ABSTRACT

In order to test the effect of diphosphonates in inhibiting bone lysis induced by human tumors, neonatal mouse calvaria are cultured in sterile conditions in different media. Osteolysis is estimated by the amount of 45Ca released from bone to medium, the mice being given injections of 45Ca on their day of birth. An increased bone lysis is observed when calvaria are incubated in the same medium conditioned from cultured tumor fragments. This effect is significantly decreased when the mice have been treated with either ethanehydroxydiphosphonate or dichloromethylendiphosphonate. These experiments represent a first step in estimating the potential effects of different drugs on malignant osteolysis.

INTRODUCTION

Osteolytic bone metastases frequently occur in patients with advanced cancer and are an important cause of morbidity and mortality. The pathogenesis of bone destruction is still not clearly understood; it has been postulated that 2 mechanisms are responsible, one mediated by osteoclasts, the other independent of osteoclast action (12). Prostaglandins are secreted by certain tumors and are able to activate the osteoclasts, as demonstrated in a few animal tumors (13, 35); indomethacin, an inhibitor of prostaglandin synthesis, can sometimes inhibit the osteolytic effect of certain tumors in animals (13, 25) and humans (1).

Diphosphonates are synthetic compounds similar to pyrophosphate, with which they share many physicochemical properties (8). Early investigations showed that the diphosphonates inhibited the precipitation of calcium phosphate efficiently in a manner similar to that of pyrophosphate (10). It was later discovered that they also slow down the dissolution of calcium phosphate crystals (9, 30). In several experimental models, the diphosphonates proved to be effective in inhibiting bone resorption when tested in organ culture or in vivo (20–22, 28, 29). Some of these compounds, first sodium etidronate (18, 31) and more recently several others (11, 19), have been shown to be effective as therapeutic agents in Paget’s disease. Bone turnover is diminished as indicated by a decrease in both urinary hydroxyproline excretion and plasma alkaline phosphatase, as well as by several studies using 47Ca kinetics and bone histology. Because little experimental work has thus far been published on the effect of these compounds on tumor-induced osteolysis (14, 16), the present study may be useful to test the protective efficiency of such compounds on the lytic action of tumors on bone.

MATERIALS AND METHODS

Tumor-conditioned medium was prepared by mincing tumor tissue, under sterile conditions, in slices 0.5 to 1 mm thick, immediately after being obtained at operation. These tumor fragments were placed on stainless steel grids (Falcon Plastics, Oxnard, Calif.) in Bigger’s medium containing 0.5% bovine serum albumin (twice crystallized; Armour Pharmaceuticals, Eastbourne, England), prepared according to the method of Reynolds and Dingle (26, 27). Seven triangular grids (each measuring 1.5 cm per side) were completely covered with tumor tissue and incubated in 25 ml of medium. The incubation lasted for 24 hr at 37° in air with 5% CO2. After incubation, the medium was filtered through Millipore filters (0.45 μm diameter), and the pH was adjusted with NaOH to 7.4 after the medium was gassed under sterile conditions for 10 min with 5% CO2 in air.

Mouse calvaria were prepared according to the method of Reynolds and Dingle (26, 27). Neonatal mice of 2 or 3 different litters were given i.p. injections, on the day of birth, of 1.0 μCi 45CaCl2 in sterile solution (Würenlingen, Switzerland). The following day, they were randomly treated by s.c. injection with either a diphosphonate (0.16 mmol/kg) or 0.9% NaCl solution. The diphosphonates used [kindly given by the Procter and Gamble Co. (Cincinnati, Ohio) and by Henkel & Co. (Düsseldorf, Germany)] were EHDP,3 or CI2MDP in a 10−2 M solution adjusted at pH 7.4 and made isosmotic with NaCl. The mice were killed 4 days after the isotope pulse. The calvaria (frontal and parietal bones) were removed aseptically by careful microdissection so as not to damage the peristeam, and each half was placed on a stainless steel grid in 5 ml Bigger’s medium adapted by Reynolds (26) and adjusted at pH 7.4 after being gassed sterile with 5% CO2 in air. After 24 hr of incubation at 37° with 95% air and 5% CO2, the medium was replaced either by 5 ml fresh medium with 0.5% bovine serum albumin or by the same amount of medium containing tumor extracts prepared as described before. The incubation was maintained for a further 48 hr. At the end of the experiment, each half-bone was dissolved in 0.5 ml concentrated hydrochloric acid. The radioactivity of bone (after adding 1 ml water) and of medium (after adding 0.5 ml concentrated HCl) was measured in an automatic liquid scintillation counter with 15 ml of scintillator (Permafluor; Packard Instrument Co., Downers Grove, III.). The bone lysis of the explant was calculated as the radioactive calcium released by the bone as the percentage of the total radioactivity of the system (bone and medium), after appropriate quench correction using an external standard.

