Association of Hypercalcemia with Tumors Producing Colony-stimulating Factor(s)

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ABSTRACT

Two human malignant tumors, which we previously reported to produce colony-stimulating factors (CSFs), were found to be accompanied by remarkable hypercalcemia. A patient with a CSF-producing lower jaw cancer (squamous cell carcinoma) developed a marked hypercalcemia (150,000/μl) and hypercalcemia (more than 15 mg/dl) and the tumor was successfully transplanted into nude mice, which developed marked hypercalcemia (300,000/μl) and hypercalcemia (20 mg/dl). White blood cell and serum calcium concentrations of these mice decreased promptly to normal levels when the tumor was excised. Treatment with prednisolone (1.5 mg/kg) or indomethacin (5 mg/kg) had no effect on the serum calcium level of these mice. Parathyroid hormone or prostaglandin E was not increased in the serum of the mice or in the tumor tissue. However, the mice bearing the tumor excreted extremely large amounts of calcium in their urine, and their bony tissues contained less calcium and phosphorus than controls. Moreover, histology of bony tissues of these nude mice clearly demonstrated the decrease in trabecular bones and cortical thickness as well as remarkable activation of osteoclasts. Another patient with a CSF-producing bronchogenic squamous cell carcinoma showed mild hypercalcemia and hypercalcemia. The biopsied tumor tissue was transplanted into nude mice, which developed marked hypercalcemia (300,000/μl) and also severe hypercalcemia (18 mg/dl). These results suggest the presence of a new syndrome of granulocytosis and hypercalcemia associated with CSF-producing tumors. The causal mechanism of the hypercalcemia was shown to be some humoral factor which activates osteoclasts other than parathyroid hormone. Neither prostaglandins nor osteoclast-activating factor seemed to be the cause of the hypercalcemia.

INTRODUCTION

Hypercalcemia associated with malignant neoplasms is one of the most frequently encountered signs in cancer patients as well as one of the most serious problems for doctors caring for them. Two causal mechanisms of the cancer-associated hypercalcemia have been postulated (12): (a) direct invasion of bone by tumor cells, which increase local bone resorption by activating osteoclasts (6) or through digestion by lysosomal enzymes (5); (b) production of various humoral factors by tumors and release of them into circulation, causing generalized bone resorption. These factors include PTH(2), PTH-like substance (3), a vitamin D-like sterol (7), prostaglandins (19, 22, 23), OAF (13, 14), and cyclic adenosine 3':5'-monophosphate-stimulating factor (20). What the role of each of these factors is in cancer-associated hypercalcemia remains to be defined, however. Furthermore, other still unknown factors are not excluded.

Nude mice transplanted with human malignant tumors provide a useful model for investigating humoral factors produced by the tumors because human malignant neoplasms transplanted into nude mice are well known to preserve their functions and rarely metastasize to vital organs. By utilizing this technique, we have been able to identify an antidiuretic hormone produced by a lung cancer (8), a cachexia-producing factor (10), human α-antichymotrypsin-like protein produced by a melanoma (9), and CSF produced by a lung cancer and a lower jaw cancer (1, 15, 18). Whether cancer-associated hypercalcemia is produced by direct bone metastasis or by humoral factors may be easily discriminated by this technique.

In our experience of heterotransplantation of various human malignant neoplasms into nude mice, 2 tumors induced severe hypercalcemia in host mice. Both of these tumors also induced a marked hypercalcemia and produced CSF (1, 15, 18). These findings prompted us to investigate the mechanism of the hypercalcemia. This paper gives an account of a new syndrome of hypercalcemia which appears to be linked with granulocytosis of CSF-producing tumors and also of a mechanism for the hypercalcemia.

MATERIALS AND METHODS

Case Reports

Case 1. A 33-year-old man was admitted to Tokyo University Hospital for detailed examination of dyspnea and bloody sputum with an abnormal lung shadow. About 5 months before admission, he had been treated by right hemi mandibulectomy with dissection of right cervical lymph nodes in Tokyo Medical Dental University Hospital for the treatment of squamous cell carcinoma of right lower jaw. Laboratory data showed moderate hypercalcemia (WBC, 37,400/μl; granulocytes, 89%) and hypercalcemia (12.0 mg/dl) with a high alkaline phosphatase level in the serum (12.1 King-Armstrong units). The other abnormal data included erythrocyte sedimentation rate (55 mm/hr) and C-reactive protein (5+). The serum phosphorus (3.5 mg/dl) was within normal value. Daily urinary excretion of calcium was increased (0.53 g/day), and that of phosphorus was within the normal range (0.69 g/day). Examination of bone marrow revealed marked granulocytosis and metastasis of tumor cells. Parathyroid hormone in the serum was within the normal range (<0.5 ng/ml). CSF was detected in the thoracic fluid. Cytological findings of sputum and thoracic fluid showed squamous cell-like malignant cells probably due to metastasis of the lower jaw cancer. As the disease progressed, granulocytosis and hypercalcemia increased up to 110,000/μl and more than 15 mg/dl, respectively, with concomitant elevation of alkaline phos-

