An Experimental Rat Model of Local Bone Cancer Invasion and Its Responsiveness to Ethane-1-hydroxy-1,1-bis(phosphonate)\textsuperscript{1}

Amalia Guaitani,\textsuperscript{2} Massimo Sabatini, Giovanni Coccioni, Silvia Cristina, Silvio Garattini, and Ivan Bartošek


ABSTRACT

Line A Walker carcinoma differs from line B in that it does not elicit hypercalcemia and hypercalciuria when implanted in rats at various sites (s.c., i.m., i.a.). However, Walker 256/A, unlike line B, may invade the tibia when implanted i.m. in the adjacent gastrocnemius muscle. This invasion was evaluated by measuring the increased weight of the bone and decreased calcium concentration per unit weight of the tibia, by reduced opacity to X-ray, and by the presence of tumor cells in the compact bone cortex.

Ethane-1-hydroxy-1,1-bis(phosphonate), a diphosphonate derivative, at a dose of 10 to 30 mg/kg/day s.c., prevented cancer cell invasion of the tibia as judged by the above criteria. This inhibition was obtained with no apparent effect on the growth of Walker 256/A carcinoma.

INTRODUCTION

Various interactions between cancers and bones have been described; some tumors (e.g., breast and prostate) metastasize to distant bones (20), others (e.g., head and neck) invade neighboring bones (18), and others induce osteolysis by producing humoral factors that stimulate osteoclasts and mobilize calcium from hydroxyapatite crystals (19). We have described a rat tumor, W 256/B,\textsuperscript{3} which induces hypercalcemia and hypercalciuria when implanted s.c. and which metastasizes to bones when injected into the aorta (12). No models appear to be available to study local bone invasion in laboratory animals, although recently Jung et al. (16) described a model of bone resorption also induced by i.a. injection of Walker carcinoma cells.

Etidronate, a member of the diphosphonate family, inhibits the formation and dissolution of hydroxyapatite crystals in vivo and in vitro (6, 7). It is utilized in the treatment of Paget's disease (1, 3) and offers some promising activities in preventing tumor-formation and dissolution of hydroxyapatite crystals in vivo and in vitro and in the study local bone invasion in laboratory animals, although recently Jung et al. (16) described a model of bone resorption also induced by i.a. injection of Walker carcinoma cells.

Etidronate, a member of the diphosphonate family, inhibits the formation and dissolution of hydroxyapatite crystals in vivo and in vitro (6, 7). It is utilized in the treatment of Paget's disease (1, 3) and offers some promising activities in preventing tumor-induced hypercalcemia (14, 15) and bone metastases (4, 16). Etidronate reduced hypercalcemia and hypercalciuria and bone metastasises induced by W 256/B (12).

The new tumor model utilized here, W 256/A, is suitable for studying local invasion of bones and the effect of etidronate on this experimental condition.

MATERIALS AND METHODS

Animals. Male CRf CD (SD)BR rats (Charles River, Italy), 6 weeks old, weighing 150 ± 2 g (SE), housed individually in Makrolon cages in standard conditions of temperature, light, and humidity, were fed an open formula standard diet (Altromin MT, Rieper, Italy) with water ad libitum.

Tumors. Two lines of Walker 256 carcinoma, A and B, were maintained through periodic s.c. transplantations as described (11, 13). For this study, about 1 g of proliferating tumor tissue was incubated in 30 ml of collagenase (390 units/ml; Sigma Chemical Co., St. Louis, MO) in Hanks' balanced salt medium (Flow Laboratories, United Kingdom) for 40 min at 37°C. The cell suspension filtered through sterile gauze and centrifuged (250 × g, 10 min), was resuspended in Dulbecco's phosphate-buffered saline (Flow Laboratories), pH 7.4, containing 5 × 10\textsuperscript{6} viable cells/ml. For i.m. transplantation, 1 × 10\textsuperscript{6} tumor cells were injected into the gastrocnemius of the rat right hind limb. For i.a. transplantation, 1 × 10\textsuperscript{6} cells were injected into the abdominal aorta after laparotomy under ether anesthesia. For s.c. inoculation, about 100 mg of proliferating tumor tissue were implanted into the right scapular region (11, 13). From Day 5, when the tumor mass became palpable, transverse and sagittal diameters were measured with calipers.

