Inhibitory Effect of L-Homoarginine on Murine Osteosarcoma Cell Proliferation

Yoshihiro Kikuchi, Minoru Takagi, Richard T. Parmley, Vithal K. Ghanta, and Raymond N. Hiramoto


ABSTRACT

An organ-specific alkaline phosphatase inhibitor, L-homoarginine, at 44.5 mM concentration inhibited [³H]thymidine uptake by C3H/He mouse osteosarcoma (OS) cells, while L-arginine, L-phenylalanine, and glycine had little effect on the uptake. This inhibitory effect by L-homoarginine persisted even after the cells were washed free of the amino acid with fresh media. L-Homoarginine did not affect [³H]thymidine uptake by mouse myeloma MOPC 104E cells. In long-term culture, 22.3 mM L-homoarginine inhibited proliferation of OS cells. L-Arginine at the same concentration inhibited the proliferation to a lesser extent. On the other hand, L-phenylalanine and glycine did not affect in vitro proliferation of OS cells. When the same number of viable OS cells was inoculated s.c. after culturing for 24 hr with 44.5 mM L-homoarginine or L-arginine, the tumor growth in mice given injections of L-homoarginine (but not L-arginine)-treated cells was delayed markedly. Electron microscopic studies indicated that the inhibiting effect on OS cell proliferation was associated with a marked increase in lysosomal granules and a decrease in virus-like structures. Similarly, biochemical assay for acid phosphatase of cell homogenates demonstrated a 2-fold increase in activity in L-homoarginine-treated cells when compared to controls and L-arginine-treated cells. Thus, L-homoarginine inhibits proliferation and alkaline phosphatase activity of mouse OS cells and appears to increase acid phosphatase activity and synthesis of lysosomal granules.

INTRODUCTION

It has been generally recognized that the activity of ALP³ is associated with various cell membranes (6, 10, 14). ALP plays an important role in the transport of various molecules across membranes (13, 15, 20, 22). Cytosochemical studies indicate that the ALP is localized in the plasma membranes of normal and malignant cells (17, 26-28). In addition, organ-specific ALP can be inhibited preferentially: L-phenylalanine and L-tryptophan inhibit intestinal and placental ALP; and L-homoarginine inhibits bone and liver ALP (7). ALP extracted from OS cells is inhibited by L-homoarginine but not by L-phenylalanine (24), similar to ALP from normal bone tissue (7). Although the mechanism of inhibition of ALP is not understood in detail, it appears to involve uncompetitive interaction of inhibitors with the enzyme (16).

Previous studies have suggested that cell surface properties, including ALP activity, are associated with the control of cell proliferation (21). OS cells used in the present study originate from osseous tissue and produce ALP (9). Since bone ALP inhibitors can affect ALP of OS cells (24), this study was undertaken to determine if ALP inhibitors also affect cell proliferation in vitro or in vivo. In this study, we investigated the effect of L-homoarginine on ALP and on the proliferation of OS cells.

MATERIALS AND METHODS

Mice. Six-week-old female C3H/HeN mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Six-week-old female BALB/c mice were obtained from Laboratory Supply Co., Indianapolis, Ind. They were maintained up to the desired age in our laboratory on standard Lab-Blox feed (Wayne Feed Co., Chicago, Ill.) and water ad libitum.

Preparation of Single-Cell Suspension of Tumor. Single-cell preparation of osteosarcoma was prepared according to the reported procedure (11). Briefly, the tumor was excised, cut into small pieces, and dispersed with 0.1% Pronase in Medium 199 (Grand Island Biological Co., Grand Island, N. Y.) at room temperature for 20 min (11). The suspension was allowed to settle, the supernatant was centrifuged at 1000 rpm for 10 min, and the pellet was washed twice with 10% FCS in Medium 199. The cell pellet was suspended in 10% FCS, and the viability was measured by the trypan blue exclusion test.

MOPC 104E Cells. MOPC 104E cells were grown in ascitic form by inoculating BALB/c mice with 1 x 10⁶ cells i.p. Cells were prepared from a 9-day-old ascitic tumor. After washing with Roswell Park Memorial Institute Tissue Culture Medium 1640, cells were suspended in the same medium.

