A Synthetic Antagonist to Laminin Inhibits the Formation of Osteolytic Metastases by Human Melanoma Cells in Nude Mice

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Abstract

The mechanisms by which tumor cells metastasize to bone are not well understood. We have investigated the role of the basement membrane glycoprotein, laminin, in bone metastasis, since antagonists to laminin have been shown to inhibit the formation of lung metastases. We studied the formation of osteolytic metastases caused by a human tumor which is known to cause osteolysis and hypercalcemia in nude mice. We found that tumor-bearing nude mice developed hypercalcemia, cachexia, and characteristic osteolytic lesions throughout the skeleton after injection of this human melanoma cell line (A375) into the left ventricle. When we gave injections to nude mice with A375 cells which had been exposed to C(YIGSR)3-NH2, a laminin-derived synthetic peptide containing three linear sequences of YIGSR with an amino-terminal cysteine which competes with laminin for its receptor, we found a decrease in the formation of detectable osteolytic bone metastases. The tumor cells were incubated with the antagonist and then inoculated into nude mice which were administered the antagonist i.p. Hypercalcemia and cachexia were also decreased in tumor-bearing mice treated with the laminin antagonist. In contrast, laminin itself increased the number of osteolytic bone metastases, as has been shown for other tumor cells. These data suggest that laminin plays a role in the formation of osteolytic bone metastases in this model and that laminin antagonists may be useful in the prevention of bone metastases in some human tumors.

Introduction

Although bone metastases are an important clinical problem in many patients with solid tumors, the mechanisms whereby tumors metastasize to bone remain poorly understood. In part, this has been due to the lack of adequate animal models for bone metastases. Recently, Arguello et al. (1) described an experimental model of bone metastases in mice which had received intracardiac injections of B-16 murine melanoma cells. These mice developed destructive osteolytic bone lesions whose distribution was similar to that found in human patients with metastatic cancer in bone. Since this approach provides a method to study some of the mechanisms by which human tumor cells metastasize to bone, we have used this model to examine mechanisms involved in bone metastases formed by a human tumor in nude mice.

The attachment of tumor cells to basement membranes is an important step in the process of tumor cell metastasis (2). The glycoprotein, laminin, serves as a bridge to assist in the attachment of tumor cells to type IV collagen in basement membranes (3, 4) and laminin receptors are increased 50-fold in some tumor cells (5). Synthetic antagonists to laminin which compete with laminin for binding to laminin receptors on tumor cells inhibit the formation of pulmonary metastases in some tumor models. One such laminin-derived peptide, YIGSR, blocked lung colonization by melanoma cells, subcutaneous growth of small cell lung carcinoma cells, and angiogenesis. This peptide is active when injected with the tumor cells and also when injected daily after i.v. injection of the tumor cells (6–11).

In this study, we have examined the role of laminin in the metastasis to bone of the human melanoma cell line, A375, following injection of the cells into the left ventricle of nude mice. These tumor cells formed characteristic lytic lesions in the skeleton which were detected radiographically. We used previously described methods to incubate tumor cells with a synthetic antagonist to laminin. When nude mice given injections of these cells were administered the synthetic laminin antagonist i.p., the formation of the detectable osteolytic bone metastases was markedly inhibited. These results show that treatment with laminin antagonists may be useful for the prevention of osteolytic bone metastases in human tumors.

Materials and Methods

A375 Cells. A375 is a human amelanotic melanoma cell line derived from a metastatic lesion in the lung. This tumor forms pulmonary metastases at a high frequency when injected s.c. or i.v. into athymic nude mice. We have previously described the effects of this tumor on bone and calcium homeostasis in tumor-bearing nude mice following s.c. injection (12). It is unknown whether the patient with this tumor had bone metastases.

The cells were cultured in Dulbecco’s modified minimal essential medium (Hazleton Biologics, Inc., Lenexa, KS) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT) and 1% penicillin-streptomycin solution (GIBCO Laboratory, Grand Island, NY) in a humidified atmosphere of 5% CO2 in air.

