurs were excised aseptically, cleaned off soft tissues and the ends of bones were removed. The marrow flushed out using culture medium expelled from a syringe through a 19-gauge needle and cell suspensions were prepared by repeatedly aspirating the cells through the needle. The cell suspension was washed with PBS and plated into a 24-well plate at 10⁶cells/cm² in an α-minimal essential medium (α-MEM) supplemented with 10% FBS, 0.2mM ascorbic acid, 10nM dexamethasone and 10mM β-glycerophosphate as described by Maniatopoulos9). After 24hr incubation, the non-adherent cells, which are mostly blood cells, were removed by discarding the media and cultured for another 13days changing the media every third day until the mineralized bone nodule were formed. On day 14, the cultures were fixed with ice-cold methanol and stained using von Kossa method to visualize the mineralized nodule. Bone-forming activity was assessed by measuring the total area of nodules in each well.

4. DNA synthesis
The bone marrow cell cultures prepared as described above were used for the cell proliferation and differentiation assessment. During the last 4hr of the experiment, the cells were pulsed with 0.5μCi/well of ³H-thymidine, and the radioactivity in the acid-insoluble fraction was counted in a liquid scintillation spectrometer.

5. Alkaline phosphatase (ALPase) activity
The alkaline phosphatase activity released in the bone marrow cell culture media was assessed using the assay kit based on Bessey-Lowry method (Wako Pure Chemical, Tokyo). Protein concentration of the cultures was determined by Lowry method after lysing the cultures with IN NaOH.
6. Statistical analysis
Quantitative data obtained for nodule formation were averaged from 6<values in replicate experiments from which mean±SD were calculated. Leven's test was used to determine homogeneity of variance and two-sample t-test was used to determine the difference between two means.

Results
1. Soft X-ray examination of S180A-bearing mice
Our previous studies have shown that both the plasma Ca and Pi levels gradually increased within several days after the tumor transplantation and on day 14, they reached 138 and 137% of those of the control mice, respectively. The plasma creatinine levels of S180A-bearing mice were not different from those of the control mice, suggesting that the increase of the plasma Pi levels was not due to the renal failure. As shown in Fig. 1, no evidence of bone metastasis of S180A was confirmed by radiographic study. Furthermore, generalized radiolucency in bone in S180A-bearing mice suggested that S180A cells produce some factors responsible for bone resorption and/or bone formation.

2. Effects of S180A CM on proliferation and differentiation of osteoblastic cells in comparison to rhTGFα and rmIL-1α
To investigate the effects of S180A CM on the bone formation, non-adherent rat bone marrow cells, which are known to sequentially expressed bone matrix proteins and mineralized matrix9), were cultured in the presence of 0.2 mM ascorbic acid, 10nM dexamethasone and 10mM β-glycerophosphate. As shown in Fig. 2, bone marrow-derived osteoblastic cells were well spread and recognized as typical fibroblastic cells with spindle shape until they reach confluent and multilayered (Fig. 2A). These morphological characteristics were not changed by the rhTGFα or rmIL-1α addition (Fig. 2B, C), although these factors affected the cell proliferation. However, the cells rounded up and formed many colony-like structures before reaching confluency by the addition of S180A CM (Fig. 2D). Further, cell rounding was not inhibited by the addition of either 10⁻⁶ M indomethacin or anti-IL-1α neutralizing antibody (Fig. 2, E and F), indicating that the effect was PGE2-independent and due to the factor other than IL-1α. Next, we examined the effects of S180A CM on the osteoblast proliferation by measuring the ³H-thymidine incorporation into the bone marrow cells. As expected from our previous results showing that S180A produces a factor with a TGFα-like activity, ³H-thymidine incorporation was significantly increased by the addition of S180A CM (Fig. 3).

Fig. 1 Soft X-ray images of femur and tibia.
Femurs and tibias were dissected from the control (A) or S180A-bearing mouse (B) 2 weeks after the tumor transplantation. Soft tissues were removed carefully after the fixation with buffered formalin and exposed to soft X-ray (35kV, 5mA) for 10sec. Note that the radiograph of S180A-bearing mouse is radiolucent indicating the severe bone loss in the animal.
Bone formation in S180A

uptake was stimulated by S180A CM significantly compared to control (Fig. 3A). The stimulation of DNA synthesis by S180A CM was observed throughout the experimental period up to day 14, whereas the values reached peak level on day 6 in the control and rhTGF α groups. Recombinant murine IL-1α inhibited the DNA synthesis on day 14 significantly compared to control.