1 Work supported by grants from the Swiss Foundation for Grants in Biology and Medicine, by the Swiss League Against Cancer, and by the Intermaritime Foundation.

2 To whom requests for reprints should be addressed, at Clinique Médicale Thérapeutique, Hôpital Cantonal, 1211 Geneva 4, Switzerland.

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ABSTRACT

Inhibition by Two Diphosphonates of Bone Lysis in Tumor-conditioned Media

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Centre for the Study of Bone Diseases, Department of Medicine, University Hospital, Geneva, Switzerland

INTRODUCTION

Osteolytic bone metastases frequently occur in patients with advanced cancer and are an important cause of morbidity and mortality. The pathogenesis of bone destruction is still not clearly understood; it has been postulated that 2 mechanisms are responsible, one mediated by osteoclasts, the other independent of osteoclast action (12). Prostaglandins are secreted by certain tumors and are able to activate the osteoclasts, as demonstrated in a few animal tumors (13, 35); indomethacin, an inhibitor of prostaglandin synthesis, can sometimes inhibit the osteolytic effect of certain tumors in animals (13, 25) and humans (1).

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In order to compare bone osteolysis induced by the tumor-conditioned medium with resorption induced by known osteolytic agents, bovine parathyroid hormone (kindly given to us by Dr. R. Felix, Berne, Switzerland) and 25-hydroxycholecalciferol (kindly given by Roche Co., Basle, Switzerland) were added to the culture medium at different concentrations. This allowed dose-response curves to be drawn.

Half of the calvaria from mice treated with either a diphosphonate or 0.9% NaCl solution were incubated in Petri dishes with 5 ml of control or tumor-conditioned medium during the same experiment.

The effects of an inhibitor of prostaglandin synthesis on the release of osteolytic factors from the tumors into the tumor-conditioned media have also been studied. Indomethacin was added at the beginning of media conditioning by tumor from a stock solution in ethanol to give a final concentration of 10 μg/ml. The release of calcium from mouse calvaria cultivated in different tumor-conditioned media in the absence or presence of indomethacin has been compared.

Each experiment was analyzed statistically by a 2-way variance analysis with an unequal number of observations in the cells (some of the observations had to be rejected because the medium was infected). The least-squares analysis used for the calculation (34) allowed the determination of the possible specific inhibition by diphosphonates of the tumor-induced osteolysis (as measured by the interaction in the variance analysis). Inasmuch as large variations in basal 45Ca release have been observed between assays conducted with different litters of mice, we have expressed the results according to most authors as the ratio of osteolysis calculated as indicated above in the experimental conditions of the osteolysis in control experiments of the same day.

Finally, some bones were prepared for histological evaluation by fixation in ethanol. They were embedded in paraffin and cut in serial cross-section (10 μm), stained with hematoxylin and eosin, and examined with light microscopy.

RESULTS

Chart 1 shows the increased osteolysis of the system when bovine parathyroid hormone or 25-hydroxycholecalcifer have been added to the medium at 3 different concentrations. Increased resorption is observed at increased concentrations of the lytic agents, although the log dose-response curves are not linear.

Twelve malignant tumors without clinical or radiological evidence of bone metastases (11 breast tumors and 1 kidney tumor) were assayed in this system. Ten breast carcinomas and one kidney carcinoma demonstrated osteolytic activity, i.e., a highly significant difference in calcium release as compared to calvaria incubated in control Biggers’s medium.

Table 1 shows the effect of indomethacin added to the medium conditioned by 4 different breast tumors. A significant inhibition of osteolysis in the presence of indomethacin is observed in experiments with Tumors C and D and is absent in experiments with Tumors A and B.