Amino Acids. L-homoarginine, L-arginine, and glycine were obtained from Sigma Chemical Co., St. Louis, Mo. L-Phenylalanine was purchased from Mann Research Laboratories, Inc., New York, N. Y. Each reagent was diluted to the desired concentration with 0.15 M NaCl, and the pH was adjusted to 7.0 with HCl or NaOH. The stock solutions were sterilized with 0.20-μm Millipore filters before use.

Cell Culture. OS and MOPC 104E cells were maintained and propagated in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% FCS, 2 mM glutamine, penicillin, and streptomycin (100 units/ml and 100 μg/ml, respectively; Grand Island Biological Co.) in a 5% CO₂ atmosphere at 37°. For long-term cultures, the medium was changed every 3 days, and when the cells reached confluency, the culture was split by dispersion with 0.25% trypsin.

Tritiated Thymidine Uptake by Tumor Cells. One-half million cells in 0.2 ml medium were incubated with 0.1 μCi tritiated thymidine (specific activity, 73.5 Ci/mM; ICN Pharmaceuticals, Inc., Irvine, Calif.) with or without addition of amino acids in a humidified atmosphere of 5% CO₂ at 37° for 24 and 48 hr from the start of culture. The cultures were harvested on glass-fiber filters with a semiautomated multiple-sample harvester. The samples were dried and transferred to 2.0 ml of counting cocktail, and the incorporation of [³H]thymidine was determined by a liquid scintillation counter.

Assay for ALP in Medium and in Plasma of Tumor-bearing Mice. Ten μl of each supernatant were added to 1.0 ml of p-nitrophenylphos-
phate disodium salt (1.0 mg/ml) in substrate buffer (0.05 M Na₂CO₃-0.001 M MgCl₂; pH was adjusted to 9.8). After the reaction mixture was incubated for 30 min at 37°C, 0.1 ml of n NaOH was added to stop the reaction (11). The samples were read at 400 nm in a Zeiss spectrophotometer. For inhibition studies of ALP, various concentrations of amino acids were added to the reaction mixture prior to incubation. Procedure for the analysis of total plasma ALP per mouse

The samples were read at 400 nm in a Zeiss spectrophotometer. For inhibition studies of ALP, various concentrations of amino acids were added to the reaction mixture prior to incubation. Procedure for the analysis of total plasma ALP per mouse has been reported (2,11).

Electron Microscopy. Cells grown in the presence of 22.3 mM L-homoarginine or L-arginine for 21 days were dispersed with 0.25% trypsin and washed twice with fresh medium. Untreated control cells were also collected as described above. The cells were then centrifuged at 1500 x g for 3 min, and the resulting cell pellet was resuspended in phosphate-buffered saline (0.15 M NaCl, 0.15 M Na₂HPO₄), pH 7.35. The cells were then again centrifuged, resuspended in 3% glutaraldehyde-0.1 M cacodylate buffer, pH 7.35, and allowed to fix for 90 min at 4°C. After rinsing the cells in 0.1 M cacodylate-7% sucrose buffer, pH 7.35, the cells were postfixed in 1% OsO₄ in 0.1 M cacodylate, pH 7.35, for 60 min. The cells were then routinely dehydrated in graded alcohols and propylene oxide and embedded in Spurr low-viscosity medium. Thin sections (60 nm thick) were counterstained with uranyl acetate and lead citrate and viewed with a Philips 300 transmission electron microscope at an accelerating voltage of 60 kV.

Determination of ACP and ALP in OS Cell Homogenates. The OS cell suspension was centrifuged at 1000 x g for 15 min. The sediment was resuspended in a 5% Triton X-100 at a final cell number of 10 x 10⁶ cells/ml, followed by 6 cycles of alternating freeze-thaw treatment in acetone-dry ice. The suspension was then centrifuged at 1000 x g for 15 min at 4°C. The supernatant was used for enzyme assay. ACP activity was measured as described by Schlosnagle et al. (23) at pH 4.9 and 30°C, using 12 mM p-nitrophenolphosphate as a substrate. One unit of activity is defined as the ability to hydrolyze 1 μmol of substrate per min at 30°C. ALP activity was measured at pH 9.8 as described previously. Protein was determined by the method of Lowry et al. (18).

Tumor Neutralization Test with L-Homoarginine. After 10 million cells were incubated with 4 ml of medium containing 44.5 mM L-homoarginine or L-arginine for 24 hr, the cells were washed 3 times with fresh medium. The cell number was counted, and 1 million viable cells were inoculated s.c. into the right flank of C3H/HeN mice. Untreated cells were also washed after 24 hr of culture and used as controls.