As shown in Fig. 3B, alkaline phosphatase activity, one of the earliest markers of the osteoblastic phenotype, released into the culture media elevated gradually with the incubation time starting on day 8 in the control, rhTGF α and S180A CM groups, while the activity failed to rise in rmIL-1α group. This elevation of alkaline phosphatase activity in the control, rhTGF α and S180A CM groups seems to reflect the transition from the replication of undifferentiated cells to committed preosteoblasts and/or early differentiated osteoblasts. However, the result (Fig. 3B) shows that the increase in the alkaline phosphatase activity in rhTGF α group is moderate (on day 10) in comparison to the control group, suggesting there must be a delay in the onset of differentiation due to a delay in the completion of proliferation. Moreover, the calculated activities of alkaline phosphatase, which were normalized to the protein content in the cultures, revealed that both S180A CM and rhTGF α greatly inhibited the alkaline phosphatase activity (32.7 and 43.9% of the control, respectively on day 14) during the differentiation stage (Fig. 3C).

3. Effects of S180A CM on mineralized nodule formation in comparison to rhTGFα and rmIL-1α

Figure 4 shows the rat bone marrow cells cultured for 14 days in the presence of ascorbic acid, dexamethasone and β-glycerophosphate, which is the same experimental condition as in Fig. 2. Von Kossa staining of these cultures at the end of the culture period revealed that the prominent mineralized nodules were observed in the control cultures. The addition of rhTGF α to the media throughout the experimental period reduced the total area of nodule, although there was no significant difference in the morphological feature of the nodule-forming cells under a phase contrast microscopy. This
Fig. 3 Effects of S180A CM, rhTGF α and rmIL-1 α on proliferation and differentiation of osteoblastic cells. Bone marrow cells were cultured in the presence of 0.2mM ascorbic acid, 10nM dexamethasone and 10mM β-glycerophosphate. Cell proliferation was measured as the 3H-thymidine incorporation into the cell cultures during the last 4hrs of the indicated incubation time (A). Alkaline phosphatase activity was examined in an aliquot of the culture media collected at the indicated incubation time when changing the media, and expressed as the amount of products per well in B or normalized to the protein content in the culture in C. Data represent the mean +/-S.D. (n=6 to 8). Open circle, vehicle alone; open triangle, S180A CM; solid triangle, rhTGF α; solid circle, rmIL-1 α.

*P<0.05 : significantly different from vehicle alone.

Bone-forming activity was completely inhibited in the presence of both S180A CM and rmIL-1 α. As shown in Fig. 4B, the colony-like structures, which were seen in Fig. 2 increased in both size and numbers in S180A group. However, their morphological characteristics were totally different from the multilayered osteoblasts which were seen in the control (Fig. 4A) and rhTGF α (Fig. 4C) groups. Moreover, mineral deposition, as assessed by von Kossa staining for phosphorus, has never occurred in the S180A CM (Fig. 4B) or rmIL-1 α (Fig. 4D) group. Collectively, these results suggest that the final stages of osteoblast differentiation which are defined by the biosynthesis and organization of the bone extracellular matrix have been inhibited by the S180A CM addition. Table 1 summarizes the effects of S180A CM, rhTGF α and rm IL-1 α added at various differentiation stages on the nodule formation. Ascites sarcoma 180 CM and rmIL-1 α inhibited the nodule formation regardless of differentiation stages at which they were added. Further, the inhibition of mineral precipitation was shown to be PGE_2-independent or not due to IL-1 α-like activity alone by using indomethacin or anti-IL-1 α
Fig. 4 Effects of S180A CM, rhTGF-α and rmIL-1α on mineralized nodule formation in bone marrow cell cultures. Bone marrow cells were cultured in the presence of 0.2mM ascorbic acid, 10nM dexamethasone and 10mM β-glycerophosphate. Vehicle alone (A), S180A CM (B), rhTGF-α (C) and rmIL-1α (D) were added to the cultures throughout the incubation time. After 14 days of incubation, the cells were fixed with ice-cold methanol and stained using von Kossa method then phase-contrast photographs were taken. No staining for mineral was observed in S180A CM and rmIL-1α groups. Arrows show the colony-like structures exclusively seen in the S180A CM group.

