Walker 256/S Carcinosarcoma Causes Osteoporosis-like Changes through Ectopic Secretion of Luteinizing Hormone-releasing Hormone

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ABSTRACT

We have shown that Walker 256/S mammary carcinoma caused osteoporosis-like changes in young female rats, accompanied by low serum estradiol and hypercalcemia without changes in the serum levels of calcium, phosphorus, and parathyroid hormone-related peptide. In this study, we investigated the cause of bone loss after Walker 256/S inoculation into female 6-week-old Wistar Imamichi rats, focusing on the sex hormone balance in the host animal. Walker 256/S-bearing rats showed characteristic osteoporosis, with a significant increase in spleen weight and a significant decrease in uterine weight by 14 days after s.c. tumor inoculation. In the in vitro bone marrow culture, mineralized nodule formation ability decreased according to the time after tumor inoculation, and tartrate-resistant acid phosphatase-positive multinucleated cell formation increased at 7 days after tumor inoculation, but it began to decrease at 14 days after tumor inoculation. This indicates that after inoculation with Walker 256/S tumor, the progenitors of osteoblasts and osteoclasts lost their balance in the bone turnover, resulting in bone loss in a short period (2 weeks) after tumor inoculation in rats. Therefore, the Walker 256/S-bearing rat is a useful model for the evaluation of drugs for osteoporosis. However, the mechanism of PTHrP-independent bone loss in Walker 256/S-bearing rats has not yet been clarified. Interestingly, after the inoculation of Walker 256/S carcinosarcoma into immature female rats, the serum concentration of estradiol gradually decreased during tumor growth (4). The decrease in serum estrogen concentration should be considered to be a cause of osteoporotic bone changes as well as postmenopausal osteoporosis.

This study deals with the histomorphology of bones and changes in sex hormones after the inoculation of Walker 256/S carcinoma into immature female rats.

MATERIALS AND METHODS

Tumor and Animals. Walker 256/S carcinosarcoma, which was initially provided by Dr. T. Sasaki (Department of Experimental Therapeutics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan), was maintained by serial (2-week intervals) s.c. transplantation in female 6-week-old Wistar Imamichi rats (Imamichi Institute for Animal Reproduction, Ibaraki, Japan). For all experiments, the animals were housed under special pathogen-free conditions at room temperature (24°C–25°C) and 55% humidity with a circa-dian light rhythm of 12 h and given standard diet pellets (Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum. Randomized female 6-week-old Wistar Imamichi rats were divided into control and Walker 256/S carcinosarcoma-inoculated groups of seven animals each. Test animals were s.c. inoculated on the back with 1 mm³ of the carcinoma from donors. At a designated period after tumor inoculation, the animals were weighed, and the tumor mass was determined by measuring the transverse and sagittal diameters with calipers. Blood (0.5 ml) was obtained from the carotid vein. The animals were killed by exsanguination from the carotid artery; the femurs and tibias of the hind limbs, tumors, and organs were then removed and measured.

Bone Analysis. Femurs and tibias were fixed in 10% phosphate-buffered formalin. The BMD was measured using a dual-energy X-ray absorptiometer (DQS-600R; Aloka, Tokyo, Japan). The femur bones were then washed with a chloroform/methanol (2:1) solution, dried at 120°C for 6 h (used for dry weight), and heated at 800°C for 6 h (used for ash weight). Undecalcified and decalcified sections were prepared from the tibia bones according to common procedures. The preparations were stained for TRAP and counterstained with toluidine blue. Morphometric parameters of the bone were then measured using a texture analyzing system (TAS plus; Ernst Leitz).

Bone Marrow Culture. Bone marrow cells from the femurs of Walker 256/S-bearing rats and age-matched healthy rats were basically cultured in α-MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Moregate, Victoria, Australia) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

For the mineralized nodule formation assay (6–8), cells were seeded into 12-well plates (Sumitomo, Tokyo, Japan) at a density of 10⁶ cells/cm². Twenty-four h after seeding, the medium was changed to fresh medium supplemented with 0.2 mM ascorbic acid phosphate ester (Wako Pure Chemical, Osaka, Japan), 1 mM β-glycerophosphate (Sigma Chemical Co., St. Louis, MO), and 10 mM dexamethasone (Sigma). The medium was changed every 2 days; at day 14, the culture was fixed with methanol and stained with Alizarin

FSH, follicle-stimulating hormone; PRL, prolactin; GH, growth hormone; LH-RH, luteinizing hormone-releasing hormone; TNF-α, tumor necrosis factor α; PIF, prolactin release-inhibiting factor; RT, reverse transcription; NIDDK, National Institutes of Diabetes, Digestive and Kidney Diseases.