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Leukocytosis and Hypercalcemia Syndrome

Chemical Industries Co., Osaka, Japan). Tibias stored in 70% ethanol as above were dried in air, and weight and length were determined. Each tibia was solubilized in 10 ml of 2 M HCl at 90° for 8 hr and after cooling was neutralized with 4 M NaOH (approximately 5 ml). White precipitate appearing at neutral pH was redissolved by acidifying the solution (pH 2.5) with 2 M HCl. The concentration of calcium and phosphorus in the solution was determined using the same reagents as described above.

Histological Examination

Tumor tissues of the lower jaw cancer and the lung cancer growing in nude mice were fixed in 10% formalin and stained with hematoxylin and eosin. The left tibia and femur fixed in 10% formalin as described above were decalcified in 10% formic acid, and transverse slices were made at the metaphysis and longitudinal slices from epiphysis to metaphysis. The sections were stained with hematoxylin and eosin.

Assay of PTH and Prostaglandins

PTH was assayed by radioimmunooassay using the PTH assay kit of the Immunochemical Institute of Eiken, Tokyo, Japan. The antisera discriminates exclusively the C-terminal region of PTH. The sensitivity of the assay is approximately 0.1 to 0.2 ng/ml (normal range in human serum, <0.5 ng/ml). Prostaglandins E and F were assayed using a radioimmunoassay kit from the Clinical Assays Division of Travenol Laboratories Inc., Mass., as described by Mest et al. (11). For determination of the serum level of prostaglandins, sera from 4 nude mice were pooled and assayed in duplicate.

Treatment with Prednisolone and Indomethacin

Prednisolone (1 μg of water-soluble Predonine per g body weight; Shionogi Pharmaceutical Co., Osaka, Japan) was injected i.p. into 3 nude mice bearing the lower jaw cancer, in which serum calcium concentration was approximately 15.0 mg/dl once a day for 7 days. About 100 μl of blood were collected from the nude mice on the first (immediately before treatment), fourth, and eighth days (24 hr after the last injection), and the sera were used for determination of calcium concentration.

Indomethacin (Sigma Chemical Co., St. Louis, Mo.) was solubilized in 99% ethanol at a concentration of 40 μg/ml and mixed with the same volume of water immediately before use, and 75 μl (150 μg indomethacin) or 50 μl (100 μg indomethacin) of the solution were administered p.o. into the stomach of tumor-bearing or control nude mice weighing about 20 g with a stainless steel gastric tube once a day for 2 days. Serum calcium was determined before treatment and 24 hr after the last administration of indomethacin. As a control experiment, 75 μl of 50% ethanol were administered to the tumor-bearing and control nude mice. About 2.5 to 5 μg of indomethacin per g body weight per day were also administered p.o. to each of 3 nude mice by feeding a food containing indomethacin (0.0015%) for 7 days. Serum calcium concentration was determined immediately before and 3, 5, and 7 days after beginning of treatment.

RESULTS

Development of Leukocytosis and Hypercalcemia in Nude Mice Transplanted with the Lower Jaw Cancer and the Lung Cancer. The lower jaw cancer and the lung cancer were successfully transplanted into nude mice for more than 5 and 8 years, respectively, preserving their histological features of squamous cell carcinoma. In nude mice transplanted with the lower jaw cancer (Chart 1), peripheral leukocyte count and serum calcium concentration started to increase soon after tumor growth occurred and gradually increased up to 109,500 ± 66,800/μl (S.D.) and 16.2 ± 1.4 mg/dl, respectively. Tumor removal by surgical operation at this
point caused a rapid decrease to normal levels within a week. Body weight in these mice decreased when the serum calcium concentration increased, but this was completely recovered after tumor removal. In nude mice transplanted with the lung cancer, the same phenomena were observed; the host mice showed marked leukocytosis (298,700 ± 63,800/µl) and hypercalcemia (17.7 ± 0.6 mg/dl), both of which decreased to normal level after tumor removal.

