Postmenopause-Like Bone Loss by Mammary Carcinoma Walker256/S Which Secretes Luteinizing Hormone-Releasing Hormone

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ABSTRACT—When Walker 256/S carcinosarcoma (W256/S) was subcutaneously inoculated into the back of mature female Wistar Imamichi rats (10-week-old), the tumor grew rapidly and caused increases in the urinary excretions of calcium and hydroxyproline, without changes in the serum concentrations of calcium and inorganic phosphorus. Furthermore, osteoporosis-like changes in the femurs and decrease in uterus weight were observed, as previously reported for W256/S-bearing young rats. In the healthy mature female rats, the estrus cycle passed through four stages (proestrus, estrus, metestrus and diestrus) within 4 to 5 days, with a peak of serum estradiol and progesterone levels in the proestrus stage. On the other hand, after subcutaneous inoculation of W256/S into the rats, the estrus cycle tended to pause upon the metestrus or diestrus stage, accompanied with significantly low estradiol and progesterone levels in serum. W256/S tumor produced and secreted luteinizing hormone-releasing hormone (LH-RH). In conclusion, it seems that the ectopic secretion of LH-RH from the tumor resulted in the decrease in the secretion of gonadotropic hormones, following low level of sex hormones and stopping the estrus cycle. Therefore, W256/S-bearing rats may be a model for osteoporosis of hypoovarianism or postmenopause.

Keywords: Walker 256/S carcinosarcoma, Osteoporosis, Estrus cycle, Menopause, Luteinizing hormone-releasing hormone (LH-RH)

Walker 256 rat carcinosarcoma (W256) is known to induce bone-metastatic cancer and humoral hypercalcemia of malignancy (1-3). It had been reported that the humoral hypercalcemia and osteolysis of malignancy are closely associated with the secretion of parathyroid hormone-related protein (PTHRP) (4, 5). We have recently shown that W256/S carcinaoma, a variant lacking bone-metastatic ability, caused osteoporosis-like changes in young female rats accompanied with hypercalciuria without influence on the serum levels of calcium, phosphorus and PTHrP (6). Moreover, in W256/S-bearing rats, the serum concentration of estradiol was gradually decreased during the tumor growth and the spleen was enlarged in the rats (6). These changes occurred within a short period (2 weeks) after the tumor inoculation into the rats. We thought that the decrease in serum estrogen concentration and enhanced immune system after inoculation of W256/S carcinoma may accelerate the osteoporotic bone changes in the rats. Therefore, W256/S-bearing rats can be a useful model for evaluating drugs for the treatment of osteoporosis (6, 7).

It is known that postmenopausal or estrogen-deficient women are at particular risk (8), and estrogen deficiency is a major contributing factor to bone loss occurring after menopause (9, 10). Generally, ovariectomized animals are used as the postmenopausal osteoporosis model, but this model needs relatively long periods (several months) to elicit significant bone loss. From this point of view, W256/S-bearing rats fall into category of animals showing osteoporosis-like changes within a short period after the tumor inoculation. Therefore, studies on the mechanism for the lowered serum estrogen concentration after W256/S inoculation are important to see whether this carcinoma is a model of postmenopausal osteoporosis.

This study deals with the relation between the bone metabolism, the estrus cycle and the serum levels of sex hormones after inoculation of W256/S carcinoma into mature female rats.

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MATERIALS AND METHODS

Tumor and animals

W256/S carcinosarcoma, which was initially provided by Dr. T. Sasaki (Department of Experimental Therapeutics, Cancer Research Institute, Kanazawa University, Kanazawa), was maintained by serial (2-week intervals) subcutaneous transplantations in female Wistar Imamichi rats (6-week-old; Imamichi Institute for Animal Reproduction, Ibaraki).

Experiments

For all experiments, animals were housed under special pathogen-free conditions at room temperature (24–25°C) and 55% humidity with a 12 hr/12 hr dark-light cycle, standard diet pellets (Oriental Yeast Co., Tokyo) and tap water ad libitum. Randomized female Wistar Imamichi rats (10-week-old) were divided into control and W256/S carcinoma-inoculated groups of 7 animals each. Animals were subcutaneously inoculated at the back with 1 mm³ of the carcinoma from donors 10 days after the inoculation. At a designated period after the tumor inoculation, the animals were weighed, and the tumor mass was determined by measuring transverse and sagittal diameters with calipers. Urine was collected for 24 hr. Blood (0.5 ml) was obtained from the carotid vein. Animals were killed 18 days after tumor inoculation by exsanguination from the carotid artery. Then, organs, tumors and femurs of hind limbs were removed and measured.