Analyses. Urine (24-h samples) and blood (0.5 ml from tail vein) were collected at intervals for calcium analysis. Tumor-bearing rats and healthy controls were killed on Day 14 (line A) or Days 15 and 19 (line B) after tumor implantation by exsanguination from the carotid artery and randomly allocated to total calcium analysis in bones or to X-ray and microscopic examination of hind limbs.

Trends in the body weight of healthy controls were compared to the net weight (without tumor) of W 256/A- or W256/B-bearing rats. For bone calcium assay, right and left tibiae were excised, weighed, and dissolved in 10 n HCl:23 n HNO\textsubscript{3} (3:1) at 80°C for 5 h; each sample was brought up to 10 ml with distilled water. Calcium was assayed by atomic absorption spectrophotometry (IL 951 apparatus, air acetylene flame, at 422.7 nm wavelength). Plasma, urine, and dissolved bones were diluted (100, 400, and 2500 times, respectively) in a solution of lithium chloride (1 g/liter) and lanthanum chloride (1 g/liter) in distilled water and compared with a calcium standard (1 to 3 mg/liter). All reagents were analytical grade.

X-Ray and Microscopic Examination. The hind limbs were fixed in buffered formaldehyde (1.33 M, pH 7.0) and coded. X-ray pictures and examinations were done in blind conditions. An arbitrary value of 1 was given for severe lesions, 0.5 for borderline lesions, and 0 for absence of lesions. The same samples were then processed for light microscopy.

Treatments. Disodium ethane-1-hydroxy-1,1-bis(phosphonate) (etidronate; Procter and Gamble, Cincinnati, OH) was dissolved in 0.9% NaCl solution and injected s.c. (2 ml/kg/day) into the abdominal region of tumor-bearing rats for 11 to 13 consecutive days. The doses are specified in the tables and figures.

Statistics. Plasma levels and urinary elimination of calcium were analyzed by Dunnett's test (17). Differences in bone weight and calcium content were assessed by split-plot design and Tukey's test (17). The incidence of X-ray-detectable bone lesions was established by Fisher's exact test (21).

RESULTS

Tumor line W 256/A, when implanted in rats at different sites (e.g., i.m., s.c., or i.a.), did not elicit hypercalcemia or increase

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\textsuperscript{3} The abbreviations used are: W 256/A, Walker carcinoma line A; W 256/B, Walker carcinoma line B; i.a., intravenously; etidronate, ethane-1-hydroxy-1,1-bis(phosphonate).

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urinary calcium excretion, whereas W 256/B did (Table 1). These measurements were made at about 75% of the mean survival time after transplantation. In the case of W 256/A, earlier (15 days) or later (30 days) measurements after s.c. implantation did not reveal any change in blood or urinary calcium levels (data not shown).

When W 256/A was implanted i.m. (gastrocnemius muscle of the right hind limb), clear invasion of the tibia was noted early. Table 2 indicates that 14 days after tumor implantation the weight of the right tibia and its total calcium content were greater than that of the contralateral bone and healthy controls. However, the increase in the tibia diameter on the side implanted with W 256/A, with zones of marginal erosion of bone cortex and diffused rarefaction and decrease of bone opacity.

The hypothesis that W 256/A invades bone was validated by X-rays and microscopic observations. Fig. 1 (A and B) shows an increase in the tibia diameter on the side implanted with W 256/A, with zones of marginal erosion of bone cortex and diffused rarefaction and decrease of bone opacity.

Fig. 2 shows light microscopy of the tumor-invaded tibiae; periosteal growth and deep invasion of bone cortex is evident, starting from the tumor implanted in the adjacent muscle [Fig. 2 (A and B)].

W 256/B (i.m.) did not significantly change tibia weight but reduced the bone calcium content and concentration compared to the contralateral tibia and healthy controls (See Table 2). Nevertheless, no changes in bone structure or tumor cell invasion could be seen on X-ray or microscopic observation (data not shown).

Having available a model to study bone invasion by a contiguous tumor, it was of interest to investigate the effect of etidronate. Table 2 shows that this drug reduced the weight of the right tibia in a dose-related manner with no significant effect on the weight of the left tibia. The rise in total calcium was counteracted by the treatment, but the calcium concentration was not affected, probably because etidronate lowers calcium in bones as shown by total calcium content and concentration in the left tibia. In fact, etidronate tended to reduce the difference between the right and the left tibia seen in W 256/A-untreated animals. The differences in tibia weight and calcium content were corroborated by X-ray and microscopic observations. As shown in Fig. 1 (C and D) and in Fig. 2 (C and D), etidronate treatment reduced the lesions and tumor invasion of the tibia, not affecting compact bone. Quantitative observations made in blind conditions showed a consistent effect of etidronate (Table 3); this inhibitory effect on bone invasion by W 256/A was not accompanied by any reduction of tumor growth, even at the highest doses of etidronate (Chart 1).

