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Osteoporosis-like Changes in Walker Carcinoma 256-Bearing Rats, Not Accompanied with Hypercalcemia or Parathyroid Hormone-related Protein Production

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Walker carcinoma 256 (W256) was reported to induce hypercalcemia dependent on bone metastasis and/or parathyroid hormone-related protein (PTHrP) in the rat, providing a model of the humoral hypercalcemia of malignancy. In this study, after the subcutaneous inoculation of cells of the W256/S line, which is maintained in this laboratory, into young female Wistar Imamichi rats (6 weeks old), serum calcium and phosphorus levels changed only within the control range, whereas serum alkaline phosphatase activity and urinary calcium level significantly increased and urinary phosphorus decreased during the tumor growth, resulting in hypercalciuria and hypophosphaturia. W256/S did not express PTHrP-mRNA, whereas LLC-W256 cells did express it. Serum PTHrP level was not changed in W256/S-bearing rats. Osteoporosis-like changes, bone weight loss, low contents of bone calcium and phosphorus, and a decrease in the bone mineral density (BMD), were observed in the femur 14 days after the tumor inoculation. There was a pronounced decrease in the serum 17β-estradiol level during the tumor growth. The reduction of BMD of femurs in W256/S-bearing rats was significantly inhibited by treatment with salmon calcitonin or 17β-estradiol. On the basis of these results, W256/S carcinoma-bearing rats seem to be a useful model for osteoporosis of hypoovarianism.

Key words: Walker 256/S carcinosarcoma — Osteoporosis — Hypercalciuria — Hypophosphaturia — Estradiol

Some tumors metastasize, invade bones, and decrease bone mass, whereas others induce osteolysis by producing humoral factors. The hypercalcemic Walker 256 rat carcinoma (W256) is a well characterized animal model of bone-metastatic cancer and humoral hypercalcemia of malignancy.[1,2] Guaitani *et al.*[3] reported that when implanted intramuscularly, line A Walker carcinoma invaded the adjacent bones without causing hypercalcemia, but another line, B, elicited hypercalcemia and osteoporosis-like changes in bone, without bone metastasis. Cancers associated with the humoral hypercalcemia of malignancy produce a parathyroid hormone-related protein (PTHrP), which may be a cause of abnormal calcium metabolism in the clinical situation.[4–6] It has been shown that a hypercalcemic W256 tumor produced and secreted an 18 kDa PTHrP.[7,8]

In this paper, we investigated the osteoporosis-like changes in rats bearing W256/S carcinosarcoma, which is a variant lacking bone-metastatic ability. The W256/S line caused osteoporosis-like changes in rats accompanied with hypercalciuria and hypophosphaturia and decreased serum estradiol level, but did not influence the serum calcium, phosphorus, and PTHrP levels.

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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. LLC-Walker 256 (LLC-W256), a reference tumor producing PTHrP, was purchased from ICN Biomedicals, Costa Mesa, CA and was maintained *in vitro* in medium 199 (Gibco Laboratories, Grand Island, NY) supplemented with 10% horse serum.

Experiments For all experiments, animals were housed under special pathogen-free conditions at room temperature (24–25°C) and 55% humidity with a circadian light rhythm of 12 h, standard diet pellets (Oriental Yeast Co., Tokyo) and tap water *ad libitum*. Randomized female Wistar Imamichi rats (6 weeks old, Imamichi Institute for Animal Reproduction, Ibaraki) were divided into control and W256/S carcinoma-inoculated groups of 10 animals each. Animals were subcutaneously inoculated at the back with 1 mm^2 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 h. Blood (0.5 ml) was obtained from the carotid vein. Animals were killed 14 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 (DEXA, DCS-600R, Aloka, Tokyo), which is a low-energy X-ray instrument modified for small animals. Thereafter, the right femur bone of each animal was washed with chloroform/methanol (2:1) solution, dried at 120°C for 6 h (used for dry weight), heated at 800°C for 6 h (used for ash weight), and dissolved in 6 *N* HCl for measurement of calcium content by atomic absorption spectroscopy (Hitachi Z 8100) or by an *o*-cresolphthalein complexone method (Calcium-TA test, Wako Pure Chemical, Osaka). Inorganic phosphorus was measured by the Fiske-Subbarow method (Inorganic Phosphorus-TA test, Wako). The left femur bone was embedded in polyester resins (Rigolac, Nisshin EM Co., Tokyo). Unmineralized ground sections (100 μm thickness) were prepared from the longitudinal sagittal plane of the distal metaphysis. They were stained with toluidine blue.