Statistical Analysis. Results are expressed as mean ± S.D. The statistical significance of differences in mean values was determined by Student's t test. Differences were considered to be significant if the probability of the observed differences occurring by chance alone was less than 5% (i.e., p < 0.05).

RESULTS

Effect of Various Concentrations of Amino Acids on OS ALP. In order to categorize ALP released from OS cells, we attempted to determine the inhibition pattern of 4 amino acids. The amino acids used were: L-homoarginine, the bone- and liver-specific ALP inhibitor; L-phenylalanine, the placental- and intestinal-specific ALP inhibitor; and L-arginine and glycine, non-ALP inhibitors (7). Each amino acid was added to the substrate mixture prior to incubation. L-Homoarginine at 11.2 mM concentration inhibited OS ALP markedly, while inhibition at 11.2 mM L-arginine and glycine was less than 20% (Table 1). Although inhibition by 11.2 mM L-phenylalanine was 42.4%, 44.5 mM L-arginine had more inhibitory effect than did 44.5 mM L-phenylalanine. L-Homoarginine showed the highest inhibitory effect at each concentration used.

Effect of L-Homoarginine on [³H]Thymidine Uptake by OS Cells. Addition of 11.2 or 22.3 mM L-homoarginine to OS cultures for 24 and 48 hr did not inhibit [³H]thymidine uptake by OS cells (Table 2). L-Homoarginine at a concentration of 44.5 mM showed [³H]thymidine incorporation of 9% of untreated controls after 24 hr and of 17% after 48 hr of incubation, respectively. Although 44.5 mM L-arginine inhibited the uptake after 48 hr of incubation (but not significantly), the other amino acids did not affect the uptake. L-Homoarginine at 44.5 mM did not affect the cell viability after incubation for 24 hr, indicating that the inhibition is not due to a decrease in cell viability. However, incubation for 48 hr with 44.5 mM L-homoarginine caused a marked decrease of the cell viability. To elucidate in detail the inhibiting effect of L-homoarginine on DNA synthesis, OS cells were cultured for 24 hr with 44.5 mM L-homoarginine. The effect of L-homoarginine on [³H]thymidine incorporation is irreversible following the removal of the amino acid. To show that the inhibition by L-homoarginine is not due to nonspecific cellular toxicity, we examined the effect on [³H] thymidine uptake by MOPC 104E cells. L-Homoarginine, L-arginine, and glycine at the 3 concentrations had no effect on [³H]thymidine uptake by MOPC 104E myeloma cells.

Effect of L-Homoarginine on In Vitro Culture of OS Cells. The results of [³H]thymidine uptake studies led us to determine the effect of L-homoarginine and L-arginine on long-term culture of OS cells. The effect of L-homoarginine on the tumor cell growth is shown in Chart 1. In untreated and 11.2 mM L-arginine-treated cells, the tumor cells entered into the growth phase 4 days after culture and reached the stationary phase by 14 days. The doubling times were 48 and 51 hr, respectively. In 11.2 mM L-homoarginine-treated culture, the tumor cell growth was delayed, and the doubling time was 88 hr. The cell growth curve of 22.3 mM L-arginine-treated culture was similar to that of 11.2 mM L-homoarginine. On the other hand, when the tumor cells were cultured in the presence of 22.3 mM L-homoarginine, the cell growth was delayed markedly (Chart 1). In both 44.5 mM L-homoarginine- and L-arginine-treated cultures, the tumor cell number decreased rapidly after 4 days of culture, and the cells could not be maintained, while L-phenylalanine and glycine at 44.5 mM did not affect the cell growth (data not shown).

Electron Microscopy. In specimens of control (untreated or
Effect of amino acids on (3H)thymidine uptake and viability of OS cells