DISCUSSION

W 256/A carcinoma shows biological features quite different from those of line W 256/B (11, 13) also as regards interactions with bones. W 256/B induces hypercalcemia and hypercalciuria when implanted in different sites (Ref. 12; present results), whereas W 256/A has no systemic effect on calcium; however, unlike W 256/B, it invades the adjacent bone when implanted i.m. Evidence of invasion is the increase in tibia weight on the tumor-invaded side. Further, osteolytic lesions are visible in X-ray pictures, and tumor cell invasion into the bone cortex is histologically detectable.

### Table 1

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Site of tumor implant</th>
<th>Plasma calcium (mg/100 ml)</th>
<th>Urinary calcium (mg/24 h)</th>
<th>Day of assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>s.c.</td>
<td>10.06 ± 0.28</td>
<td>1.47 ± 0.37</td>
<td>20</td>
</tr>
<tr>
<td>W 256/A</td>
<td>i.m.</td>
<td>9.99 ± 0.17</td>
<td>1.32 ± 0.57</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>l.a.</td>
<td>9.91 ± 0.14</td>
<td>0.54 ± 0.06</td>
<td>14</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tibia wt (mg)</th>
<th>Calcium/tibia (mg/bone)</th>
<th>Calcium concentration (mg/g bone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right</td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>Non-treated rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy controls</td>
<td>361 ± 16</td>
<td>373 ± 22</td>
<td>48.5 ± 1.8</td>
</tr>
<tr>
<td>W 256/A</td>
<td>568 ± 43</td>
<td>349 ± 15</td>
<td>61.9 ± 4.6</td>
</tr>
<tr>
<td>W 256/B</td>
<td>378 ± 34</td>
<td>368 ± 26</td>
<td>39.3 ± 3.3</td>
</tr>
<tr>
<td>Etidronate treatment (W 256/A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/kg s.c.</td>
<td>469 ± 35</td>
<td>359 ± 12</td>
<td>54.1 ± 2.7</td>
</tr>
<tr>
<td>20 mg/kg s.c.</td>
<td>365 ± 4</td>
<td>327 ± 7</td>
<td>40.2 ± 1.4</td>
</tr>
<tr>
<td>30 mg/kg o.c.</td>
<td>322 ± 10</td>
<td>315 ± 12</td>
<td>37.1 ± 1.5</td>
</tr>
</tbody>
</table>

- Mean ± SE.
- Difference between right and left tibia, P < 0.01.
- Difference between tumor-implanted animals and healthy controls, P < 0.01.
- Difference between tumor-implanted animals and healthy controls, P < 0.05.
- Difference between right and left tibia, P < 0.05.
- Difference between etidronate and W 256/A, P < 0.05.
- Difference between etidronate and W 256/B, P < 0.01.
etidronate. 10 (A), 20 (D), and 30 (X) mg/kg/day s.c. from Day 1 to Day 13. Points, mean tumor diameter of 5 rats; bars, SE.

implanted i.m. in the right hind limb of rats treated with 0.9% NaCl solution (O) or an invasive rather than a metastatic process. Osteolytic lesions can thus be characterized as representative of from hematogenous dissemination of W 256/A cancer cells. In cultured cells of Walker 256 carcinoma together with a factor tissue, partly because of bone rarefaction due to tumor invasion. D'Souza et al. (2) identified a bone résorption factor released by X-rays and light microscopy, respectively. That the effect of etidronate is due to a reduction in tumor growth leading to decreased bone invasion can be reasonably ruled out because there was no indication of change in the growth of W 256/A even at the highest dose utilized.

The mechanisms by which etidronate inhibits tumor invasion of bones are not yet understood. It may be hypothesized that etidronate binds to hydroxyapatite and makes bone insensitive to the action of osteoclasts and/or tumor cells. Osteoclasts may be activated by the tumor cells or by principles secreted by the tumor which might attack bone matrix (8, 9). Whatever mechanism is involved, these observations provide a method for studying bone invasion by cancer cells, showing in addition that etidronate is potentially useful in reducing the invasion.