Intracardiac Injection of Cells with Laminin and Antilaminin Peptide into Nude Mice. Laminin, C(YIGSR)3-NH2 (14), and a control peptide YOSH-4 (residue 1363–1383 of laminin B1 chain; KLQSLDL-SAAAQMTCGTPPGA) (7) were generously supplied by Dr. Hynda K. Kleinman and Dr. Maura C. Kibbey (National Institute of Dental Research, Bethesda, MD). Nude 4- to 6-wk-old, male, BALB/c-nu/nu mice (Harland Sprague-Dawley, Indianapolis, IN) were given laminin, C(YIGSR)3-NH2 or YOSH-4 (50 µg/mouse in 0.1 ml of PBS administered i.p. 1 day before intracardiac injection of A375 cells, according to techniques previously described (9). On the day of injection, A375 cells (1 × 10^6) were suspended in 0.1 ml of PBS containing 100 µg of laminin, C(YIGSR)3-NH2, or YOSH-4; incubated for 5 min as described previously (7); and then injected with 27-gauge needles into the left cardiac ventricle of nude mice (7 mice/treatment group) under anesthesia with pentobarbital (0.05 mg/g) according to the methods referenced for the experimental model of bone metastases in mice which had received intracardiac injections of B-16 murine melanoma cells.

The abbreviations used are: C(YIGSR)3-NH2, a cyclic peptide from the B1 chain of laminin with an amino-terminal cysteine for a total of 16 amino acids; KLQSLDL-SAAAQMTCGTPPGA (7) were generously supplied by Dr. Hynda K. Kleinman and Dr. Maura C. Kibbey (National Institute of Dental Research, Bethesda, MD). Nude 4- to 6-wk-old, male, BALB/c-nu/nu mice (Harland Sprague-Dawley, Indianapolis, IN) were given laminin, C(YIGSR)3-NH2 or YOSH-4 (50 µg/mouse in 0.1 ml of PBS administered i.p. 1 day before intracardiac injection of A375 cells, according to techniques previously described (9). On the day of injection, A375 cells (1 × 10^6) were suspended in 0.1 ml of PBS containing 100 µg of laminin, C(YIGSR)3-NH2, or YOSH-4; incubated for 5 min as described previously (7); and then injected with 27-gauge needles into the left cardiac ventricle of nude mice (7 mice/treatment group) under anesthesia with pentobarbital (0.05 mg/g) according to the methods...
ANTILAMININ PEPTIDE INHIBITION OF OSTEOLYTIC BONE METASTASES

Fig. 1. Radiograph of a nude mouse given an injection of A375 cells. Arrows indicate osteolytic metastatic foci which were visible as well-circumscribed radiolucent lesions in the spine, pelvis, and hind limbs. The radiograph was taken at 5 wk after tumor injection.

Fig. 2. Decalcified sections of bone from A375-injected nude mice treated with laminin (A) and C(YIGSRh-NH2) (B). In A, multinucleated osteoclasts (arrowed) are resorbing the cortical bone of a tibia. Metastatic melanoma cells (M) are present close by and have replaced the normal bone marrow cells. In B, multinucleated osteoclasts (arrowed) are resorbing the cortical bone of a tibia. Metastatic melanoma cells (M) are present close by and have replaced the normal bone marrow cells. In C, multinucleated osteoclasts (arrowed) are resorbing bone on both sides of the epiphyseal plate of the tibia with metastatic melanoma cells (M) close by. H & E, × 100.

previously described (1). From the same day, animals were given laminin, C(YIGSR)2-NH2, or YOSH-4 (50 µg/mouse in 0.1 ml of PBS) i.p. twice a day for 6 days. A control group of animals given A375 injections received an equal volume of PBS given in the same manner. The experiments were carried out 3 times. Animals were kept in our animal facilities for 4 to 7 wk as described (13).

Scoring of Bone Metastases. The number of osteolytic bone metastases was determined on radiographs. At the end of the experiments, animals were anesthetized deeply, laid down in a prone position against the films (22 x 27 cm; X-OMAT AR; Kodak, Rochester, NY), and exposed with an X-ray at 35 kVp for 6 s using a Faxtron radiographic inspection unit (Model 8050-020; Field Emission Corporation, Inc., McMinnville, OR). Films were developed using a RP X-OMAT processor (Model M68; Kodak). All of the radiographs of the bones in nude mice were evaluated extensively and carefully by 3 different individuals including one radiologist who were without knowledge of the experimental groups. From the radiographs, osteolytic metastatic foci as small as 1 mm in diameter, which were recognized as demarcated radiolucent lesions in the bones, were enumerated. It is possible that micrometastases were present in the bones but were not detected on the radiographic films. These were not quantitated in the present study.