One-half million cells were cultured with various concentrations of amino acids and 0.1 μCi of (3H)-thymidine in microtiter plates for 24 and 48 hr.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>25 hr</th>
<th>Viability (%)</th>
<th>48 hr</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>34,882 ± 4,422</td>
<td>89.7</td>
<td>29,602 ± 5,119</td>
<td>79.7</td>
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<tr>
<td>11.2 mM L-homoarginine</td>
<td>32,878 ± 5,609</td>
<td>97.1</td>
<td>28,810 ± 1,993</td>
<td>69.2</td>
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<tr>
<td>22.3 mM L-homoarginine</td>
<td>32,133 ± 4,235</td>
<td>81.8</td>
<td>33,987 ± 2,801</td>
<td>55.6</td>
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<td>44.5 mM L-homoarginine</td>
<td>3,112 ± 516</td>
<td>87.8</td>
<td>4,956 ± 617</td>
<td>25.0</td>
</tr>
<tr>
<td>11.2 mM L-arginine</td>
<td>35,744 ± 5,095</td>
<td>87.0</td>
<td>33,744 ± 1,698</td>
<td>89.3</td>
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<tr>
<td>22.3 mM L-arginine</td>
<td>36,082 ± 2,158</td>
<td>88.7</td>
<td>37,345 ± 1,476</td>
<td>70.8</td>
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<tr>
<td>44.5 mM L-arginine</td>
<td>34,438 ± 2,537</td>
<td>81.8</td>
<td>21,938 ± 4,225</td>
<td>42.9</td>
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<tr>
<td>1.2 mM L-phenylalanine</td>
<td>35,770 ± 3,077</td>
<td>78.9</td>
<td>46,130 ± 7,441</td>
<td>80.0</td>
</tr>
<tr>
<td>22.3 mM L-phenylalanine</td>
<td>29,889 ± 2,627</td>
<td>80.0</td>
<td>35,510 ± 2,362</td>
<td>88.2</td>
</tr>
<tr>
<td>44.5 mM L-phenylalanine</td>
<td>27,308 ± 4,116</td>
<td>88.9</td>
<td>30,337 ± 2,850</td>
<td>72.7</td>
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<tr>
<td>11.2 mM Glycine</td>
<td>35,419 ± 1,718</td>
<td>84.4</td>
<td>33,299 ± 5,012</td>
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<tr>
<td>22.3 mM Glycine</td>
<td>29,697 ± 1,574</td>
<td>90.5</td>
<td>37,316 ± 3,287</td>
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<tr>
<td>44.5 mM Glycine</td>
<td>31,529 ± 1,706</td>
<td>88.9</td>
<td>39,485 ± 7,098</td>
<td>72.7</td>
</tr>
</tbody>
</table>

Mean ± S.D. of triplicate cultures.

Biochemical Changes of OS Cells Treated with Amino Acids. To study the effect of L-homoarginine on lysosomal enzyme and ALP synthesis in OS cells, we determined ACP and ALP in the cell homogenates after 21 days of exposure to 22.3 mM L-homoarginine. ALP activity in cells treated with L-homoarginine and L-arginine was significantly lower than in control cells. On the other hand, ACP increased markedly (p < 0.01), about 2-fold, in L-homoarginine-treated cells when compared to that in untreated and L-arginine-treated cells (Table 3).

Effect of Pretreatment of OS Cells with L-Homoarginine or L-Arginine In Vitro on the Tumor Growth In Vivo. Pretreatment with L-homoarginine resulted in a marked delay of tumor growth as measured by total circulating ALP levels (Chart 2). The relationship between the circulating ALP and number of tumor cells in vivo has been established previously (11). Using the reported mathematical relationship, doubling time of the tumor was calculated. The doubling time was 12 hr for the untreated control group, 14 hr for the L-arginine-treated group, and 26 hr for the L-homoarginine-treated group. Furthermore, we examined the effect of pretreatment with L-homoarginine on the survival time. Median survival time of untreated and L-arginine-treated groups was 52 and 55 days, respectively (Chart 3). On the other hand, 3 of 5 mice in the L-homoarginine-treated group were still alive after 126 days. Although 2 had an initially palpable tumor, the tumor disappeared 56 days postinoculation. Another mouse still has a palpable tumor, and it appears that the tumor growth is arrested, based on the value of ALP, which is between 200 and 300 units/mouse as compared to the normal ALP values of non-tumor-bearing mice of 50 units/mouse (9, 11).

DISCUSSION

Previous studies have demonstrated that OS cells produce bone-specific ALP and that this enzyme is inhibited by L-homoarginine (9, 11, 16, 24). The present study has extended these observations by demonstrating that L-homoarginine in-
Effect of L-Homoarginine on Tumor Growth

Table 3
Effect of L-homoarginine and L-arginine on ALP and ACP activity in OS cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Untreated</th>
<th>L-Homoarginine</th>
<th>L-Arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>816.1 ± 65.2</td>
<td>589.2 ± 104.5</td>
<td>593.2 ± 132.5</td>
</tr>
<tr>
<td>ACP</td>
<td>5.7 ± 0.5</td>
<td>10.6 ± 2.4</td>
<td>5.4 ± 1.0</td>
</tr>
</tbody>
</table>

* Mean ± S.D. from 3 separate experiments.

