Inhibition by Diphosphonates of Bone Resorption Induced by the Walker Tumor of the Rat

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ABSTRACT

An animal model is described to test the effect of diphosphonates, which are powerful antosteolytic agents, against bone tumors. This model consists of injecting Walker tumor cells into one iliac artery of a series of rats while the contralateral artery is clamped during the injection, and waiting 7 days to obtain a significant destruction of the femur and tibia of the rats. In most of the animals, after this delay, extensive lesions are observed macroscopically by X-ray and histologically.

The parenteral administration of three diphosphonates, dichloromethylene diphosphonate, ethanehydroxydiphosphonate, and aminopropanediphosphonate, at 16 and 160 µmol/kg/day, protects the bones by decreasing the extent of osteolysis. This protective effect is seen both in the tumor-injected leg and in the contralateral leg and is significant when compared to nontreated animals. The most active of the drugs was dichloromethylene-diphosphonate; ethanehydroxydiphosphonate and aminopropanediphosphonate were less active, especially when given at the higher dosage. All diphosphonates produce a marked decrease of the number of osteoclasts; ethanehydroxydiphosphonate at the higher dosage, induced a large increase of nonmineralized bone.

These results are discussed in light of recent clinical work, showing that this animal model is a useful tool to test the effect of new drugs against osteolysis of cancer.

INTRODUCTION

Osteolytic bone metastases are frequently seen in patients with a variety of malignant tumors and are an important cause of morbidity and mortality. Although the pathogenesis is still not clearly understood, it is generally accepted (7, 8, 25) that tumor cells produce osteolytic substances, prostaglandins among others, which induce osteoclastic proliferation and bone destruction. The diphosphonates, which inhibit growth (6) and dissolution (5, 22) of hydroxyapatite crystals, are effective in inhibiting bone resorption in a variety of experimental systems and in vivo (18, 21). Some of these compounds have been used successfully to treat Paget's disease (16, 23) as well as to control hypercalcemia in a few patients with malignant diseases (1, 2, 10, 11, 26).

There are, however, few experimental models available for studying the effect of pharmacological agents against bone metastases. Galasko et al. (9), as well as our group (13), have described a tissue culture method adapted from the system of Reynolds and Dingle (20). These studies have shown that some diphosphonates actually inhibit the release of calcium from mouse calvaria in tumor-conditioned media. To test if these in vitro observations are relevant to the in vivo behavior of tumors, we have used the intraarterial injection of Walker carcinosarcoma cells into the rat, because this tumor produced osteolytic deposits in a high proportion of cases in a short period of time (19). This report shows that some diphosphonates, C₂₅MDP,² EHDP, and APD, are active in inhibiting the bone destruction induced by this tumor.

MATERIALS AND METHODS

Animals and Tumor Cells. Male Wistar rats, weighing 200 to 250 g, were given injections i.p. of Walker carcinosarcoma tumor cells; the rats were kindly given to us by the Institute for Tumor Research in Zurich. As soon as the ascites was apparent (usually 3 to 5 days after injection), the hemorrhagic ascites was sampled under ether anesthesia by i.p. puncture. One ml of this malignant ascitic fluid was either reinjected i.p. into healthy male rats to propagate the tumors, or it was frozen with glycerol and kept in liquid nitrogen for further use, or it was used directly for the experiments. We confirmed that the number of malignant cells was fairly constant from one experiment to the other (mean of 7 different ascites, 41.74 million-cells/cm³ ± 5.59 million cells (S.D.)).

Experimental Protocol. In preliminary experiments, 0.4 ml of undiluted ascites was injected intraarterially using 24-gauge needles in the abdominal aorta after laparotomy under ether anesthesia. The rats were killed after 7 days. X-rays of the hind limbs were taken, and the bones were dissected and examined macroscopically. Histology of undercalcified longitudinal and transversal sections of the tibia and femurs was performed. Bone samples were fixed in 70% alcohol, embedded in methylnethacrylate, and stained with both Solochrome cyanine R (for evaluation of osteoid) and Goldner's method (for examination of osteoclasts and resorption lacunae).