Bone analysis

The femurs were fixed in 10% phosphate-buffered formalin. The bone mineral density (BMD) was determined using a dual-energy X-ray absorptiometer (DXA, DCS-600R; Aloka, Tokyo). Thereafter, the bones were washed with chloroform/methanol (2:1) solution, dried at 120°C for 6 hr, and weighed.

Biochemical analyses

Calcium and inorganic phosphorus in serum and urine were measured by an O-cresolphthalein complexone method (Calcium-TA test; Wako Pure Chemical, Osaka) and a Fiske-Subbarow method (Inorganic Phosphorus-TA test, Wako), respectively. Alkaline phosphatase (ALP) in serum and creatinine in urine were measured by an Alkaline-Phosha-TA test kit (Wako) and a Creatinine-test Wako, respectively. Hydroxyproline (HP) in urine was assayed according to the method reported by Kivirikko et al. (11). 17β-Estradiol (estradiol) and progesterone were assayed using a radioimmunoassays kit (Diagnostic Products Co., Los Angeles, CA, USA). Luteinizing hormone-releasing hormone (LH-RH) was measured using an enzyme immunoassy, Endokit RedTM LHRH, developed by CYT Immune Sciences, Inc. (Greenmead Drive College Park, MD, USA).

Estrus cycle

The estrus cycle was cytologically observed in the vaginal smear under a microscope and divided into four stages, proestrus, estrus, metestrus and diestrus, according to the description by Gorbman and Bern (12).

Reverse transcription and polymerase chain reaction

mRNAs were prepared from rat cerebral, hypothalamus, pituitary, Walker256/S carcinoma from the tumor-bearing rat and from Walker256/SH, established as a cultured cell line, by a QuickPrep micro mRNA purification kit (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions. Reverse transcription (RT) reactions were carried out in 40 mM KCl; 50 mM Tris-HCl (pH 8.3); 6 mM MgCl₂; 1 mM dithiothreitol; 0.1 mg/ml bovine serum albumin (BSA); 1 mM each of dATP, dCTP, dGTP and dTTP; 10 units RNase inhibitor (Promega, Madison, WI, USA), 100 pmol of random hexamer, mRNA and 200 units of the Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Berlin, Germany) in a final volume of 50 µl at 37°C for 60 min. Polymerase chain reactions (PCR) were carried out in a final volume of 20 µl containing 5 µl of RT reaction mixture; 50 mM KCl; 20 mM Tris-HCl (pH 8.3); 2.5 mM MgCl₂; 0.1 mg/ml BSA; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 10 µM each of the mixed oligonucleotide primer; and 1 unit of Taq DNA polymerase (Gibco-BRL). Each cycle consisted of 1 min at 94°C, 2 min at 55°C and 2 min at 72°C. RT reactions were done with each 1 µg of mRNA, and the PCR reaction was done at 35 cycles for the RT-PCR reaction. The sequences of rat LH-RH primers were placed in the first exon and in the third exon, as presented by Azad et al. (13). The primer sequences were 5'-CACTATGGTCAC CAGCCGGG-3' and 5'-AGAGCTCTTCGCAGATCCC TAAGA-3'. The predicted fragment amplified by PCR is 375 base pairs (bp) in cDNAs encoding a 92 amino acid precursor protein for the decapetide LH-RH and the 56-residue-long prolactin release-inhibiting factor. The primers for rat β-actin were 5'-TTCAATGAAGCTG CGTGTGGC-3' and 5'-CTC(A/G)TAGCTCTTCTCCA GGGAGGA-3' (6).

Statistics

Data are the mean±S.D. of seven rats. Statistical significance was measured with Student's t-test, two way ANOVA and Duncan's new multiple range test.
RESULTS

**Organ weight and bone analyses**

When W256/S tumor was subcutaneously inoculated into the rats, the tumor grew well at the inoculation site (Fig. 1) and became about 23 g wet weight by 18 days, without change in the body weight (Table 1). Table 1 also shows that in the tumor-bearing rats, the spleen weight was significantly higher and the uterus weight was significantly lower than in the healthy control rats, as previously reported in immature rats (6), without any marked changes in the weight of ovary and other organs (data not shown) of the tumor-bearing rats, compared with the healthy control group. Table 2 shows the analytical data of femurs. The bone length was not changed, but the bone dry weight and BMD were significantly lower in W256/S-bearing rats than in the age-matched healthy control rats.