Chart 2. The effect of pretreatment of tumor cells with L-homoarginine or L-arginine on the tumor growth as determined by circulating ALP in vivo. After tumor cells were cultured with 44.5 mm L-homoarginine or L-arginine for 24 hr, the cells were washed 3 times with fresh medium, and 1 million viable cells were inoculated s.c. After tumor implantation, the animals were bled from the tail vein every week, and plasma ALP per mouse was determined as described in "Materials and Methods." Each experimental group consisted of 5 mice. Untreated (x), L-homoarginine (c), and L-arginine (o). Dotted line shows normal level of ALP per mouse.

Chart 3. Survival profile of mice given injections of OS cells as described below. Tumor cells were cultured with 44.5 mm L-homoarginine or L-arginine for 24 hr and washed 3 times with fresh medium before tumor inoculation, and 1 million viable cells were inoculated into the right flank s.c. Each experimental group consisted of 5 mice. Untreated (x), L-homoarginine (c), and L-arginine (o).

The increased ACP activity in L-homoarginine-treated cells could result from increased ALP synthesis or activation of a previously synthesized enzyme. The ultrastructural demonstration of a marked increase in lysosomal granules and Golgi elements in L-homoarginine-treated cells strongly suggests that increased synthesis of ACP is responsible for the increased ACP reactivity observed biochemically.

The OS cells reported in this study appear capable of producing osteoid and contain virus-like particles similar to those initially reported for Dunn osteosarcoma (8, 25). Osteoclast is the only bone cell which produced a significant number of lysosomes (3, 12). Previous histological studies of Dunn osteosarcoma have demonstrated tumor cells which appear to derive from osteoblasts, osteoclasts, and fibroblasts (5) similar to those observed in humans (4). The appearance of numerous cells resembling osteoclasts after L-homoarginine treatment could result from increased differentiation of pluripotential tumor cells to osteoclasts or from selective inhibition of osteoblastic cells resulting in a relative increase in osteoclastic cells. Regardless of the mechanism, the end result appears to be an increase in cells with apparent osteoclastic nature.

The decrease in virus-like particles in L-homoarginine-treated cells corresponds to an increase in lysosomal and phagocytic activity in these cells. Phagocytic cells have been demonstrated previously to be beneficial in host defenses against viral invasion (1, 19). The increase in lysosome activity and phagocytosis of viral particles observed in L-homoarginine-treated cells may then reflect an increase in the ability of the cells to destroy the virus.

In conclusion, L-homoarginine treatment of OS cells inhibits ALP activity, cellular proliferation, and production of virus-like particles and stimulates lysosomal activity, phagocytosis, and ACP activity. It is not clear whether L-homoarginine directly initiates all of these events or if they occur as complex secondary phenomena following ALP inhibition. The latter hypothesis would seem more likely, but it requires further investigation.

REFERENCES
7. Fishman, W. H., and Sie, H-G. Organ specific inhibition of human alkaline phosphatase isoenzymes of liver, bone, intestine, and placenta; L-phenylal-
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Fig. 1. This control cell (not treated with l-arginine) contains abundant virus-like particles (V), mitochondria (M), and cytoplasmic vacuoles (CV). Nucleus (N). Bar = 0.5 μm. Uranyl acetate and lead citrate, × 10,000.

Fig. 2. In untreated control cells, virus-like particles can be seen in hyaloplasm and cisternae of rough endoplasmic reticulum (ER). Lysosome (L). Bar = 0.5 μm. Uranyl acetate and lead citrate, × 22,500.

Fig. 3. In A, in this l-homoarginine-treated cell, virus-like particles (V) are decreased in hyaloplasm and cisternae of rough endoplasmic reticulum (ER). Heterophagic granules or secondary lysosomes (L) containing virus-like particles and tubulovesicular structures (TVS) are increased significantly. Golgi apparatus (G), mitochondria (M). Bar = 0.5 μm. Uranyl acetate and lead citrate, × 15,600. B, secondary lysosomes. × 31,300.
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