Tables 2 and 3 show that the 2 diphosphonates EHDP and

![Chart 1. Dose-response curves for parathyroid hormone (PTH) and 25-hydroxy cholecalcifer (25-OH-D3). Values are expressed as the ratio of the osteolysis of mouse calvaria in medium with addition of parathyroid hormone or 25-hydroxycholecalcifer, respectively, to the osteolysis in control medium. Points, means of 6 values in all experiments; bars, S.D.](image-url)

**Table 1**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumor medium without indomethacin</th>
<th>Tumor medium with indomethacin</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.32 ± 0.23 (6)</td>
<td>1.37 ± 0.16 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>B</td>
<td>1.68 ± 0.23 (6)</td>
<td>1.77 ± 0.28 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>C</td>
<td>3.38 ± 0.39 (5)</td>
<td>2.49 ± 0.16 (5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>D</td>
<td>2.43 ± 0.22 (4)</td>
<td>1.54 ± 0.33 (4)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Mean ± S.D.  
* Numbers in parentheses, number of observations.  
* NS, not significant.

**Table 2**

<table>
<thead>
<tr>
<th>Tumor (breast)</th>
<th>Control medium</th>
<th>Tumor-conditioned medium</th>
<th>Significance of interaction (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-treated mice</td>
<td>EHDP-treated mice</td>
<td>Non-treated mice</td>
</tr>
<tr>
<td>E</td>
<td>1.00 ± 0.14 (2)</td>
<td>0.82 ± 0.04 (4)</td>
<td>1.88 ± 0.56 (3)</td>
</tr>
<tr>
<td>F</td>
<td>1.00 ± 0.05 (2)</td>
<td>0.89 ± 0.07 (4)</td>
<td>2.88 ± 0.23 (3)</td>
</tr>
<tr>
<td>G</td>
<td>1.00 ± 0.05 (2)</td>
<td>0.89 ± 0.07 (4)</td>
<td>1.89 ± 0.09 (3)</td>
</tr>
<tr>
<td>H</td>
<td>1.00 ± 0.09 (9)</td>
<td>0.81 ± 0.04 (9)</td>
<td>2.01 ± 0.15 (9)</td>
</tr>
<tr>
<td>I</td>
<td>1.00 ± 0.14 (7)</td>
<td>0.86 ± 0.08 (7)</td>
<td>2.56 ± 0.29 (7)</td>
</tr>
</tbody>
</table>

* Mean ± S.D.  
* Numbers in parentheses, number of observations.  
* NS, no significant interaction at the 0.05 level.
CI₂MDP significantly inhibited the lytic activity induced by extracts of different human tumors. As is seen in these tables, EHDP, as well as CI₂MDP, also acted on bone lysis occurring in control medium. The analysis of variance used here allowed discrimination between a general inhibitory effect of diphosphonates on naturally occurring lysis of bone in the medium and a specific effect on tumor-induced osteolysis, which was significant in 4 of 5 experiments where EHDP was used and in all of the 6 experiments where CI₂MDP was used.

Fig. 1 shows the histological appearance of bones in 4 different cases: control; tumor-conditioned medium; parathyroid hormone (60 nm); and 25-hydroxycholecalciferol (130 nm). Although comparison between bones is not easy, resorption seems increased when calvaria have been incubated in tumor-conditioned media compared to the control. The amount of bone present may have decreased slightly and a few osteoclasts were seen, whereas no osteoclast was observed in control bones taken in equivalent sections.

**DISCUSSION**

As shown by other groups (14, 15, 23, 24), if tumor tissue is incubated in nutrient media, an increased osteolysis of fetal or neonatal bones is observed when these bones are cultivated in these conditioned media or directly in contact with fragments of tumor tissue. The cause of the increased osteolysis is not clear: (a) it is possible that lysis is due to stromal cells such as leukocytes and fibroblasts, which may produce bone-resorbing factors. These stromal cells were present in various amounts, and the same holds true for tumor fragments subjected to histological study; (b) if Greaves et al. (15) have found substantial amounts of prostaglandin in extracts of malignant breast and renal tumors, this has not been the case in all tumors that have assayed. Further, they have observed that indomethacin has significantly reduced release of calcium in some but not all the tumors that they have tested. This is in accordance with the results of Powles et al. (24) using aspirin and of Galasko et al. (14) using different prostaglandin synthesis inhibitors, as well as with our results. When 4 different tumors were tested, in only 2 of them was a significant reduction of osteolysis observed after incubation of tumor fragments with indomethacin. This would suggest that, at least in certain cases, non-prostaglandin materials are responsible for bone resorption, as suggested by Dowsett et al. (4) and by Galasko et al. (14). Some breast carcinomas can produce considerable quantities of proteolytic enzymes in vitro (5), and bone resorption by the supernatant from an established breast cancer line independent of osteoclastic activity has also been described (6). Although the mechanisms are probably multiple and are not fully elucidated, an apparent correlation has been found between these in vitro experiments and the clinical development of bone metastases (25), although this point remains controversial.