Excretion of Calcium and Phosphorus in the Urine of Nude Mice Bearing the Lower Jaw Cancer. As shown in Table 1, the urinary excretion of calcium and phosphorus in tumor-bearing mice was comparable to that of control nude mice, when the tumor was small (0.9 ± 0.4 sq cm) and when serum calcium levels were not increased (10.6 ± 0.5 mg/dl). When the tumor grew large (2.0 ± 0.4 sq cm) and the serum calcium level increased (14.7 ± 3.1 mg/dl), however, the tumor-bearing mice excreted significantly larger amounts of calcium than did controls, whereas excretion of phosphorus was not increased.

Bone Mass and Mineral Content in the Bones of Tumor-bearing Mice. The tibia of the tumor-bearing mice was lighter and contained significantly less calcium and phosphorus than did that of controls (Table 2). The tibia used in this experiment was obtained from the left side of the mice, while the tumor was growing in the right side.

Histological Analysis of the Bones. A decrease of spongiosa as well as irregularity and thinness of the growth plate was observed at the metaphysis of the tibia (Fig. 1 and 2) and femur in the mice bearing the lower jaw cancer. Proliferation of myeloid cells in the bone marrow cavity was also observed (Fig. 2). The osteoclasts often presented along the endosteal surface in Howship’s lacunae at the diaphysis of the tumor-bearing mouse (Fig. 4). In the cortex of diaphysis of the tumor-bearing mouse, cystic resorption with numerous osteoclasts was observed (Fig. 4) while, in that of the control nude mouse, no such features were observed (Fig. 3). These osteoclasts were recognizable light microscopically as lightly stained multinuclei with conspicuous nucleoli and abundant eosinophilic cytoplasm often with fine vacuoles. It should be pointed out that this osteoclastic bone resorption was observed in both the left tibia and the left femur of the tumor-bearing mouse when the tumor was growing in the right flank. No tumor cells were seen around the bone tissues.

Determination of PTH and Prostaglandins E and F. Serum concentration of PTH was not increased in nude mice bearing the lower jaw cancer (0.19 ± 0.11 ng/ml) compared to controls (0.11 ± 0.00 ng/ml). Tissue homogenate of the lower jaw cancer (1.15 ng/g wet tissue) did not contain larger amounts of PTH.

### Table 1

<p>| nude mice Bearing the lower jaw cancer and controls |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Tumor size (sq cm)</th>
<th>Serum calcium (mg/dl)</th>
<th>Urinary calcium (µg/day)</th>
<th>Urinary phosphorus (µg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bearing small tumors</td>
<td>0.9 ± 0.4a</td>
<td>10.6 ± 0.5</td>
<td>152 ± 108</td>
</tr>
<tr>
<td>Bearing large tumors</td>
<td>2.0 ± 0.4a</td>
<td>14.7 ± 3.1b,c</td>
<td>1,126 ± 542b,c</td>
</tr>
<tr>
<td>Control</td>
<td>10.5 ± 0.6</td>
<td>70 ± 24</td>
<td>377 ± 90</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* b p < 0.05 compared to nude mice bearing small tumors.
* c p < 0.005 compared to control.

### Table 2

<table>
<thead>
<tr>
<th>nude Mice</th>
<th>Length (cm)</th>
<th>Weight (mg/tibia)</th>
<th>Calcium content (mg/tibia)</th>
<th>Phosphorus content (mg/tibia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bearing the lower jaw cancer</td>
<td>1.7 ± 0.1a</td>
<td>24 ± 4</td>
<td>3.7 ± 0.6</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Control</td>
<td>1.7 ± 0.1</td>
<td>31 ± 2</td>
<td>5.0 ± 0.6</td>
<td>2.8 ± 0.3</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* NS, not significant.
Leukocytosis and Hypercalcemia Syndrome

A lower jaw cancer induced severe hypercalcemia and marked granulocytosis not only in the patient but also in nude mice into which the cancer was transplanted. The other lung cancer also induced severe hypercalcemia as well as marked granulocytosis especially in nude mice bearing it. Both tumors were histologically squamous cell carcinomas and had been shown to produce CSF (1, 15, 18). Since our original reports, there have been 2 reports from other workers which described marked granulocytosis with demonstration of CSF in nude mice transplanted with human malignant neoplasms. One was a squamous cell carcinoma of thyroid origin and was also accompanied with severe hypercalcemia both in the cancer patient and in nude mice bearing the tumor (17). The other was a poorly differentiated squamous cell carcinoma of the lung, but serum calcium levels were not mentioned in the report (21). It is very interesting that all 4 CSF-producing tumors with marked granulocytosis were histologically squamous cell carcinomas. In addition, these facts suggest that these 2 phenomena, granulocytosis with CSF production and hypercalcemia, are closely related to each other and that these 2 signs might be grouped into a new syndrome of leukocytosis and hypercalcemia associated with human malignant tumors.