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The abbreviations used are: PTHrP, parathyroid hormone-related protein; BMD, bone mineral density; TRAP, tartrate-resistant acid phosphatase; LH, luteinizing hormone-

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estradiol and progesterone were assayed using the respective RIA kits (Diagnostik Products Co., Los Angeles, CA). TNF-α was assayed using an enzyme-linked immunosorbent kit (Genzyme, Cambridge, MA).

**Statistics.** Statistical analyses were done by using Student’s t test or Welch’s t test.

**RESULTS**

**Organ Weight and Bone Analyses.** When a Walker 256/S tumor was s.c. inoculated into the back of 6-week-old female rats, the tumor grew rapidly (Fig. 1). Table 1 shows the organ weight of age-matched healthy control rats and Walker 256/S-bearing rats at 14 days after tumor inoculation. In the tumor-bearing rats, the spleen weight was significantly higher and the uterine weight was significantly lower than those of the healthy control rats, although the change in uterine weight is less pronounced when compared to the change in the spleen weight. The body weight and other organ weights did not differ between the healthy control group and the Walker 256/S-bearing group. Table 2 shows that the wet weight and BMD of the femur and tibia bones were significantly lower in Walker 256/S-bearing rats than in the age-matched healthy control rats at day 14, without changes in the bone length. Both dry and ash weights and the BMD of femurs were significantly decreased in the Walker 256/S-bearing rats compared with the healthy control rats, but the ash:dry ratio was maintained during tumor growth (Fig. 2).

**Histomorphological Study.** The frontal and transverse sections of the proximal tibia of Walker 256/S-bearing and nonbearers at day 14 were analyzed by quantitative bone histomorphometry, and the results are shown in Table 3. The secondary trabecular bone mass (bone volume: tissue volume) was significantly decreased in the Walker 256/S-bearing rats compared with the healthy control group, but the trabecular number was maintained. An increment in the resorption surface per bone surface was observed together with an increase in the osteoclast number per bone surface of the bone from the Walker 256/S-bearing rats. In contrast, the osteoblast number per bone surface was significantly decreased in the tumor-bearing group compared with that of the healthy control group.

**Bone Marrow Culture.** To determine bone cell recruitment in the femur bones after inoculation with Walker 256/S tumor, we examined the ability of bone marrow cells to form mineralization nodules and osteoclast-like cells in the in vitro culture systems. As shown in Fig. 3, in a culture of bone marrow cells derived from Walker 256/S-bearing rats, mineralized nodule formation decreased with time after inoculation with the Walker 256/S tumor. On the other hand, the formation of TRAP-positive multinucleated cells increased at 7 days.

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**Table 1** Organ weights of age-matched healthy control rats and Walker 256/S-bearing rats 14 days after tumor inoculation

<table>
<thead>
<tr>
<th>Organ</th>
<th>Healthy control rats</th>
<th>Walker 256/S-bearing rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>164 ± 11</td>
<td>161 ± 14</td>
</tr>
<tr>
<td>Brain (g)</td>
<td>1.29 ± 0.16</td>
<td>1.30 ± 0.07</td>
</tr>
<tr>
<td>Pituitary (mg)</td>
<td>4.61 ± 1.02</td>
<td>5.07 ± 0.92</td>
</tr>
<tr>
<td>Thyroid (mg)</td>
<td>9.73 ± 2.58</td>
<td>10.2 ± 2.0</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>7.23 ± 0.45</td>
<td>8.06 ± 0.85</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>440 ± 55</td>
<td>975 ± 228(^b)</td>
</tr>
<tr>
<td>Adrenal (mg)</td>
<td>22.6 ± 5.7</td>
<td>23.5 ± 2.1</td>
</tr>
<tr>
<td>Kidney (mg)</td>
<td>694 ± 36</td>
<td>658 ± 47</td>
</tr>
<tr>
<td>Uterus (mg)</td>
<td>346 ± 52</td>
<td>286 ± 22</td>
</tr>
<tr>
<td>Tumor (g)</td>
<td>24.0 ± 8.3</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Data are the mean ± SD of seven rats.

\(^b\) Significantly different from healthy control rats at *P* < 0.01.

\(^c\) Significantly different from healthy control rats at *P* < 0.05.

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**Fig. 1.** Walker 256/S tumor growth in rats. Each point with a bar represents the mean ± SD of seven rats.
after tumor inoculation but was markedly decreased at 14 days after tumor inoculation.