EHDP and CI₂MDP have been able to inhibit the lytic activity observed in the tumor-conditioned media, as was the case in the experiments of Galasko et al. (14). These authors have also shown that diphosphonates are more potent than are prostaglandin synthesis inhibitors and that the combination of both agents is not greater than the individual components. Our results are not strictly comparable with those of Galasko et al. (14), but our conclusions are the same. This protective effect of diphosphonates is probably dependent on the activity of living bone cells, because they are not active on the lysis of dead bones in culture (28). Furthermore, recent studies have shown that the diphosphonates have profound effects on cellular metabolism; thus, CI₂MDP inhibits lactate production (21) and glycolysis (7) in vitro. Some of these compounds, in particular those with long alkyl side chains as well as amino-propanediphosphonate, also demonstrate cellular toxicity (32).

Although this model is so far from the clinical situation that it can be questioned if tumor-induced osteolysis is what is actually being measured, it is encouraging to observe a direct relation between the inhibition of lysis observed in vitro, the effect of these diphosphonates on the osteolysis produced by the Walker tumor of the rat (16), and a few recent clinical reports (2, 3, 17, 33, 36). These have shown that CI₂MDP, EHDP, and aminopropanediphosphonate can inhibit the hypercalcemia induced by different tumors. Further, some of these compounds are probably able to decrease bone resorption in myeloma and bone metastases as measured by reduced hydroxyproline excretion (33) or calcium kinetic and balance studies. This experimental approach might therefore be useful for testing in vitro other compounds which could be active against tumor osteolysis in humans.

**ACKNOWLEDGMENTS**

We would like to thank Dr. T. J. Powles, Dr. G. C. Easty, Dr. R. G. G. Russell, Dr. J. Reeve, and Dr. R. Felix for their helpful advice; Mrs. D. Turnill for technical assistance; and Mr. P. Carraux for having performed the photographs. The tumors were kindly donated by Dr. A. M. Schindler and her colleagues.

**Table 3**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Control medium</th>
<th>Tumor-conditioned medium</th>
<th>Significance of interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CI₂MDP-treated mice</td>
<td>Non-treated mice</td>
<td>CI₂MDP-treated mice</td>
</tr>
<tr>
<td>F (breast)</td>
<td>1.00 ± 0.05 (2) 0.65 ± 0.08 (2)</td>
<td>2.88 ± 0.23 (3) 0.96 ± 0.27 (3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>G (breast)</td>
<td>1.00 ± 0.05 (2) 0.65 ± 0.08 (2)</td>
<td>1.89 ± 0.09 (3) 1.26 ± 0.05 (3)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>I (breast)</td>
<td>1.00 ± 0.14 (7) 0.85 ± 0.04 (6)</td>
<td>2.56 ± 0.29 (7) 1.80 ± 0.07 (6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>J (breast)</td>
<td>1.00 ± 0.08 (10) 0.86 ± 0.11 (9)</td>
<td>1.81 ± 0.26 (10) 1.02 ± 0.08 (10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>K (kidney)</td>
<td>1.00 ± 0.05 (6) 0.81 ± 0.08 (6)</td>
<td>2.65 ± 0.20 (6) 1.44 ± 0.09 (6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>L (breast)</td>
<td>1.00 ± 0.12 (4) 0.82 ± 0.06 (4)</td>
<td>2.43 ± 0.22 (4) 1.23 ± 0.12 (4)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

* Numbers in parentheses, number of observations.
REFERENCES


Fig. 1. Histological picture of mouse calvaria treated in 4 different media. A, control medium; B, tumor-conditioned medium (Tumor K); C, addition of parathyroid hormone (60 nM); D, addition of cholecalciferol (130 nM). × 480.
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