Previously, we demonstrated that the granulocytosis was due to the production of CSF by tumors (1, 15, 18). In this report, we investigated the nature of the hypercalcemia by using mainly the lower jaw cancer transplanted into nude mice. We clearly demonstrated that the hypercalcemia in tumor-bearing mice was abolished by tumor removal. The finding strongly suggested that this paraneoplastic syndrome was humorally mediated but not due to bone metastasis. Bony tissues on the side contralateral to the transplanted tumor showed marked osteopenia (decreased bone mass, decreased bone weight, and low content of calcium and phosphorus) with apparent activation and proliferation of osteoclasts, which gave rise to histological features similar to osteitis fibrosa cystica seen in hyperparathyroidism of primary or secondary origin. In addition, the hypercalcemic mice demonstrated hypercalcuria. Taken together, these findings suggest that the tumor produced some humoral factor which stimulates osteoclastic bone resorption, causing marked hypercalcemia in the nude mice and probably in the patients.

Although the histological examination suggests an excess of PTH, this humoral factor is not PTH; the serum concentration of PTH in the patient or in the tumor-bearing mice was not increased. Furthermore, the tumor did not contain an increased amount of PTH. In addition, there was no increased phosphaturia in spite of marked calcuria, suggesting that PTH is suppressed rather than increased in the patient as well as in tumor-bearing nude mice.

Prostaglandins are also not likely to be the candidate. Neither tumor tissues nor the serum in nude mice bearing the lower jaw cancer contained larger amounts of prostaglandins than the other tumor tissues or control serum of nude mice. Indomethacin (5 µg/g body weight/day) which did not induce emaciation in host mice did not decrease serum calcium concentration. In addition, a continuous administration of food containing indomethacin (2.5 to 5 µg/g body weight/day) was not effective in decreasing the serum calcium concentration. According to the recent report of Doppelt et al. (4), the restriction of only dietary calcium promptly restored the hypercalcemia of VX2 carcinoma-bearing rabbits to the normal range. Therefore, although administration of larger...
amounts of indomethacin (7.5 μg/g body weight/day) seemed effective in lowering the serum calcium concentration, the effect of the emaciation and probably the fasting of the host animals on the serum calcium level was not excluded in these experiments. In fact, tumor-bearing nude mice showed marked hypercalcemia without hyperplastaphasia. As suggested from clinical data, these mice do not seem to develop hyperthrombocytopenia. If this excess excretion of calcium in the urine is supplied only from bones, excess phosphorous liberated from bones should be excreted in the urine or stored in the body. In order to explain this disparity, marked suppression of phosphorus intake via gastrointestinal canal must be considered. Regulation of intake of calcium and phosphorus via gastrointestinal canal, therefore, is thought to play another important role in the pathophysiology of this syndrome.

Lastly, the humoral factor does not seem to be OAF; treatment with prednisolone had no effect on the hypercalcemia in tumor-bearing nude mice as well as in the cancer patient. Raisz et al. (16) and Mundy et al. (14) demonstrated in vitro and in vivo, respectively, that bone resorption and hypercalcemia induced by OAF were very susceptible to glucocorticoid hormones. In conclusion, it is suggested that the hypercalcemia seen in this syndrome is caused by some humoral factor which directly or indirectly activates osteoclasts to induce bone resorption other than PTH, prostaglandins, and OAF.

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REFERENCES


Fig. 1. Longitudinal section of tibial metaphysis and diaphysis of the control nude mouse. No abnormalities were observed. H&E × 48.5.
Fig. 2. Longitudinal section of tibial metaphysis and diaphysis of the tumor-bearing nude mouse. Note the decrease of spongiosa at the metaphysis, irregularity and thinness of the growth plate, and proliferation of the myeloid cells in the marrow cavity. H&E × 48.5.
Fig. 3. Cortex and endosteal surface of tibia at diaphysis of the control nude mouse. No abnormalities were observed. H&E × 48.5.
Fig. 4. Cortex and endosteal surface of tibia at diaphysis of the tumor-bearing nude mouse. Note cystic resorption with proliferating osteoclasts. H&E × 48.5.
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