Biochemical analysis of serum and urine Calcium and inorganic phosphorus in serum and urine were measured as described above. Alkaline phosphatase (ALP) in serum was measured using an Alkaline-Phospha-TA test kit (Wako). Serum PTHrP was measured using a radioimmunoassay kit, employing a rabbit anti-human PTHrP (1–34) (Peninsula Laboratories, Belmont, CA), which exhibits cross reactivity with rat PTHrP and W256-induced PTHrP, but not with rat PTH.[7,8] 17β-Estradiol (estradiol) was also assayed using a radioimmunoassay kit (Diagnostic Products Co., Los Angeles, CA).

Reverse transcriptase-polymerase chain reaction (RT-PCR) Direct isolation of polyadenylated RNA using a QuickPrep Micro mRNA Purification kit (Pharmacia, Uppsala) was carried out from LLC-W256 cells of a confluent culture and from W256/S tumor surgically enucleated from a tumor-bearing rat 2 weeks after the inoculation. Synthesis of cDNA from the isolated mRNA was carried out with RNase H^- reverse transcriptase (SuperScript II, Gibco BRL, Gaithersburg, MD), RNase inhibitor (Nippon Gene Co., Tokyo) and 0.5 m*M* oligo-dT primer (Pharmacia) in a volume of 20 μl. Two oligonucleotides used as PCR primers for detecting PTHrP expression were devised from the published sequence of PTHrP gene[9,10]: PTHrP-1, 5′-AAACCAACAAGGTGGAGACG-3′ (cDNA sequence 393–412); and PTHrP-2, 5′-TGTCCTTGGAAGATCTTCGG-3′ (cDNA sequence 635–616). The PCR with PTHrP-specific primers produced a DNA fragment of 243 base pairs (bp). The integrity of mRNA isolated from the samples was checked by RT-PCR with primers for rat β-actin: β-actin-1, 5′-TTCTACAATGAGCTGCGTGTGGC-3′; and β-actin-2, 5′-CTC(A/G)TAGCTCTTCTCCAGGGAGGA-3′ (Nippon Gene). A PCR mixture consisted of 10 μl of 10 × PCR buffer (500 m*M* KCl, 15 m*M* $MgCl_2$, 0.01% gelatin, 100 m*M* Tris-HCl, pH 8.3), 0.2 m*M* dNTP mixture, 100 pmol of each oligonucleotide primer, 2.5 units of Taq DNA polymerase (Gibco BRL), and 1.0 μl of template solution (0.1 μg of the mRNA) in a final volume of 100 μl. Finally, 70 μl of paraffin oil was added. Twenty-six of the following incubation cycles were performed in a DNA Thermal Cycler 480 (Takara Shuzo Co., Kyoto): 60 s denaturation step at 94°C, 50 s annealing step at 58°C and 120 s extension at 72°C. PCR products were electrophoresed on 2% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME) with *Hae*III-digested pHY DNA molecular weight standards (Takara) and visualized by ethidium bromide staining. PTHrP-cDNA was confirmed by digestion with *Taq*I and *Hae*III to

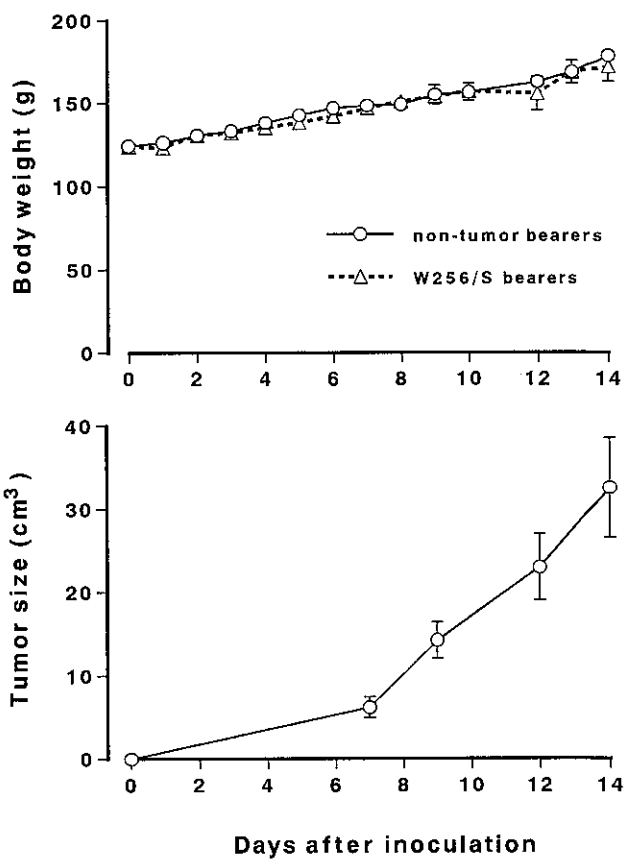

Fig. 1. Changes in body weight and tumor size in W256/S-bearing rats. Data are the mean±SD of ten rats.

produce 63 and 180 bp fragments and 101 and 122 bp fragments, respectively.

Treatment with salmon calcitonin or estradiol of W256/S-bearing rats Salmon calcitonin (10 IU/kg), dissolved in 20 m*M* sodium acetate buffer containing 0.1% bovine serum albumin (pH 6.0), was intramuscularly injected at every second day, or estradiol in olive oil (100 μg/kg) was subcutaneously injected at every third day from Days 1 to 13 after the tumor inoculation. The BMD of femurs of the rats was determined 14 days after the tumor inoculation.

Statistics Data are represented as the mean±SD. Statistical analyses were done by using Student's *t* test or Duncan's test.

RESULTS

When subcutaneously inoculated with W256/S, rats died from Day 17, and the mean survival time was about 20 days. The body weight of the tumor-bearing rats apparently increased in the same manner as that of the healthy control rats up to Day 14. Tumor growth was slow up to Day 7, then rapidly increased by Day 14 after the tumor inoculation (Fig. 1). Table I shows the weights of major organs of tumor bearers and non-tumor bearers on Day 14. The weights of the spleen and liver significantly increased, but that of the uterus significantly decreased, without any marked change in other organs of the tumor-bearing rats, compared with those of the healthy control rats. Both calcium and inorganic phosphorus levels in serum of the tumor-bearing animals changed only within the control range, but serum ALP increased and maintained high levels up to Day 7, followed by a gradual decrease (Fig. 2). Fig. 3 shows the changes of calcium and inorganic phosphorus in urine after the tumor inoculation. The course of urinary calcium excretion was very complex; a transient decrease at Day 1, a peak at Day 5 and a trough at Day 9, followed by an increase again. Urinary phosphorus excretion appeared to follow an opposite course to urinary calcium.

Table I. Wet Weight of Organs from Age-matched Healthy Control Rats and W256/S-bearing Rats 14 Days after Subcutaneous Tumor Inoculation

Item		Healthy controls	W256/S bearers
Body weight (g)		185.8±6.0	180.0±8.6
Brain (g)		1.42±0.16	1.43±0.07
Pituitary (mg)		5.07±2.00	5.58±1.92
Thyroidea (mg)		10.7±3.1	11.2±2.0
Liver (g)		7.95±0.84	8.87±1.00[a)]
Spleen (mg)		500±99	1210±240[c)]
Adrenal (mg)	right	24.8±10.7	25.8±2.1
	left	26.7±5.1	28.0±1.9
Kidney (mg)	right	764±49	724±53
	left	716±62	712±48
Ovary (mg)	right	48.1±6.9	50.0±9.5
	left	41.0±6.5	44.7±2.8
Uterus (mg)		371±56	315±18[b)]
Tumor (g)		—	22.3±14.8

Data are the mean±SD of ten rats.
a), *b*), *c*) Significantly different from the healthy control at $P<0.05$, 0.01, and 0.001, respectively.

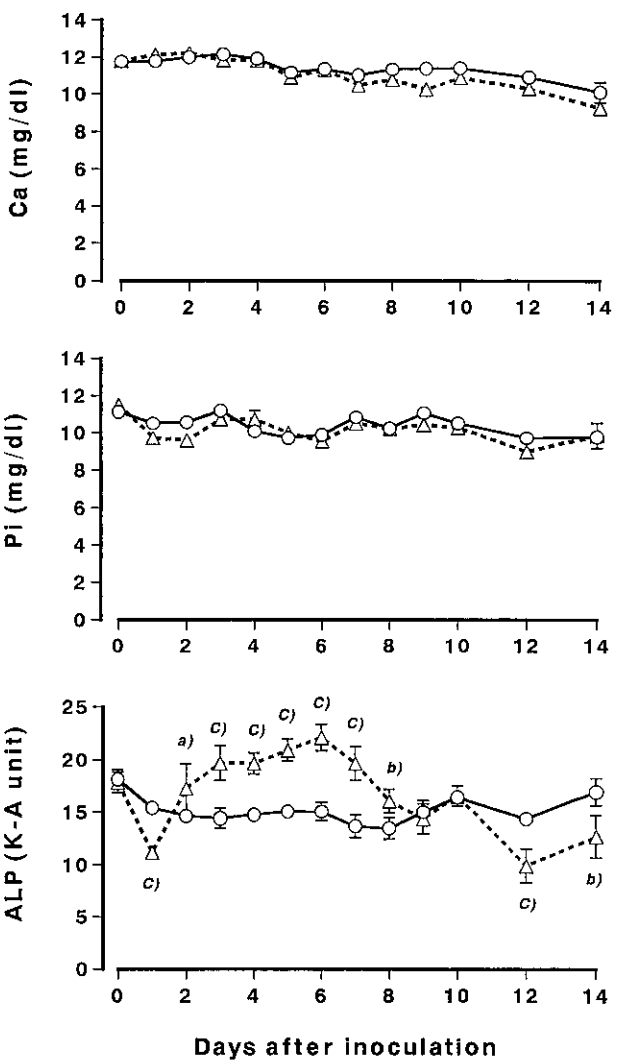

Fig. 2. Changes of serum calcium (Ca), inorganic phosphorus (Pi), and alkaline phosphatase (ALP) in rats. Sera were obtained from age-matched healthy control rats (○) and W256/S-bearing rats (△). Data are the mean±SD of ten rats. *a*), *b*), *c*) Significantly different from the control at $P<0.05$, 0.01, and 0.001, respectively.

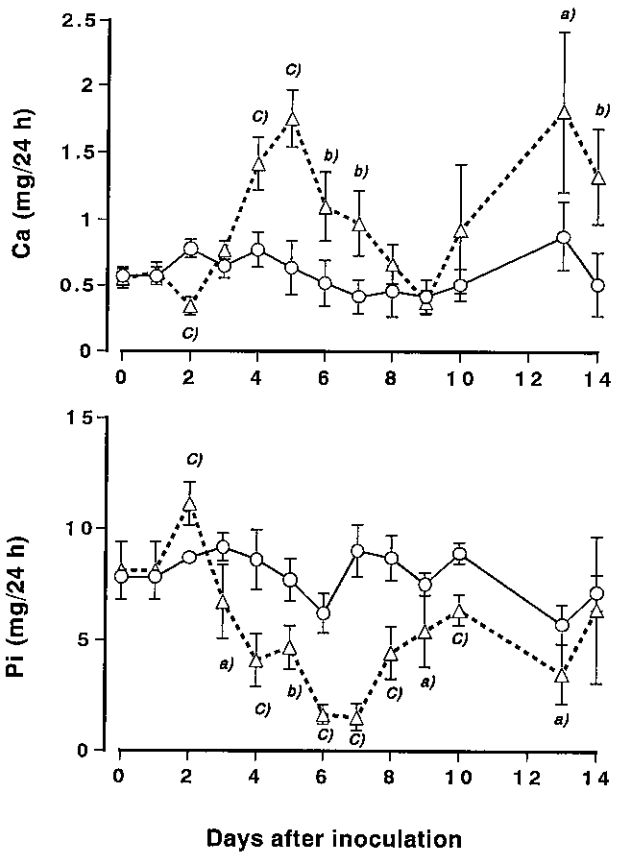

Fig. 3. Changes of urinary calcium (Ca) and inorganic phosphorus (Pi) in rats. Urine samples were collected for 24 h from age-matched healthy control rats (○) and W256/S-bearing rats (△). Data are the mean±SD of ten rats. *a*), *b*), *c*) Significantly different from the control at $P<0.05$, 0.01, and 0.001, respectively.

No tumor invasion or metastasis into the bone of W256/S-bearing rats was found by macroscopic and microscopic histopathological observations. Fig. 4 and Table II show the histopathology and the analytical data of femural bones 14 days after subcutaneous inoculation of W256/S carcinoma. A decrease in cancellous bone in the secondary trabecula was noted in the tumor-bearing rats (Fig. 4). The dry weight and the bone calcium and inorganic phosphorus contents in the right femur bones significantly decreased in W256-bearing rats, compared with the healthy controls (Table II). Moreover, the BMD of femur was significantly lower in the tumor-bearing rats than in non-tumor bearers (Fig. 5 and Table II). The decrease of BMD was evident in the midshaft to the distal end of the femur.

Fig. 6 shows the serum PTHrP level in W256/S-bearing rats. The serum PTHrP level in W256/S-bearing rats during the tumor growth was not different from that in the healthy control rats, but showed a tendency to increase during the experimental period. Fig. 7 shows PTHrP mRNA expression of W256/S tumor and LLC-W256 cells. W256/S tumor did not express detectable PTHrP mRNA, while LLC-W256 cells clearly showed PTHrP-mRNA expression. Although the female rats used in this study had an immature sex cycle during experiments (6 to 8 weeks old), the estradiol level in serum of the tumor-bearing rats was significantly decreased (Fig. 8), without any weight change of the ovaries (Table I), compared with the non-tumor bearers.

Table III shows the effects of salmon calcitonin and estradiol on the BMD of femurs of W256/S-bearing rats. Treatment with salmon calcitonin or estradiol significantly inhibited the decrease of the BMD induced by W256/S.

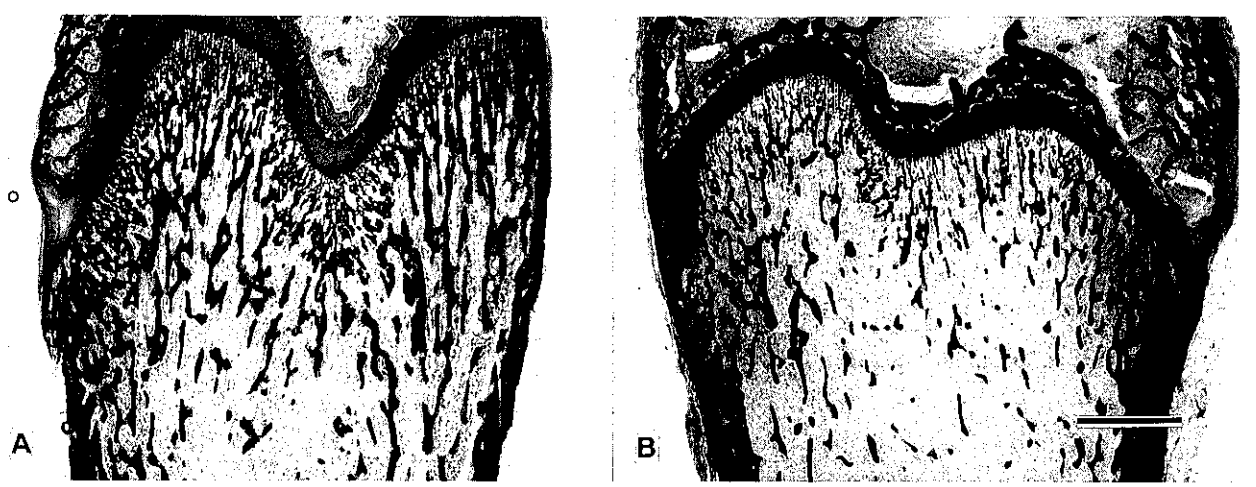

Fig. 4. Microscopy of unmineralized sections of the proximal metaphysis of the left femurs from age-matched healthy control (A) and W256/S-bearing rats 14 days after the tumor inoculation (B). A decrease in the amount of darkly stained cancellous bone spicules in W256/S-bearing rats was noted. Toluidine blue stain. Bar=1 mm.

Table II. Analytical Data of Right Femurs of Rats

	Length (mm)	Dry weight (mg)	Ca (mg/bone)	Pi (mg/bone)	BMD (mg/cm^2)
Healthy controls	29.3±0.2	281.2±2.6	52.5±0.7	28.3±8.2	72.3±1.3
W256/S bearers	28.7±0.5	247.6±9.0[a)]	45.7±1.6[b)]	25.5±0.8[a)]	63.2±1.2[c)]

Right femur bones were from age-matched healthy control rats and W256/S-bearing rats 14 days after the subcutaneous tumor inoculation. The bone length, dry weight, calcium (Ca), inorganic phosphorus (Pi), and total bone mineral density (BMD) were measured. Data are the mean±SD of ten rats.
a), *b*), *c*) Significantly different from the healthy control at $P<0.05$, 0.01, and 0.001, respectively.

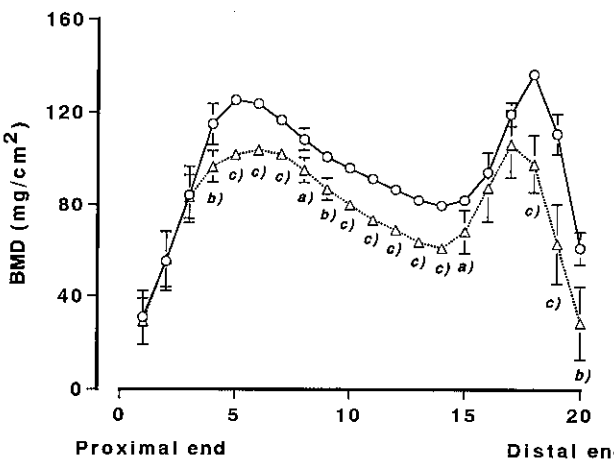

Fig. 5. Scanning profiles of the BMD of femurs. Femur bones were obtained from age-matched healthy control rats (○) and W256/S-bearing rats (△) 14 days after subcutaneous tumor inoculation. Data are the mean±SD of ten rats. *a*), *b*), *c*) Significantly different from the control at $P<0.05$, 0.01, and 0.001, respectively.

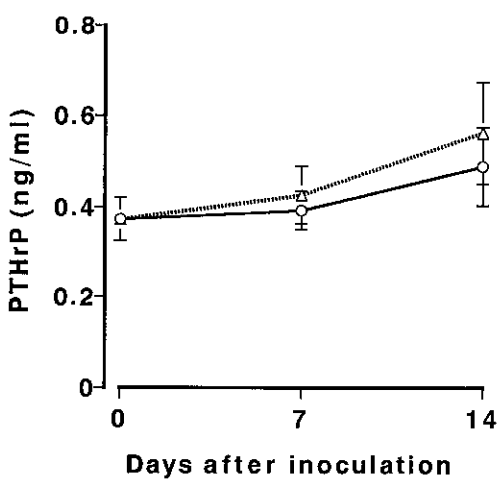

Fig. 6. Changes of serum PTHrP immunoreactive to anti-human PTHrP(1–34) antibody. Sera were collected from age-matched healthy control rats (○) and W256/S-bearing rats (△). Data are the mean±SD of ten rats.

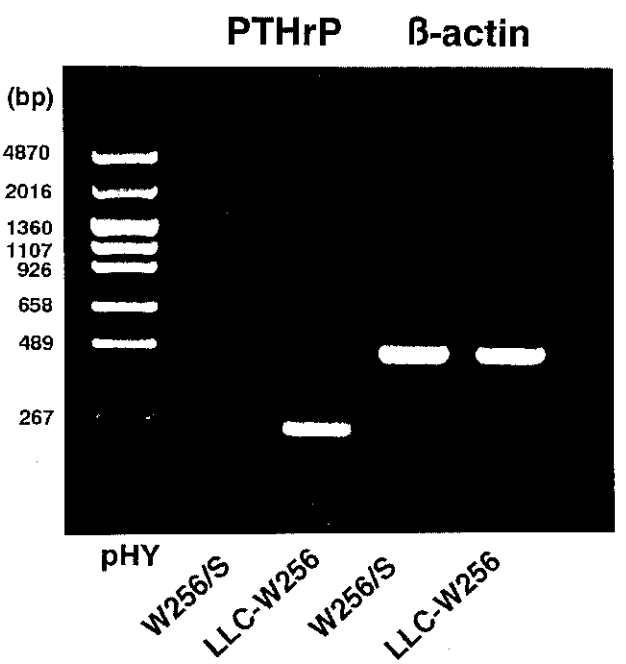

Fig. 7. Detection of PTHrP-mRNA by RT-PCR in W256/S tumor and LLC-W256 cells. Left lane: *Hae*III-digested pHY DNA molecular standards.

DISCUSSION

W256/S carcinoma used in this study showed quite different biological features from W256 lines previously reported by other investigators.[1–3, 7, 8] W256/S had no effect on serum concentrations of calcium and phosphorus (Fig. 2) and did not produce PTHrP (Fig. 7), but induced hypercalciuria and hypophosphaturia (Fig. 3). Despite these different features, W256/S induced typical osteoporosis-like bone changes; a decrease in the secondary trabecular bone in the metaphysis, loss of bone weight, calcium and phosphorus contents, and BMD, within a short period (14 days) after the tumor inoculation (Figs. 4 and 5, Table II). It has recently been reported that the humoral hypercalcemia of malignancy

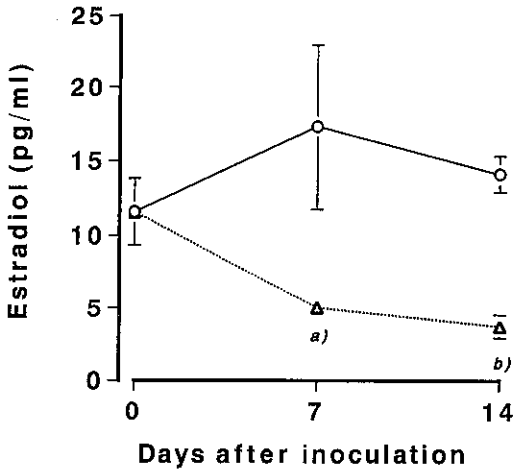

Fig. 8. Changes of serum estradiol in rats. Sera were from age-matched healthy control rats (○) and W256/S-bearing rats (△). Data are the mean±SD of ten rats. *a*), *b*) Significantly different from the control at $P<0.05$ and 0.001, respectively.

Table III. Effects of Salmon Calcitonin (sCT) and Estradiol on Femur BMD of W256/S-bearing Rats

Treatment		BMD (mg/cm^2)
Healthy controls		77.4±3.7[a)]
W256/S bearers		
Control		66.5±3.4
sCT	10 IU/kg	70.3±4.3[b)]
Estradiol	100 μg/kg	73.6±1.4[a)]

W256/S-bearing rats were treated with sCT at every second day or estradiol at every third day. Fourteen days after the tumor inoculation, the BMD of paired femur bones was measured using DEXA. Data are the mean±SD of ten rats. *a*), *b*) Significantly different from the W256/S-bearer control at $P<0.001$ and 0.05, respectively.

is closely associated with the secretion of PTHrP, resulting in osteolysis.[4-8] The systemic calcium metabolism is regulated by thyroid and parathyroid hormones, calcitonin and PTH, under physiological conditions. Although the influence of W256/S carcinoma on secretion of these hormones is not understood, the effect of the tumor on the calcium metabolism is at least not mediated through PTHrP produced by the tumor.

A key finding was the decrease in the serum estradiol level during growth of W256/S (Fig. 8). This is the first report to indicate a correlation between change in serum estrogen level and osteoporotic bone changes in Walker carcinoma-bearing rats. The weight loss of the uterus (Table I) may result from the decrease in estrogen secretion in the tumor-bearing rats. The W256/S line may release some antiovarial factor(s), causing bone resorption. It has been reported that estrogens inhibit bone resorption through stimulation of osteoblasts to produce transforming growth factor-β[11, 12] and suppression of release of osteolytic cytokines, interleukin-1, interleukin-6, and tumor necrosis factor-α.[13-16] Therefore, the decrease in serum estrogen concentration in W256-bearing rats could be a possible mechanism of osteoporotic bone changes, as in postmenopausal osteoporosis.[17, 18] Generally, relatively long periods (several months) are needed to elicit osteoporosis after ovariectomy or hypoovarianism in rats.[19] In this study, a decrease of bone mass was obvious by 14 days after tumor inoculation. Therefore, it may also be assumed that the W256/S carcinoma stimulates the immune system, resulting in overproduction of osteolytic cytokines, because the spleen was enlarged after the tumor inoculation (Table I). We also observed actual body weight loss and increase in liver weight in the tumor-bearing rats (Table I). These effects may result from changes in the hormonal and immune balance in the animals. Since serum ALP activity is a marker of bone-forming activity,[20] the early increase in serum ALP after W256/S inoculation (Fig. 2) may be a result of stimulation of osteoblasts. At the same time, it is thought that osteoclast activity increases with decrease in estrogen secretion. Such a high turnover of bone metabolism seems to induce bone loss after tumor inoculation. This hypothesis is supported by evidence that the osteoporosis-like changes were improved by treatment with estradiol or calcitonin (Table III).

In conclusion, W256/S carcinoma-bearing rats seem to be a useful model of osteoporosis caused by hypoovarianism or postmenopause, though studies on the mechanisms of osteolysis and of the decrease in estrogen secretion in W256/S-bearing rats are still needed.

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