ACKNOWLEDGMENTS

The authors wish to thank Procter and Gamble for providing etidronate. Thanks are due to Stefano Dallorida for technical help in total calcium assay and to Dr. Luciano Morasca for his helpful interpretation of microscopic studies.

REFERENCES


Table 3

Effect of etidronate on bone lesions in rats implanted i.m. with W 256/A carcinoma

<table>
<thead>
<tr>
<th>Etidronate* (mg/kg s.c. daily)</th>
<th>Rats showing tibia lesions b</th>
<th>Av. severity of the lesion c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls d</td>
<td>7/9</td>
<td>0.77</td>
</tr>
<tr>
<td>10</td>
<td>2/5</td>
<td>0.30</td>
</tr>
<tr>
<td>20</td>
<td>1/5</td>
<td>0.20</td>
</tr>
<tr>
<td>30</td>
<td>1/5</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Rats were treated from Day 1 to Day 13 after W 256/A implantation i.m.

b Determined by X-rays.

c Scored as 1 for severe lesions, 0.5 for borderline lesions, and 0 for absence of lesions.

d Controls were given 0.9% NaCl solution, 2 ml/kg/day s.c. from Day 1 to Day 13 after W 256/A implantation i.m.

* Statistical significance compared to controls, P < 0.07 (1-tailed Fisher’s exact test).

† Statistical difference compared to controls, P < 0.01 (1-tailed Fisher’s exact test).

The fact that these lesions are limited to the tumor-implanted side rules out the possibility of the osteolytic lesions resulting from hematogenous dissemination of W 256/A cancer cells. In addition, light microscopic observations indicate erosion of the bone contiguous to the edge of the growing tumor. These osteolytic lesions can thus be characterized as representative of an invasive rather than a metastatic process. Further studies are needed to clarify the biochemical and cellular mechanisms involved in bone invasion by cancer cells. The lower calcium concentration per unit of bone weight compared to the contralateral tibia and to tibiae of healthy rats may in part depend on reduced calcium deposition close to the tumor tissue, partly because of bone rarefaction due to tumor invasion. D’Souza et al. (2) identified a bone resorption factor released by cultured cells of Walker 256 carcinoma together with a factor activating adenylyl cyclase in cells.

The bone invasiveness of W 256/A is specific to this tumor line, inasmuch as it is not seen with another line, W 256/B. In this model, etidronate was tested in view of its not yet identified mechanism of osteolysis inhibition (5) and in relation to experimental and clinical evidence indicating that it prevents tumor-induced hypercalcaemia and bone metastases (10, 12). Indeed, etidronate at different doses did prove to be effective in preventing the increase of weight, size, and calcium content of the tibia as well as osteolytic lesions and cancer cell invasion as detected by X-rays and light microscopy, respectively. That the effect of etidronate is due to a reduction in tumor growth leading to
Fig. 1. X-ray pictures of rat hind limbs. A, right tibia (left) examined 14 days after i.m. inoculation of W 256/A carcinoma into the gastrocnemius muscle. Bone swelling is evident with marginal thickening lengthwise in both the tibia and fibula. The left limb (right) is compared. B, right tibia (left) examined 14 days after i.m. inoculation of W 256/A carcinoma into the gastrocnemius muscle. Bone swelling with localized marked erosion at the proximal middle third is evident. Contralateral limb (right) for comparison. C and D, right tibiae (left), close to i.m. implanted W 256/A, from rats treated with etidronate (20 and 30 mg/kg for 13 days). W 256/A-bearing rats treated with etidronate show morphological symmetry of right and left tibia and no signs of structural alterations.

Fig. 2. Sections of tibiae from rats with W 256/A carcinoma implanted i.m. in the right hind limb. Periosteal growth (A, x 250) as well as deep invasion of the bone cortex (B, x 625) is evident. Etidronate administration (30 mg/kg/day) reduces tumor cell invasion. The compact bone appears not to be affected (C, x 250; D, x 625). All sections from decalcified osseous tissue were stained with H & E for light microscopy.
Monoclonal Antibodies to Cell Surface Antigens Shared by Chemically Induced Mouse Bladder Carcinomas