**Biochemical analyses of serum and urine**

Although the serum levels of calcium, inorganic phosphorus and ALP were not different between the healthy control group and the tumor-bearing group (Table 3), the urinary excretion of calcium was significantly higher in the tumor bearers than in the healthy animals, and the urinary phosphorus tended to be lower in the tumor bearers than in the healthy animals (Fig. 2). The HP excretion was increased according to the tumor growth.

**Estrus cycle and serum hormone levels**

Since we previously observed in immature rats that the serum level of β-estradiol was lowered in W256/S-bearing rats (6), the estrus cycle was measured in mature animals after tumor inoculation. Figure 3 shows the changes of estrus stages of individual rats of the healthy control group and W256/S-bearing group during the experimental period (18 days after tumor inoculation, 10- to 12-week-old). While in healthy rats the estrus cycle passed through four stages within 4 to 5 days, that of the tumor-bearing rats became irregular until about 1 week after the tumor inoculation and tended to pause upon the metestrus or diestrus stage in the later period of the experiment (4 days before finish). Figure 4 shows the courses of the serum concentration of estradiol and progesterone

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**Table 1. Wet weight of organs from rats**

<table>
<thead>
<tr>
<th>Item</th>
<th>Healthy control</th>
<th>W256/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (g)</td>
<td>259±7</td>
<td>263±8</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>706±101</td>
<td>1364±348**</td>
</tr>
<tr>
<td>Ovary (mg)</td>
<td>94±19</td>
<td>96±17</td>
</tr>
<tr>
<td>Uterus (mg)</td>
<td>474±89</td>
<td>334±76**</td>
</tr>
<tr>
<td>Tumor (g)</td>
<td>—</td>
<td>23±8</td>
</tr>
</tbody>
</table>

Data from rats 18 days after tumor inoculation. Data are the mean±S.D. of seven rats. **Significantly different from the healthy control at P<0.01.

**Table 2. Analytical data of femurs of rats**

<table>
<thead>
<tr>
<th>Item</th>
<th>Healthy control</th>
<th>W256/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>left</td>
<td>33.1±0.5</td>
<td>32.6±0.5</td>
</tr>
<tr>
<td>right</td>
<td>33.0±0.5</td>
<td>32.6±0.6</td>
</tr>
<tr>
<td>Dry weight (mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>left</td>
<td>433±21</td>
<td>395±28*</td>
</tr>
<tr>
<td>right</td>
<td>429±19</td>
<td>390±27**</td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>left</td>
<td>111±4</td>
<td>101±4**</td>
</tr>
<tr>
<td>right</td>
<td>109±3</td>
<td>101±2**</td>
</tr>
</tbody>
</table>

Data from rats 18 days after tumor inoculation. Data are the mean±S.D. of seven rats. *Significantly different from the healthy control at P<0.05 and P<0.01, respectively.

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**Table 3. Serum calcium (Ca), inorganic phosphorus (Pi) and ALP of rats**

<table>
<thead>
<tr>
<th>Item</th>
<th>Healthy control</th>
<th>W256/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca (mg/dl)</td>
<td>8.4±0.2</td>
<td>9.4±0.2</td>
</tr>
<tr>
<td>Pi (mg/dl)</td>
<td>6.1±0.2</td>
<td>9.7±0.3</td>
</tr>
<tr>
<td>ALP (B-L unit)</td>
<td>4.2±1.5</td>
<td>5.4±2.1</td>
</tr>
</tbody>
</table>

Data from rats 18 days after tumor inoculation. Data are the mean±S.D. of seven rats.

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![Fig. 1. Change in tumor size in W256/S-bearing rat. Data are the mean±S.D. of seven rats.](image-url)
Fig. 2. Changes in urinary excretion of calcium (Ca), inorganic phosphorus (Pi) and hydroxyproline (HP) in age-matched healthy rats (○) and W256/S-bearing rats (●). Urine was collected for 24 hr, and each data value represented the ratio to the creatinine (Cr) excretion. Data are the mean ± S.D. of seven rats. * and **Significantly different from the healthy control group at \( P < 0.05 \) and \( P < 0.01 \), respectively.