To obtain quantitative results, the protocol was slightly modified: 0.4 ml of the suspension of tumor cells was injected into the left iliac artery instead of into the aorta. During the time of injection, and 1 min after injection, the aorta, above the puncture site, and the right iliac artery were clamped in order that the cells might be distributed only in the left leg. The rats were killed after 7 days. Femur and tibia of either side were dissected and examined. They were rinsed first with demineralized water, then placed in 0.02 M Tris-HCl buffer, pH 7.4, for 1 hr and then rinsed again. They were dried, minced, and ground with a mill (Spex Freezer Mill, Metuchen, NJ) in liquid nitrogen. The bone powders were weighed, two 5-µg samples of each powder were dissolved in concentrated HCl, and the calcium content of the bone powder was analyzed by atomic absorption spectrophotometry. This allowed the amount of calcium of the femur and tibia of each leg to be calculated at the end of the experiment. The results are expressed as g of Ca per 100-g rat in both legs of one rat as well as per 100-g rat in both femur and tibia of each leg taken together. In Table 1, the results are expressed as a percentage of the sham-inoculated nontreated rats. Rats were randomized into different groups, including treated and nontreated animals, as well as tumor-bearing animals and sham-injected animals. The latter were operated on as described previously, but received an injection of 0.4 ml of 0.9% NaCl solution instead of tumor cells into one leg.

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2 Present address: Service de Médecine, Hôpital-1260 Nyon, Switzerland. To whom requests for reprints should be addressed.

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3 The abbreviations used are: C₂₅MDP, dichloromethylene diphosphonate; EHDP, ethanehydroxydiphosphonate; APD, aminopropanediphosphonate.
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diphosphonates.

and transversal cutting of undecalcified sections. Eleven nonconsecutive ethanol and later embedded in methyl methacrylate before longitudinal

adjusted at 7.4. Control rats were given 0.9% NaCl solution instead of Co. (Cincinnati, OH) were dissolved in distilled water; the pH was

seventh day. The compounds kindly supplied by the Procter & Gamble at a dose of 160 μmol/kg/day or 16 μmol/kg/day by s.c. injection from iliac artery. The treated rats were given either EHDP, Cl₂MDP, or APD

injected with Walker tumor cells in one iliac artery, with the contralateral artery clamped during the injection.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sham-inoculated animals</th>
<th>Tumor-inoculated legs</th>
<th>Contralateral legs</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (9%)</td>
<td>100 ± 7.5(6)</td>
<td>46.2 ± 5.3(7)</td>
<td>71.3 ± 4.5(7)</td>
</tr>
<tr>
<td>Cl₂MDP (160 μmol/day)</td>
<td>109.1 ± 9.0 (6)</td>
<td>90.0 ± 21.6(7)</td>
<td>93.7 ± 18.4(7)</td>
</tr>
<tr>
<td>Cl₂MDP (16 μmol/day)</td>
<td>112.9 ± 8.4 (6)</td>
<td>76.8 ± 9.7(7)</td>
<td>94.2 ± 6.2(7)</td>
</tr>
<tr>
<td>EHDP (160 μmol/day)</td>
<td>83.9 ± 10.3 (6)</td>
<td>63.5 ± 4.2 (7)</td>
<td>68.9 ± 10.6(7)</td>
</tr>
<tr>
<td>EHDP (16 μmol/ day)</td>
<td>99.1 ± 6.9 (6)</td>
<td>65.8 ± 12.3(7)</td>
<td>87.2 ± 8.6(7)</td>
</tr>
<tr>
<td>APD (160 μmol/ day)</td>
<td>81.3 ± 3.9 (6)</td>
<td>61.4 ± 8.6 (7)</td>
<td>67.5 ± 5.6 (7)</td>
</tr>
<tr>
<td>APD (16 μmol/ day)</td>
<td>108.5 ± 6.6 (6)</td>
<td>80.0 ± 16.8(8)</td>
<td>86.2 ± 18.1(8)</td>
</tr>
</tbody>
</table>

* Mean ± S.D. of the content of calcium in both tibia and femur taken together of each leg divided by the weight of each rat.