Measurement of Ca²⁺ and Body Weight. Animals were anesthetized, and Ca²⁺ was determined using a Ciba-Corning calcium pH analyzer (Model 634; Corning, Medfield, MA) as described (13). Body weight was measured using a Lum-O-Gram (Ohaus Scale Corporation, Florham Park, NJ). These measurements were carried out at 9:00 a.m. twice a week from the beginning of the experiments.

Histological Examination. Bones from one animal in each group were fixed in buffered 10% formalin and decalcified in a 14% EDTA solution to confirm the presence of metastatic tumor and to examine the relationship between the tumor cells and the adjacent bones. Paraffin sections were cut following conventional processing and stained with hematoxylin and eosin.

Statistical Analysis. Statistical significance of the data was analyzed by Duncan's new multiple-range test (14).

Results

Nude mice inoculated with A375 cells into the left ventricle of the heart started to show a marked loss of muscle, adipose tissue, and body weight (cachexia) from 4 to 5 wk after tumor cell inoculation. Radiographic examination of these animals clearly demonstrated osteolytic metastases in the lower extremities, spine, and pelvic bone (Fig. 1), mandible, and upper extremities (not shown). Unlike C57BL/6 mice given injections of B16 murine melanoma cells (1), no macroscopic metastases to organs other than bones were observed at autopsy in our experiments, but we have seen occasional micrometastases to muscle in A375 tumor-bearing mice that were not treated with laminin or laminin antagonists. Histological examination of bones from
Fig. 3. Radiographs of osteolytic bone metastases in A375-bearing nude mice treated with PBS (A), C(YIGSR)3-NH2 (B), YOSH-4 (C), and laminin (D). Radiographs were taken at 5 wk after the injection of tumor cells. Note the differences in the number of osteolytic bone metastatic foci in B and D as compared to A.
the animals confirmed the presence of metastatic tumor and associated osteolysis due to osteoclastic bone resorption (Fig. 2).

Since osteolytic lesions in the bones had relatively clear margins and were well separated from each other on radiographs, we quantified the number of osteolytic bone metastases which were radiographically recognizable in each treatment group. The antilaminin peptide C(YIGSR)3-NH2 significantly decreased the number of osteolytic bone metastatic foci, whereas laminin increased them (Fig. 3; Table 1). There were no morphological differences observed in the appearance of the tumors and of the osteoclasts which were resorbing the adjacent bones between each treatment group (Fig. 2), suggesting that the laminin antagonist was not inhibiting osteoclastic bone resorption. Animals given injections of A375 cells manifested hypercalcemia and severe cachexia in parallel with the formation of osteolytic bone metastases (Table 2). The C(YIGSR)3-NH2 peptide significantly decreased Ca2+ loss and body weight. In contrast, administration of laminin enhanced the development of hypercalcemia and cachexia. Control peptide YOSH-4 showed no effects on either osteolytic bone metastasis formation, Ca2+, or body weight. Laminin, C(YIGSR)3-NH2, and YOSH-4 at 1 μg/ml showed no growth-inhibitory effects on A375 in culture (data not shown).

Discussion

In the present study, we have used a recently developed model for studying tumor cell metastasis to bone and found that a synthetic laminin antagonist C(YIGSR)3-NH2, a more potent inhibitor of pulmonary metastases than YIGSR-NH2 (15), inhibits the formation of osteolytic bone lesions by human amelanotic A375 melanoma cells. YIGSR-NH2 is a synthetic laminin pentapeptide which inhibits formation of pulmonary metastases (7) and angiogenesis (11, 16). We have also found that laminin increases osteolytic bone metastases. Laminin has already been shown to increase pulmonary metastasis presumably due to stimulation of type IV collagenase production (6, 17). Our results indicate that laminin plays an important role in tumor cell metastasis to bones as well as to the lungs and suggest that laminin antagonists could be used to inhibit the formation of not only pulmonary but also skeletal metastases in patients with cancer.

Bone metastasis presents notoriously difficult clinical problems, particularly when patients become hypercalcemic. Once solid cancers become housed in bone, they are typically unresponsive to antitumor agents, and the prognosis for such patients is very poor. Trials of drugs such as bisphosphonates are now being undertaken, but this approach is directed only at inhibition of osteoclastic bone resorption. It presumably will not prevent the spread of tumor cells to bone, although it should inhibit the capacity of the tumor cells in the microenvironment to destroy bone. In the treatment of cancers which involve the skeleton, a combined therapeutic approach based on prevention of the attachment of tumor cells at the bone site together with the inhibition of osteoclastic resorption may provide better results than either approach alone.