Healthy control rats

W256/S-bearing rats

No. 1

No. 2

No. 3

No. 4

No. 5

No. 6

No. 7

10 w
11 w
12 w

10 w
11 w
12 w

Age

Tumor inoculation

Fig. 3. Courses of estrus stages after W256/S inoculation into rats. Number (No. 1 to 7) corresponds to an individual rat used in each group. Columns indicate the proestrus (I, □□), estrus (II, ■■■), metestrus (IV, ♦♦) and diestrus (V, □□).
Fig. 4. Changes in serum estradiol (○) and progesterone (△) in rats. The serum concentrations of hormones from the healthy control rats are expressed as the value in the respective estrus stages (see legend for Fig. 3). Data are the mean ± S.D. of seven rats.

in the rats. The serum levels of estradiol and progesterone in healthy control rats were synchronously changed with the estrus cycle and reached a peak at the proestrus stage. In W256/S-bearing rats, these hormone levels were hardly changed and were maintained at very low concentrations at two weeks after the tumor inoculation.

**LH-RH production by W256/S tumor**

It was clear from data using the RT-PCR method that W256/S tumor expressed LH-RH mRNA, as well as the hypothalamus and also the culture cell line W256/SH (Fig. 5). Figure 6 confirms that W256/SH cells secreted LH-RH into the culture medium.

Fig. 5. Detection of LH-RH mRNA by RT-PCR. The size of the PCR fragment of LH-RH was 375 bps.

Fig. 6. Changes in LH-RH concentration in culture medium of W256/SH cells. W256/SH cells (5 × 10⁶ cells/ml) were incubated in α-MEM supplemented with 10% fetal calf serum, and the medium was exchanged every day and LH-RH concentration in the media was measured. Data are the mean ± S.E.M. of three experiments.
DISCUSSION

In general, osteolysis of malignancy has been believed to be dependent upon PTHrP secreted by the tumor. We previously showed that W256/S mammary carcinoma caused osteoporosis-like changes in immature female rats such as a decrease in cancellous bone in the secondary trabecula, lowered bone dry weight without change in the ratio of calcium content and decreased BMD (6). This study also confirmed in mature female rats that W256/S carcinoma induced the osteoporosis-like changes, including decreased femur BMD. Surprisingly, we found that W256/S-bearing rats showed a pause in their estrus cycle, accompanied with decreased serum sex hormone levels and hyperexcretion of calcium and hydroxyproline in urine without change in the serum calcium concentration. These changes are very similar to those observed in postmenopausal women (14, 15). In this study, the urinary phosphorus excretion was lower in W256/S-bearing rats than in the healthy control rats. There are few reports describing the low excretion of phosphorus in an early stage of postmenopause and after ovariectomy. Riggs et al. (16) reported that estrogens modulate or stimulate the production and function of PTH. Thus, the hypophosphataturia and hypercalciuria in W256/S-bearing rats appear due to a presumed reduction of PTH function under the low estrogen situation.

Moreover, W256/S tumor and W256/SH cells, which are established as a culture line from the tumor, expressed LH-RH mRNA and secreted the hormone. It may be expected that LH-RH continuously secreted from the tumor inhibits the release of LH and follicle-stimulating hormone (FSH) through the negative feedback system in the host animal, as it was reported that exogenously administered LH-RH inhibited the release of LH and FSH from the pituitary (17). This should be the cause of stopping the estrus cycle and lowered level of estradiol and progesterone after the tumor inoculation, resulting in an involutional uterus and bone loss. It had been formerly believed that W256 tumor caused the humoral hypercalcemia and osteolysis of malignancy by secreting PTHrP (4, 5). In this study, we revealed the W256/S carcinoma, which does not produce PTHrP (6), ectopically secretes LH-RH and causes osteoporosis-like changes.

It has been reported that estrogens inhibit bone resorption through stimulation of osteoblast to produce transforming growth factor-β (18, 19) and suppression of release of osteolytic cytokines (20–23). The mechanisms of the osteoporosis-like changes in W256/S-bearing rats are not completely clarified, but the changes in the bone metabolism may be related with the hormonal and immune balance in the tumor-bearing rats. Because enlarged spleen in W256/S-bearing rats suggests a rise of the immune system in the host animal, it may be considered that cytokines, such as interleukin 1 and tumor necrosis factor α, secreted from immune cells escape the control of estrogens and activate osteoclasts, resulting in the bone loss in a short period.

This study indicates that W256/S carcinosarcoma induced osteoporosis-like changes through ectopic secretion of LH-RH. W256/S-bearing rats exhibit a disorder in their hormone balance, particularly stopping of the estrus cycle. Consequently, the W256/S-bearing rats may be a model for postmenopausal osteoporosis.

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