Nonstatistical Analysis. In these experiments, the aim is to find possible main effects, i.e., the influence of different treatments at different doses and the influence of the site of the tumor invasion [sham/direct tumor invasion (left legs)/metastatic tumor invasion (right legs)]. Furthermore, it is examined if there exist interactions between these main effects. The 21 cells of these experiments were composed of 6, 7, or 8 rats. To do this, we used the method of contrasts (15), which consists of comparing linear combinations of means of each individual cell:

\[ L = \lambda_1 x_1 + \lambda_2 x_2 + \cdots + \lambda_n x_n \]

with the condition that the sum of the coefficients, \( \lambda_i \), is equal to zero:

\[ \sum \lambda_i = 0 \]

This method allows a comparison between groups of individuals either taking into account all the cells (for calculating the residual variance) or choosing arbitrarily a certain number of cells between which a difference should be accepted or rejected at the desired probability level (effect of different drugs, for instance). This consists mathematically of making none, one, or some of the \( \lambda_i \) in the preceding equation equal to zero.

RESULTS

Preliminary Experiments. The preliminary experiments, which consist of injecting Walker tumor cells into the aorta, show important destructive osteolytic lesions of both femurs and tibias in about 80% of the animals. The X-rays taken postmortem show the rarefaction of bones in these animals (Fig. 1). By contrast, when animals have been treated with one of the diphosphonates at high doses (160 μmol/kg/day), the X-rays do not show osteolytic lesions (Fig. 2), and at autopsy only small lesions can be seen. In these, as well as in further experiments, only the bones of the treated or nontreated rats which showed unequivocal macroscopic lesions were retained for our statistics, which means that some treated animals presenting with micrometastases may have been excluded (this was the case for 2 rats treated with EHDP). The rats tolerated the injections quite well, none, one, or some of the \( \lambda_i \) in the preceding equation equal to zero.

Comparison between Treatments. Table 1 shows the means and S.D. of the groups of animals treated with different drugs at 2 dosages. The results are expressed as a percentage of the nontreated sham-injected rats.

The statistical analysis shows a highly significant (p < 0.001) difference between the treated and the nontreated animals, and a more favorable effect (p < 0.001) of Cl₂MDP compared to that of EHDP and APD taken together. There were no differences between the effect of EHDP and the effect of APD. Furthermore, EHDP and APD were significantly more active when used at the low dosage (16 μmol/kg/day) instead of the high dosage (160 μmol/kg/day). Cl₂MDP seems slightly more active when used in higher concentration, but the difference was not significant.

When comparing the legs of sham-injected animals with the tumor-injected legs, statistical analysis reveals a significant difference (p < 0.01). This is also true when one compares the legs of sham-injected animals with the contralateral legs of tumor-inoculated animals. There was a significant interaction between the effect of treatment and the direct tumor invasion (left leg); the difference between treatment and absence of treatment was significantly more important on the left leg than on the sham-injected animals. No other significant interaction has been detected.

Bone Histology. The marrow space of rats inoculated with tumor cells was invaded by large islands of sarcomatous cells inducing an important increase of the osteoclast population in the vicinity of the malignant cells. These changes were seen both in the directly inoculated leg and in the contralateral leg. In both Cl₂MDP- and EHDP-treated rats, an obvious and dramatic reduction in the number of osteoclasts was observed when compared to untreated animals given injections of Walker sarcoma (Fig. 3). In animals treated with Cl₂MDP, the osteoid seam thickness was identical to the one in untreated rats (Fig. 4) regardless of whether they were injected with sarcoma. In contrast, in EHDP-treated rats (Fig. 5), a gross increase in osteoid seam thickness was evident in all sections.

