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Original Paper

Morphology of Bone Metastasis

T. Hiraga,[1,2] S. Tanaka,[3] M. Ikegame,[4] M. Koizumi,[5] H. Iguchi,[6] T. Nakajima[1] and H. Ozawa[2]

[1]First Department of Oral and Maxillofacial Surgery; [2]First Department of Oral Anatomy, School of Dentistry, Niigata University, Niigata; [3]Clinical Pharmacology Research Laboratories, Institute for Drug Development Research, Yamanouchi Pharmaceutical Co., Ltd, Ibaragi; [4]Department of Pharmacology, School of Dentistry, Niigata University, Niigata; [5]Japanese Foundation for Cancer Research, Cancer Institute Hospital, Tokyo; and [6]Department of Biochemistry, National Kyushu Cancer Center, Fukuoka, Japan

INTRODUCTION

BONE IS one of the most common sites of tumour metastasis. