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N^G-Nitro-L-arginine Methyl Ester Inhibits Bone Metastasis after Modified Intracardiac Injection of Human Breast Cancer Cells in a Nude Mouse Model

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We investigated the effects of N^G-nitro-L-arginine-methyl ester (L-NAME), a nitric oxide synthase (NOS) inhibitor, on bone metastasis of human breast cancer, MDA-231 cells. Tumor cells (2×10^5 cells in 0.2 ml of phosphate-buffered saline; PBS) were injected through the diaphragm into the left ventricle of the heart of laparotomized nude mice (male 5-week-old ICR-*nu/nu*). L-NAME (2 mg/mouse/injection in 0.1 ml of PBS) was given intraperitoneally to mice 6 h and 3 h before and immediately, 3 h, 6 h, 18 h and 21 h after the intracardiac injection of tumor cells. As a control, 0.1 ml of PBS was injected instead of L-NAME. The effect of N^G-nitro-D-arginine-methyl ester (D-NAME; 2 mg/mouse/injection), an inactive analogue of L-NAME, was also investigated to evaluate the specificity of L-NAME action. Radiographical examination 31 days after the tumor-cell injection showed that the incidence and number of osteolytic bone metastases and the number of bones with metastasis in L-NAME-treated mice were significantly reduced compared with those in PBS-treated mice ($P<0.05$). The differences between PBS-treated and D-NAME-treated mice were not significant. Our findings suggest that specific and appropriate NOS inhibitors may represent a new pharmacological approach to therapy for cancer patients at risk of developing osteolytic bone metastases.

Key words: Nitric oxide (NO) — N^G-Nitro-L-arginine methyl ester (L-NAME) — Bone metastasis — Human breast cancer — Nude-mouse model

Bone metastasis, substantially regarded as bone marrow metastasis, is a frequent clinical problem in patients with breast, prostate and other cancers. It causes considerable morbidity including bone pain, pathologic fractures, spinal cord compression and hypercalcemia. Relief from these symptoms and improvement in quality of life are major goals in the treatment of metastatic bone diseases.

Recent studies on the pathophysiology of bone metastasis have shown that the most important mechanism of bone destruction is the release from tumor cells of paracrine osteoclast-activating factors such as interleukin (IL)-1 and IL-6, parathyroid hormone-related protein and prostaglandins, which stimulate osteoclasts to resorb bone. Bone is itself a significant source of growth factors released during bone resorption, and these further stimulate tumor growth, forming a vicious circle in the bone microenvironment.[1,2] However, the pathophysiology of bone metastasis is still incompletely understood.

Nitric oxide (NO) has recently been shown to have manifold functions, including regulation of immune function by mediating tumoricidal and bactericidal functions in macrophages, vasodilation, and neurotransmission in the central and peripheral nervous systems.[3] NO is synthesized from L-arginine by NO synthase (NOS). NOS is competitively inhibited by N^G-monomethyl-L-arginine (L-NMMA) or by N^G-nitro-L-arginine-methyl ester (L-NAME) and other L-arginine derivatives substituted on the guanidino nitrogen atoms.

NO also plays important roles in tumor pathophysiology. Prolonged and excessive production of NO by tumor cells sustains the enhancement of blood flow and vascular permeability in tumor tissues, and thereby promotes tumor growth, angiogenesis and invasiveness.[4–7] From the viewpoint of tumor metastasis, it was reported that the production of NO by tumor cells was inversely correlated with their metastatic potential,[8,9] but that NO was involved in cytokine-induced enhancement of tumor-cell adhesion to endothelial cells.[10] Thus, the effects of NO or NOS inhibitors on tumor metastasis remain to be fully established, especially in relation to bone metastasis.

In order to understand the pathophysiology of bone metastasis, an appropriate animal model is indispensable.

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A murine model in which B16 melanoma cells were injected into the left ventricle, and modified models using nude mice and human cancer cells have been established.[11-14] In these models, tumor cells can form osteolytic bone metastases. Since intracardiac injection of tumor cells is performed blindly through the anterior chest wall in these models, accurate injection of tumor cells requires considerable skill. Accordingly, we have developed a modified technique in which tumor cells are injected through the thin diaphragm into the left ventricle of laparotomized nude mice. This method allows tumor cells to be introduced more easily and reproducibly.

In the present study, we investigated the effects of L-NAME, a non-metabolizable NOS inhibitor, on bone metastasis of human breast cancer cells using our nude-mouse model with a modified technique for intracardiac injection of the tumor cells.

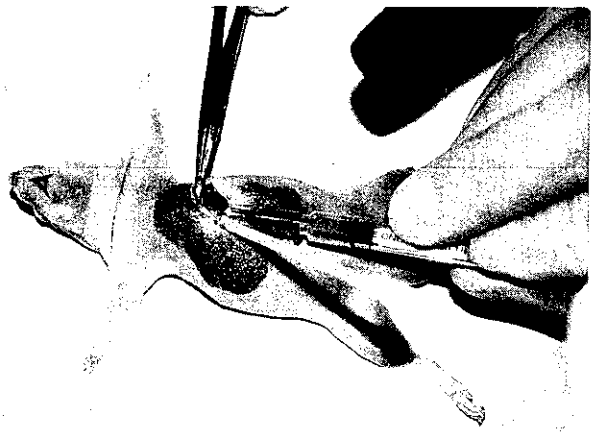

Fig. 1. A modified technique for intracardiac injection. The xiphoid process was pulled ventrally by an assistant and the liver was slightly pulled caudally with a swab. MDA-231 cells were injected using a 27-gauge needle into the left ventricle in the laparotomized nude mouse through the diaphragm.

MATERIALS AND METHODS

Animals and tumor cells Male 5-week-old athymic nude mice (ICR-*nu/nu*; Charles River Japan Inc., Yokohama) were used for experiments. Mice were maintained in a specific pathogen-free environment. Water and food were given *ad libitum*. A human breast cancer cell line, MDA-231, was found to be estrogen receptor-negative and was cultured in modified Eagle's minimum essential medium (Nissui, Tokyo) containing a 2-fold concentration of amino acids and vitamins, supplemented with 10% fetal bovine serum (CSL Ltd., Victoria, Australia) in a humidified atmosphere of 5% CO_2 in air at 37°C.[13] When cells were subconfluent, they were harvested by treatment with 0.25% trypsin and 0.02% EDTA. Cell counts and cell viability were determined by using a hemocytometer and the Trypan-blue exclusion test, respectively. The production of NO by MDA-231 cells was undetectable when measured with the Griess reagent (Nitrate/Nitrite assay kit; Cayman Chemical, Ann Arbor, MI). The *in vitro* proliferation of MDA-231 cells was not influenced by L-NAME (Wako Pure Chemical Industries, Osaka; 50 ng/ml–5 μg/ml, $IC_{50}=135$ ng/ml).

Intracardiac injection of tumor cells into nude mice Each nude mouse was anesthetized with pentobarbital (35 mg/kg) and placed on its back. The upper abdomen of the mouse was sterilized with iodine and alcohol swabs, then a small epigastric transverse incision was made and the xiphoid process was exposed. The xiphoid process was pulled ventrally by an assistant and the liver was slightly pulled caudally with a swab. Then the left ventricle, beating between the lungs, appeared semi-transparently through the thin diaphragm. MDA-231 cells (2×10^5 cells in 0.2 ml of phosphate-buffered saline; PBS) were injected precisely into the left cardiac ventricle using a 27-gauge needle (Fig. 1). Pulsatile back-flow from the left ventricle into the syringe was confirmed every time tumor-cell injection was completed. The abdominal incision was closed with 5–0 absorbable sutures in one layer.

Experimental schedule L-NAME (2 mg/mouse/injection in 0.1 ml of PBS) was injected intraperitoneally into mice 6 h and 3 h before and immediately, 3 h, 6 h, 18 h and 21 h after intracardiac injection of tumor cells so that NO production was reduced until about 24 h after tumor-cell injection. A similar dosage of L-NAME has pharmacological activity in mice[15] and is effective in decreasing the production of NO and the diameter of mesenteric blood vessels in mice (Takashi Yamamoto in our institute, unpublished data). As a control, 0.1 ml of PBS was injected instead of L-NAME. In an additional experiment, we investigated the effect of N^G-nitro-D-arginine-methyl ester (D-NAME; Wako Pure Chemical; 2 mg/mouse/injection), an inactive analogue of L-NAME, in order to evaluate the specificity of L-NAME action.

Bone metastases were determined on radiographs 31 days after inoculation of tumor cells. Osteolytic metastatic foci were defined as macroscopically demarcated radiolucent lesions in the bone on the original films (25.4 $\times$30.5 cm; X-OMAT TL; Kodak, Rochester, NY). The incidence and number of osteolytic metastases and the number of bones with osteolytic metastasis were evaluated. We considered mice with hindlimb paralysis to have bone metastasis in the vertebrae even if radiological examination failed to detect osteolytic lesions. All mice were killed and other organs were examined carefully for macroscopic metastases.

Statistical analysis The significance of differences in incidence was assessed with the chi-squared or Fisher's exact probability test. That of other differences was assessed with the Mann-Whitney or Kruskal-Wallis rank sum test, as appropriate.

RESULTS AND DISCUSSION

Intracardiac injection of tumor cells was straightforward using our modified technique, and there were no major complications, such as bleeding and pneumothorax.

The majority of nude mice given intracardiac injections with MDA-231 cells showed osteolytic metastases in limbs, vertebrae and scapulae 31 days after the tumor inoculation, as previously reported.[13] Some mice had hindlimb paralysis, probably due to spinal cord compression induced by metastasis to vertebrae. No macroscopic metastases to organs other than bones were observed at autopsy, except in one mouse as mentioned below, but micrometastases to adrenal glands were occasionally detected, as previously reported.[13] Moreover, some mice suffered cachexia with a marked loss of body weight approximately 3 weeks after the tumor injection.

The results of experimental bone metastases in PBS-, L-NAME- and D-NAME-treated nude mice are summarized in Table I. The incidence of metastasis in L-NAME-treated mice was significantly lower than that in PBS-treated mice ($P<0.05$ in each experiment). The number of bone metastases and the number of bones with metastasis in L-NAME-treated mice tended to decrease ($P=0.06$ and 0.07, respectively in Experiment 1) or were significantly reduced as compared with those in PBS-treated mice ($P<0.05$ in Experiment 2). The differences between PBS-treated and D-NAME-treated mice were not significant. Representative radiographs of PBS-treated and L-NAME-treated nude mice are shown (Fig. 2). While many osteolytic lesions were seen in PBS-treated mouse, no remarkable lesion was detected in L-NAME-treated mouse. Histological examination of osteolytic lesions confirmed the replacement of spongiosa and bone marrow by tumor cells, and the occurrence of osteoclast-mediated bone resorption[2, 12, 13] (Fig. 3).

While bone is one of the major metastatic targets of breast, prostate or other cancers, the frequency of its

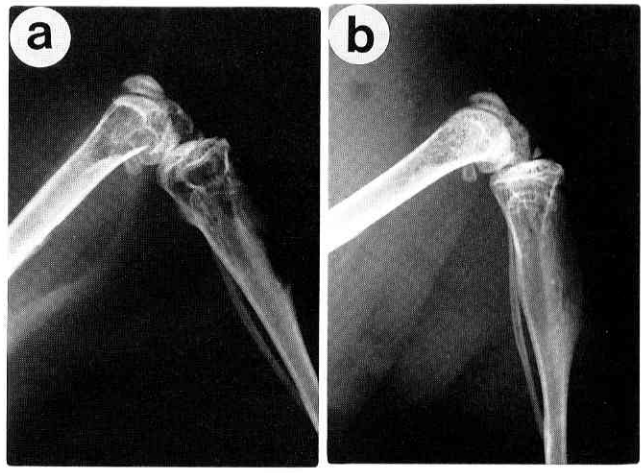

Fig. 2. Representative radiographs of osteolytic bone lesions in hindlimb. The radiographs were taken 31 days after tumor injection. a, PBS-treated mouse. Distinct osteolytic lesions are present in the distal femur and proximal tibiae. b, L-NAME-treated mouse. Osteolytic lesions are invisible.

Table I. Inhibitory Effects of L-NAME on Bone Metastasis of MDA-231 Cells

Treatment (No. of mice)	Incidence of bone metastasis (%)	No. of bone metastases Median (range)	No. of bones with metastasis Median (range)
Experiment 1			
PBS	10/13 (76.9)	0,0,0,1,1,1,1,1,2,2,3,7,13	0,0,0,1,1,1,1,1,1,2,3,5,7
($n=13$)		1 (0–13)	1 (0–7)
L-NAME	3/11 (27.3)[a]	0,0,0,0,0,0,0,0,2,2,4	0,0,0,0,0,0,0,0,2,2,4
($n=11$)		0 (0–4)[b]	0 (0–4)[c]
Experiment 2			
PBS	10/11 (90.9)	0,1,2,4,5,6,7,7,8,10,11	0,1,2,4,5,5,6,6,7,8,8
($n=11$)		6 (0–11)	5 (0–8)
D-NAME	12/13 (92.3)	0,1,1,2,2,2,2,5,6,8,8,9,11	0,1,1,2,2,2,2,4,4,6,7,7,7
($n=13$)		2 (0–11)	2 (0–7)
L-NAME	3/8 (37.5)[a]	0,0,0,0,0,1,5,6	0,0,0,0,0,1,5,6
($n=8$)		0 (0–6)[a]	0 (0–6)[a]

a) Significantly different from PBS-treated group at $P<0.05$.
b) $P=0.06$ vs. PBS-treated group.
c) $P=0.07$ vs. PBS-treated group.

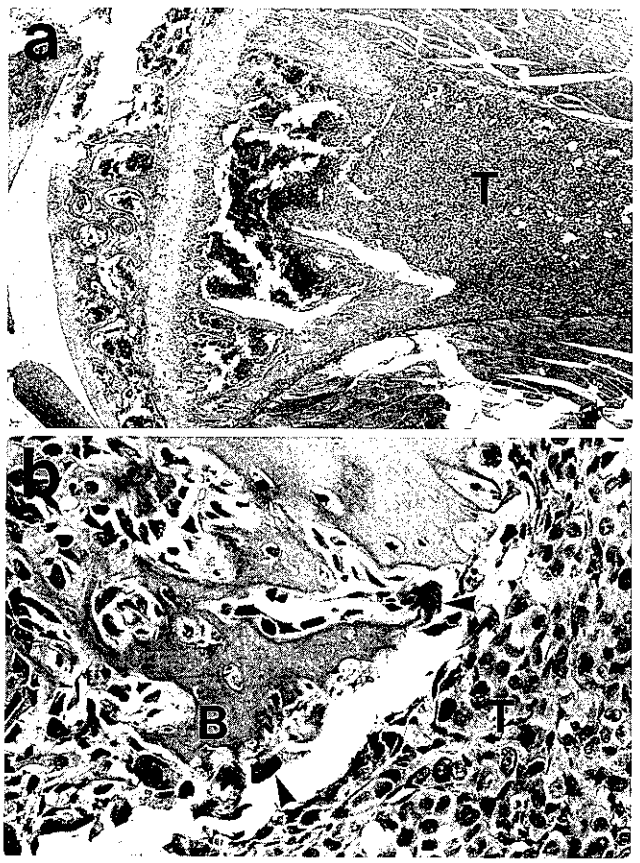

Fig. 3. Histological view of the proximal tibiae of PBS-treated nude mouse bearing MDA-231 cells. a, The majority of spongiosa and the marrow cavity are replaced by metastatic tumor (T). Tumor cells are extending into the metaphysis. HE staining, ×34. b, Osteoclastic bone resorption along the endosteal surface. Osteoclasts (arrowheads) are present between metastatic MDA-231 cells (T) and bone (B). HE staining, ×240.

involvement would not be predicted on the basis of its modest blood supply, estimated at 5–10% of cardiac output.[16, 17] Hence, L-NAME-induced inhibition of bone metastasis cannot necessarily be attributed to reduction of blood flow to the bone. The direct introduction of tumor cells into the systemic arterial circulation simulates hematogenous bone metastasis, a process consisting of arrest, extravasation and proliferation of tumor cells in the bone. Arrest and extravasation in the highly specialized and morphologically modified microvasculature of bone are likely to be very different from those of other organs. The arterioles become sinuses without an intervening capillary. Since bone marrow sinuses are lined by endothelial cells lacking a basement membrane, blood cells, or possibly tumor cells can easily complete transendothelial migration in the sinuses.[1, 18]

Apart from this, NO was reported to exert a powerful inhibitory effect on the bone-resorbing activity of osteoclasts.[19] In the present study, however, L-NAME was administered within 24 h after tumor-cell injection, so our findings probably reflected NO effects on the processes of arrest and extravasation by tumor cells in the bone microenvironment, before the development of osteoclast-mediated osteolytic metastasis.[20] Besides, L-NAME could not inhibit either the proliferation *in vitro* of MDA-231 cells, which themselves have no ability to produce NO *in vitro*, or the progression of established bone metastasis as judged from X-ray films in a preliminary experiment (data not shown).

Thus, L-NAME-induced reduction of bone metastasis appears to be at least partially due to inhibition of arrest and transendothelial migration (extravasation) by tumor cells.

As mentioned above, only one L-NAME-treated mouse had macroscopic liver metastasis without bone metastasis. The effects of L-NMMA on regional blood flows to numerous tissues, except bone, were previously examined.[21] The NOS inhibitor reduced blood flows to various organs, whereas only blood flow to the liver increased. Based on our limited data, liver metastasis in the L-NAME-treated mouse in the present study might be a result of enhanced blood flow to the liver.

As to other systemic influences of L-NAME, L-NAME-treated nude mice showed a transient loss in body weight 24 h after the tumor-cell injection, but this recovered 48 h after the tumor inoculation (data not shown). We considered that this was chiefly due to L-NAME-induced anorexia, as previously reported,[22] although we did not measure food intake.

To confirm the involvement of NO in bone metastasis, we should examine further, for example, the ability of L-arginine to reverse the L-NAME-induced effects on bone metastasis. However, the ability of L-arginine to reverse the vasoconstriction induced by an NOS inhibitor varies between vascular beds for unknown reasons.[23] Indeed, a synergistic effect of L-NAME and L-arginine on tumor-cell retention in the lungs in a murine experimental metastasis model was reported.[24] This is the reason why we did not examine the effect of L-arginine, but rather that of D-NAME on bone metastasis as a negative control in this study. Even though we still need to investigate the effects of other NOS inhibitors or their dose-dependency, our results show clear inhibitory effects of L-NAME on bone-metastasis development, probably through the reduction of endogenous NO or possibly through other unknown mechanism(s).

Future therapeutic opportunities for treatment of bone metastasis should be based on an understanding of tu-

mor-host interaction in the bone microenvironment. For example, bisphosphonates, which are taken up preferentially by bone and inhibit osteoclast-mediated bone resorption, have beneficial effects on skeletal complications including bone pain, pathologic fractures and hypercalcemia.[25] These effects, however, remain unsatisfactory, and additionally, the potential for impairing mineralization of newly forming bone was reported. Thus, a new strategy for treatment of bone metastasis is required.

Our findings suggest that specific and appropriate NOS inhibitors without unfavorable systemic influence may represent a new pharmacological approach to therapy for cancer patients at risk of developing osteolytic bone metastases.

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