DISCUSSION

These results show clearly that the 3 diphosphonates, Cl₂MDP, EHDP, and APD, are able to inhibit bone lysis induced by a particular type of tumor, the Walker carcinosarcoma of the rat. The etiology of bone destruction by this tumor is not established; Minne et al. (17) have proposed that a hyperproduction of parathyroid hormone was present in rats in which the tumor
was inoculated s.c., but the rats of their studies showed a generalized tissue necrosis with kidney tract obstruction so that these measurements should be interpreted with caution. Powles et al. (19), using this cancer in similar experiments, have shown an inhibition by aspirin and indomethacin of the tumor osteolytic activity, which would mean that prostaglandins might be responsible. Whatever the cause, the histological examination of bone has shown an intense osteoclastic activity, which is certainly responsible for the bone destruction observed in nontreated animals. The destruction of bone by invasion of malignant cells has been greater in the legs in which cells have been injected directly than it has been in the contralateral leg. It is, however, not possible to state if this is metastatic dissemination or simply residual cells injected in the blood stream which disseminate everywhere.

It is not surprising that diphosphonates are active in this model, since they have been shown to inhibit bone resorption in a variety of models in vitro as well as in vivo (18, 21). However, these agents have rarely been tested in experimental models of tumor osteolysis. Galasko et al. (9) have shown in elegant experiments that fragments of mammary cancer of different patients as well as of an experimental tumor of the rabbit, the VX2 carcinoma, induced an osteolysis in vitro which is prevented by prostaglandin synthesis inhibitors as well as by 2 diphosphonates. Minne et al. (17) have shown somewhat conflicting results using the Walker tumor with a different protocol. Finally, our group (13) has shown in vitro an inhibitory effect of the 3 diphosphonates on the osteosclerosis induced by human tumors cultured in a tumor-conditioned medium.

The mechanisms by which diphosphonates inhibit bone lysis are still controversial (23, 24). In this study, we have shown that high doses of EHDP or Ca\textsubscript{2}MDP drastically reduce the number of osteoclasts induced by the tumor. Further, EHDP seems as active in inhibiting the osteoclastic effect, but produces an important inhibition of calcification. This probably explains why Ca\textsubscript{2}MDP is more active than EHDP as well as APD in this model. This phenomenon is probably also responsible for the higher activity of EHDP and APD at the lower dosage (16 μmol/kg weight) than at the higher dosage; it is likely that the inhibition of bone destruction is largely counterbalanced by an inhibition of bone formation, as has been demonstrated for the treatment of Paget’s disease with low and high doses of EHDP (14).

It has been shown in several recent articles that Ca\textsubscript{2}MDP, EHDP, and APD are efficient in malignant hypercalcemias (1, 2, 10, 26) and are able to inhibit osteolysis in a few normocarcinoma tumors (24). Currently undertaken metabolic and calcium kinetic studies (12) show that there is a parallelism between the inhibition of bone destruction seen in this experimental model and the decrease of bone destruction calculated using a calcium kinetic model. This proves that, although this tumor is different from the normal human carcinomas, it allows the activity of different drugs against malignant osteolysis to be tested.

REFERENCES
Fig. 1. X-ray of a nontreated rat 7 days after injection with 0.4 ml of a suspension of Walker tumor cells into the aorta.

Fig. 2. X-ray of a rat 7 days after injection of the same amount of Walker tumor cells as in Fig. 1. This rat was given CI-MA (160 μmol/kg/day) s.c. since the day of inoculation of tumor cells.
Fig. 3. Histological picture of the femur of a nontreated rat 7 days after intraarterial injection of Walker tumor cells. Notice the large number of osteoclasts which erode the bone trabeculae. Goldner stain, × 150.

Fig. 4. Histological picture of the femur of a CI-MDP-treated rat 7 days after intraarterial injection of Walker tumor cells. This rat has received CI-MDP 160 μmol/kg/day since the day of inoculation of tumor cells. Although the bone marrow is invaded by malignant Walker cells, no proliferation of osteoclasts is observed. Goldner stain, × 85.

Fig. 5. Histological picture of the femur of an EHDP-treated rat 7 days after intraarterial injection of Walker tumor cells. This rat has received EHDP 160 μmol/kg/day since the day of inoculation of tumor cells. As in Fig. 4, no osteoclasts are seen. Notice the enormous increase of osteoid in the Haversian spaces. Goldner stain, × 100.
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