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ABSTRACT

Rats were immunized with cultured cells from chemically induced transitional cell carcinomas of the mouse urinary bladder, and their spleen cells were hybridized with NS-1 mouse myeloma cells. Following initial screening of antibodies made by hybridoma clones, the tissue distribution of antigens defined by the antibodies was established by using a peroxidase-antiperoxidase technique with frozen sections of a variety of mouse tumors, as well as normal adult and embryonic tissues. Two antibodies were identified which detected antigens with bladder carcinoma specificity. One antibody (3B12) reacted weakly with epithelial cells from several sources, including normal bladder, while the second antibody (6.10), which bound strongly to bladder carcinoma cells, was negative on bladder epithelium and bound (weakly) to only a small fraction of all epithelial cells tested except for epidermal cells and periosteum from embryos. Both antibodies should be useful to assess the immunotherapeutic and immunoprophylactic effects of monoclonal antibodies to tumor-type specific oncofetal antigens.

INTRODUCTION

Chemically induced transitional cell bladder carcinomas in mice (or rats) share tumor-type associated surface antigens, to which lymphocyte-mediated immune responses can be demonstrated in vitro (1), and they express individually unique, tumor-specific antigens which are detectable by transplantation tests in vivo (2). Taranger et al. (1) have demonstrated that, if rats are immunized with bladder tumor tissue, there is a longer latency period before they develop bladder tumor after exposure to a chemical carcinogen. This suggests but does not prove that an immune response to the shared bladder tumor antigens can be induced in vivo to the benefit of the host.

Tumor-type associated antigens, like the shared bladder carcinoma antigens referred to, have attracted much attention, in view of the fact that most of the human tumor antigens so far detected by using xenogeneic (mouse) monoclonal antibodies belong to this category (3). It is an open question, however, whether or not an immune response to such monoclonal antibody-defined antigens can be induced in syngeneic hosts and, if so, what its effects would be on the tumor and on the host.

Mouse bladder carcinomas appear to provide suitable models for studying such problems. For this to be feasible, however, one needs monoclonal antibodies with a degree of specificity for tumor which is closely similar to that of the mouse anti-human tumor antibodies (3). We have reported previously on 2 monoclonal rat antibodies to mouse bladder carcinoma antigens (4). Their degree of specificity for bladder carcinoma was, however, much less than that of the more specific mouse antibodies to human tumor antigens, and the expression of the relevant antigens in vivo was not well established.

This paper gives data on 2 monoclonal rat antibodies to cell surface antigens which are strongly expressed in mouse bladder carcinoma as tested by immunohistological assays of tumor samples (5, 6). The specificity of these antibodies for tumor approaches that of the human melanoma-associated antigen p97 (3). Like most of the human tumor-associated antigens (3), the antigens defined by the 2 antibodies are also expressed in some embryonic tissues. These antibodies should be very useful for "model" experiments aimed toward tumor therapeutics and immunoprophyaxis.

MATERIALS AND METHODS

Animals. Six- to 10-week-old BALB/c mice were used for tumor induction and propagation. They were bred by brother-to-sister mating at the Fred Hutchinson Cancer Research Center. One-month-old Wistar/Furth and Sprague-Dawley male rats were obtained from Fred Hutchinson Cancer Research Center animal facilities and immunized to produce hybridomas.

Tumors. Transitional cell carcinomas of the urinary bladder were induced in BALB/c female mice, using either MCA or FANFT, as described previously (4). The MCA-induced tumors comprise three histologically different types of neoplasms in approximately equal amounts (2). One type is a carcinoma, composed of transitional cells; squamous metaplasia with pearl formation is sometimes seen. The other type is characterized by highly anaplastic cells that are spindle-shaped with abundant cytoplasm around the nuclei and which grow in undifferentiated sheets. By classical histological criteria, these tumors are most similar to sarcomas.

Four MCA-induced BALB/c transitional cell carcinomas of the bladder were used, 1656, 1657, 1660, and 1670 (Fig. 1); most of these were transplanted in mice, but primary specimens of some tumors were investigated as well. Occasionally, the same tumor comprised areas of both carcinomas and sarcomas (Fig. 2 shows this for a transplant of Bladder Tumor 1660). One transplanted FANFT-induced transitional-cell carcinoma, 1682, was also tested. All of the above-mentioned bladder tumors have also been established in culture.

Other tumors studied include a spontaneous C3H mammary carcinoma (1651), the B16 melanoma, 3 thymoblastomas (1490, 1511, and 1639), and a lymphosarcoma (1647) as listed in Table 1. These tumors were all transplanted. All immunohistological studies were done

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