We chose the well-characterized A375 human melanoma to study in this model, since these tumor cells have the capacity to stimulate osteoclastic bone resorption in vivo (12). We also used conditions which seemed to favor bone metastasis over other organ metastases. Kozlowski et al. (18) had previously demonstrated that A375 cells have heterogeneous metastatic potential and that metastasis to some organs may depend on natural killer cell activity. Not all tumors, even those which can stimulate bone resorption, cause osteolytic lesions when injected in the manner we describe in this manuscript. The A375 tumor also causes a number of paraneoplastic syndromes in nude mice, namely, cachexia, hypercalcemia, and leukocytosis (12). These paraneoplastic syndromes provide a guide to total tumor burden in the nude mice. The A375 cells produce TGF-α, and human TGF-α was originally purified from conditioned medium from this tumor (19). The tumor cells also secrete granulocyte-macrophage colony-stimulating factor which induces TNF production by host immune cells (12). We have not investigated the factors responsible for the osteolysis in the metastatic lesions in the present study, but our histological studies indicate that they are due to osteoclastic bone resorption adjacent to invading A375 tumor cells. This could be due to either TGF-α produced by the A375 tumor cells or by TNF produced by host cells at the tumor site (or both). Although parathyroid hormone-related protein is not produced by these cells (12), it might be responsible for osteolysis in other tumors, as proposed in cases of bone metastases in patients with breast cancers (20). Thus, this bone metastasis model should produce important information to increase our understanding of the mechanisms of bone metastasis and possibly to develop a therapeutic regimen for treatment of patients with cancers that metastasize to bone.

Acknowledgments

We are grateful to Dr. Hynda K. Kleinman and Dr. Maura C. Kibbey for the generous gifts of laminin, (YIGSR)3-NH2, and YOSH-4 and to Nancy Garrett and Thelma Barrios for their help in the preparation of this manuscript and for their excellent secretarial skills.

References


Table 1 Effect of laminin, (YIGSR)3-NH2, and YOSH-4 on the formation of the osteolytic bone metastatic foci in A375-bearing nude mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median no. of osteolytic bone metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375 bearing</td>
<td>24(14–37)</td>
</tr>
<tr>
<td>Laminin</td>
<td>50(40–78)</td>
</tr>
<tr>
<td>(YIGSR)3-NH2</td>
<td>10(0–27)</td>
</tr>
<tr>
<td>YOSH-4</td>
<td>34(19–45)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, range.
* Significantly different from untreated A375 tumor-bearing nude mice (P < 0.05).

Table 2 Effects of laminin, (YIGSR)3-NH2, and YOSH-4 on Ca2+ and body weight in A375 tumor-bearing nude mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ca2+ (mmol/liter)</th>
<th>Body wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wk 0</td>
<td>Wk 4</td>
</tr>
<tr>
<td>Non-tumor-bearing</td>
<td>1.18 ± 0.05*</td>
<td>1.23 ± 0.04*</td>
</tr>
<tr>
<td>A375 bearing</td>
<td>1.15 ± 0.03</td>
<td>1.48 ± 0.05*</td>
</tr>
<tr>
<td>Laminin</td>
<td>1.20 ± 0.04</td>
<td>1.54 ± 0.03*</td>
</tr>
<tr>
<td>(YIGSR)3-NH2</td>
<td>1.18 ± 0.04</td>
<td>1.26 ± 0.04*</td>
</tr>
<tr>
<td>YOSH-4</td>
<td>1.21 ± 0.04</td>
<td>1.44 ± 0.05*</td>
</tr>
</tbody>
</table>

* Mean ± SE of three separate experiments. Normal Ca2+ ranges are 0.98 to 1.38 mmol/liter.
* Significantly different from Wk 0 (P < 0.01).
* Significantly higher than non-tumor-bearing nude mice at Wk 4 (P < 0.01).
* Significantly different from non-tumor-bearing nude mice at Wk 4 (P < 0.005).
* Significantly different from PBS-treated A375-bearing nude mice at Wk 4 (P < 0.01).

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