**LH-RH mRNA Expression and Serum Hormone and TNF-α Levels.** Fig. 4 shows the results of RT-PCR for LH-RH mRNA in brain specimens from healthy rats, a tumor specimen from Walker 256/S-bearing rats, and the Walker 256/SH cultured cell line. A LH-RH transcript was clearly detected in both Walker 256/S tumor and Walker 256/SH cells, as well as in the hypothalamus. Moreover, the serum level of LH-RH significantly and gradually increased from 3 days after tumor inoculation, whereas the level was very low in the healthy control rats (Fig. 5). Fig. 6 shows the serum pituitary hormone levels 14 days after Walker 256/S tumor inoculation. The serum levels of FSH, LH, and PRL were significantly decreased, but the GH level was not changed in the tumor-bearing group. Also, the serum concentration of estradiol rapidly decreased with time after tumor inoculation (Fig. 7). The serum level of progesterone also decreased, but the change was delayed. During tumor growth, the serum level of TNF-α increased after tumor inoculation (Fig. 8).

**DISCUSSION**

Walker 256/S mammary carcinoma-bearing rats began to show osteoporosis-like changes with an ateliotic uterus and an enlarged spleen. Although we used young (age, 6 weeks) female rats, the involutorial change in the uterus observed after Walker 256/S tumor inoculation appeared to be similar to the changes observed in ovariec-tomized animals or postmenopausal women. The bone loss in ovariec-tomized animals and postmenopausal women can generally be treated by estrogen replacement therapy. We have previously reported that bone loss in Walker 256/S-bearing rats is prevented by estradiol (4), suggesting the involvement of estrogen deficiency in this bone loss. Thus, in this study, we investigated the relationship between bone loss and the sex hormone balance in Walker 256/S-bearing rats.

Osteoporotic bone loss in Walker 256/S-bearing rats was characterized by a decreased BMD of the tibia and femoral bones and a decrease in the bone dry weight and the bone ash weight without a change in the ash weight:dry weight ratio (Table 2; Fig. 2). Moreover, histomorphometric studies revealed a decrease in osteoblastic cells in Walker 256/S-bearing rats (Table 3). Mineralized nodule formation in bone marrow cell culture decreased when the cells were prepared from tumor-bearing rats (Fig. 3), which indicates the decrease in osteoprogenitor cells in these animals. From these results and the evidence, it is suggested that the recruitment of osteoblast progenitors is impaired in Walker 256/S-bearing rats.

The number of osteoclasts increased at 14 days after the tumor inoculation (Table 3). In the bone marrow culture system, more osteoclast-like cells were formed in marrow cell culture when the cells were prepared 7 days after tumor inoculation (Fig. 3). Therefore, it is obvious that differentiation and activation of osteoclasts are remarkably stimulated in the tumor-bearing rats. However, less osteoclast-like cells were formed in the culture from the animal at the late stage (14 days) of tumor growth (Fig. 3). This might be due to the decrease in osteoprogenitors or the impairment of osteoblasts, because the differentiation of osteoclasts is regulated by some humoral factors from osteoblasts and cell-cell contact with osteoblasts (12, 13).

With regard to carcinomatous osteoporosis, except for bone metastasis and myeloblastoma, there are few reports on adrenocorticotropin hormone-secreting medullary carcinoma (14) and prolactinoma (15, 16). It has been widely accepted that humoral hypercalcemia and osteolysis of malignancy are closely associated with the secretion of PTHrP (17). Stiegler et al. (15) reported that in patients with hyperprolactinemia plasma, the PTHrP level was elevated, and most of the tumor cells were strongly PTHrP positive. Similarly, Walker 256 tumor has also been believed to cause osteolysis by secreting PTHrP.

<table>
<thead>
<tr>
<th>Item</th>
<th>Healthy control rats</th>
<th>Walker 256/S-bearing rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone volume/tissue volume (%)</td>
<td>12.8 ± 1.9</td>
<td>7.60 ± 4.18</td>
</tr>
<tr>
<td>Trabecular number (no./mm)</td>
<td>5.46 ± 0.65</td>
<td>5.04 ± 1.22</td>
</tr>
<tr>
<td>Resorption surface/bone surface (%)</td>
<td>26.5 ± 1.7</td>
<td>51.0 ± 10.26</td>
</tr>
<tr>
<td>Osteoclast number/bone surface (cells/mm)</td>
<td>9.7 ± 1.7</td>
<td>18.6 ± 1.7</td>
</tr>
<tr>
<td>Osteoblast number/bone surface (cells/mm)</td>
<td>193 ± 31</td>
<td>38.5 ± 15.5</td>
</tr>
</tbody>
</table>

*Measurements were done in a ~1.7-mm² area. Data are the mean ± SD of seven rats.

^a^ Significantly different from healthy control rats at P < 0.05.

^b^ Significantly different from healthy control rats at P < 0.01.