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<th>Proliferation stage</th>
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Bone marrow cells were cultured in the presence of 0.2mM ascorbic acid, 10nM dexamethasone and 10mM β-glycerophosphate. S180A CM, rhTGF-α and IL-1α were added to the culture throughout the incubation time. In some cultures, 10^{-4}M indomethacin was added concomitant with S180A CM, and in the group of S180A CM + anti-IL-1α, the reconstituted S180A CM was pretreated with anti-IL-1α neutralizing antibody at 37°C for 1hr then added into the media. After 14 days of incubation, the cultures were fixed with ice-cold methanol and stained using von Kossa method then the total area stained black was measured and expressed as percentage of that of control (average of six individual cultures).
neutralizing antibody, respectively. On the other hand, rhTGF α inhibits the late differentiation stage (day 8 to day 14), but not completely unlike S180A CM or rmIL-1α.

Discussion

The pathogenesis of cancer-associated hypercalcemia is not yet completely understood, despite intense investigation. This syndrome is believed to be a consequence of the tumor production of humoral factors, mainly PTHrP. However, patients with humoral hypercalcemia of malignancy have features suggesting that factors other than PTHrP might play a role in this syndrome. We have been studying the factors responsible for the tumor-induced hypercalcemia by using S180A, which is a transplantable non-PTHrP-producing tumor maintained in mice, as an animal model. We have previously reported that plasma Ca concentration of S180-bearing mice increased within 2 weeks after the tumor transplantation6). Also, serum-free conditioned media of the ascites cell cultures, which was shown to be contaminated with only trace amount of macrophages, if any, contain potent bone-resorbing activity which coeluted with either TGF α-like activity or IL-1α-like activity in Bio-Gel P-100 column chromatography7). Further, S180A CM contains bone-resorbing factors other than TGF α and IL-1α based on the result that the bone resorption was not completely inhibited by the simultaneous addition of 10⁻⁶ M indomethacin, which is known to block the TGF α-induced bone resorption7,10) and anti-IL-1α neutralizing antibody and acts synergistically on bone resorption7). Recently, Niida et al. have reported that vascular endothelial growth factor (VEGF) could support the osteoclastogenesis and there was no difference in the bone-resorbing activity between M-CSF- and VEGF-induced osteoclasts11). On the other hand, the evidence that significant amount of biologically active VEGF accumulated in the ascites fluid of tumors, particularly in tumors of sarcoma and carcinoma origin as has been reported by Shibuya et al.12). Thus, the possibility that VEGF, in addition to TGF α and IL-1α, has a role on accelerating bone resorption in S180A-bearing mice has been raised. These observations suggest that various local factors produced by tumor cells and/or secondary by immune cells may be responsible for the disrupted bone homeostasis, which is regulated strictly in a normal bone.

Most researchers are now focusing on the treatment of HHM by using the therapeutic agents such as bisphosphonates13-16), osteoprotegerin, a decoy receptor for receptor activator of nuclear factor-kappa B (RANK) ligand17), soluble murine RANK-human immunoglobulin fusion protein18) and calcitonin19). Preventing osteolysis in these patients, who are experiencing skeletal complications including bone pain, fractures, hypercalcemia and spinal cord compression, significantly improve their quality of life. For this purpose, it is very important to understand the mechanism by which the hypercalcemia occurs and what factor(s) are responsible for each step of the onset of the lesions in order to figure out the better therapeutic candidates. Tumor-induced hypercalcemia is thought to be essentially due to a marked stimulation of osteoclast-mediated bone resorption and an inhibition of bone formation and an enhanced tubular reabsorption of Ca play an important contributory role. There are several investigations which show that the bone formation is impaired in myeloma20) and in solid tumors such as breast cancer and prostate cancer21). Impaired bone formation in patients with myeloma can be manifested clinically by measurement of serum alkaline phosphatase, which is not increased, unlike most other patients with osteolytic bone lesions, and radioisotope bone scans that show no evidence of increased skeletal uptake of the isotope5). Ibbotson et al. have reported that human recombinant TGF α inhibited alkaline phosphatase activity in a dose-dependent manner in a rat osteogenic sarcoma UMR-106 cells and suppressed collagen synthesis in fetal rat calvaria22). Using the same model, TGF α has been shown to stimulate DNA synthesis and PGE2 production, whereas collagen synthesis and procollagen mRNA expression was decreased by TGF α23). In contrast, infusion of TGF α for 6 days by osmotic minipumps in normal mice increased mineral apposition rate and bone formation rate as determined by bone histomorphometry24).