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Table 2: Analytical data for bones in rats

<table>
<thead>
<tr>
<th></th>
<th>Healthy control rats</th>
<th>Walker 256/S-bearing rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Item</td>
<td>Length (mm)</td>
<td>Wet weight (mg)</td>
</tr>
<tr>
<td>Femurs</td>
<td>28.9 ± 0.8</td>
<td>455 ± 22</td>
</tr>
<tr>
<td>Tibias</td>
<td>32.8 ± 0.6</td>
<td>371 ± 18</td>
</tr>
</tbody>
</table>

^a^ Data from rats 14 days after tumor inoculation represent the mean ± SD of seven rats.

^b^ Significantly different from healthy control rats at P < 0.01.
However, we have previously demonstrated that Walker 256/S, a variant lacking bone-metastatic ability, does not express PTHrP mRNA (4).

In the previous study, we have revealed the low serum level of estradiol and the decreased uterine weight in Walker 256/S-bearing rats (4). It has been reported that treating women with LH-RH agonists leads to estrogen deficiency-induced bone loss (20–22). Our previous findings, together with this evidence, suggest the implication of LH-RH in several phenomena in Walker 256/S-bearing rats. As expected, Walker 256/S tumor expressed LH-RH mRNA, and the serum concentration of LH-RH was markedly increased from an early stage of tumor growth (Figs. 4 and 5). This long-term continuance of a high level of LH-RH should lead to lowered serum levels of LH and FSH without changing the level of another pituitary hormone, GH (Fig. 6). These changes in the LH-RH, LH, and FSH levels could explain the decrease in estradiol and progesterone (Fig. 7). In addition, the PRL level was also lowered in tumor-bearing rats (Fig. 6).

The LH-RH gene exists on the long PIF gene (11). Because the mRNA amplified by RT-PCR in this study contained whole LH-RH and a part of PIF, it is possible that the Walker 256/S tumor also expresses and secretes PIF peptide. This is the first report that the tumor ectopically secretes LH-RH and PIF and that it induces osteoporosis-like changes based on hypoestrogenism. Estradiol acts directly on osteoblast-like cells, following the inhibition of the secretion of osteolytic cytokines, such as interleukin-1, TNF-α, and interleukin-6 (12) and the stimulation of the secretion of insulin-like growth factor I, which enhances osteoblast proliferation and activities by an autocrine mechanism (23, 24). Estrogens also inhibit bone resorption by stimulating osteoblasts to produce transforming growth factor β (25, 26) and suppressing the release of osteolytic cytokines (27–30). The elicitation of significant bone loss in rats notably required several months after ovariectomy or hypoovarianism (31) and at least 30 days after the administration of buserelin, a LH-RH agonist (32). On the contrary, the osteoporosis-like changes in Walker 256/S-bearing rats were observed within a very short time (14 days) after tumor inoculation. Therefore, it is unlikely that overexpression of LH-RH alone causes the rapid bone loss in Walker 256/S-bearing rats.

The enlarged spleen in Walker 256/S-bearing rats (Table 1) suggests the activation of the immune system in the host. The serum level of TNF-α increased in the host along with the tumor growth (Fig. 8). Although the TNF-α level was measured in this study, other immunoresponsible osteolytic cytokines are also presumed to increase in the host. It is possible that the increased cytokines caused the regression of the ovarian tissue, particularly the follicles. Either way, it is
assumed that in Walker 256/S-bearing rats, estrogen deficiency and the elevation of TNF-α and probably other osteolytic cytokines synergistically induce a very rapid bone loss.

In conclusion, Walker 256/S mammary carcinoma ectopically secretes LH-RH, resulting in a decrease in pituitary gonadotropic hormone secretion followed by a low level of sex steroid hormones in female rats. The osteoporosis-like changes were caused in a short period of time after inoculation with a Walker 256/S tumor, due to the additional effects of hypoestrogenism and the high levels of osteolytic cytokines such as TNF-α on osteoprogenitors.

Fig. 6. Serum concentrations of FSH, LH, PRL, and GH in age-matched healthy control rats (□) and Walker 256/S-bearing rats (■) at 14 days after tumor inoculation. Each column with a bar represents the mean ± SE of seven rats. * and **, significantly different from the healthy control rats at P < 0.05 and 0.01, respectively.

Fig. 7. Changes in serum concentration of estradiol and progesterone in age-matched healthy rats (○) and Walker 256/S-bearing rats (●). Each point with a bar represents the mean ± SE of seven rats. * and **, significantly different from the healthy control rats at P < 0.05 and 0.01, respectively.

Fig. 8. Changes in serum concentration of TNF-α in age-matched healthy rats (○) and Walker 256/S-bearing rats (●). Each point with a bar represents the mean ± SE of seven rats. * and **, significantly different from the healthy control rats at P < 0.05 and 0.01, respectively.
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