In the present study, S180A CM increased the osteoblast proliferation and alkaline phosphatase activity, however, completely inhibited the mineralized nodule formation in bone marrow cultures. It is well accepted that linkage of phenotypic gene induction to the downregula-
tion of cell cycle and cell growth genes to support quiescence is the hallmark of differentiation in various cell types. In osteoblasts, postconfluent proliferation subsequently supports focal multilayering of bone forming cells and biosynthesis of the type I collagen bone extracellular matrix. Further, the determination of peak expression levels of osteoblastic phenotype-related genes in total RNA prepared at 3-day intervals, define the growth period (histone, TGFβ, fibronectin, collagen), the postproliferative matrix-maturation stage (alkaline phosphatase) and the mineralization stage (osteocalcin, osteopontin and bone sialoprotein). Additionally, several possible nucleators of bone mineral formation, including collagen, osteopontin, bone sialoprotein and bone acidic glycoprotein-7525-29).

Although it remains to be clarified how, and exactly on which differentiation stage, S180A causes the defect in osteoblast function, the results clearly show that the inhibition of bone formation by the growth factors and/or cytokines produced by tumor cells may be involved in the hypercalcemia cooperating with the stimulation of bone resorption. And this may be the primary reason why the hypercalcemia and bone loss have developed in a short time course, i.e., within 2 weeks, in S180A-bearing mice. Furthermore, our previous study6) revealed that the plasma alkaline phosphatase activity greatly decreased with the time after tumor transplantation (42% of the control mice on day 14), while the plasma acid phosphatase activity, which is produced by bone-resorbing osteoclasts, significantly increased in S180A-bearing mice. This result in vivo strongly supports our conclusion based on the impaired mineralized nodule formation observed in the present study, taking together with the fact that many of the bone phenotypic markers (alkaline phosphatase, osteocalcin) can be monitored in serum and are useful markers of bone function20).

In summary, S180A CM delayed or disrupted the sequential development of osteoblastic phenotype, which in turn caused the complete abolishment of mineral deposition in bone marrow cultures. It is likely that the inhibition of bone formation by tumor-produced factors contributes to hypercalcemia cooperating with the stimulation of bone resorption in S180A-bearing mice.

References
10) Tashjian, A. H. Jr., et al.: α and β human transforming growth factors stimulate


骨形成能を有するS180A由来の腫瘍細胞の石灰化抑制

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S180A由来の腫瘍細胞にはParathyroid hormone-related protein非産生性の腫瘍であるが、
高Ca血症を惹起する。また腫瘍細胞を血清非存在下で培養して得られた上清（S180A CM）中にはマウス頭蓋骨からのCa溶離を促進する因子が存在することが確認されている。本研究では
S180Aの骨形成への影響について知るためにラット骨髄細胞をascorbic acid, dexamethasone, β-glycophosphateの共存下で培養し、石灰化抑制形成について以下の検討を行った。S180A CM添加により
3H-thymidineの取り込みはコントロール群に比べて有意に増加し（培養10日目で約5.5倍）、この細胞増殖促進作用は14日間の実験期間中を通して観察された。一方、コントロール群では細胞増殖の完了に伴い細胞分化の指標であるalkaline phosphatase活性が上昇するが、S180A群ではこの活性上昇が有意に抑制された（培養10日目と14日目でそれぞれコントロール群の44.1%および70.8%）。また培養14日経過後、コントロール群では多数の石灰化結節が形成されるのに対し、S180A添加群ではその形成は完全に抑制された。以上の結果からS180Aでは骨吸収促進と骨形成抑制が同時に起こり、腫瘍動物において短期間（2週間以内）で高Ca血症および著しい骨欠損が観察される可能性が示された。

キーワード：ザルコーマ180, 高Ca血症, 薬形成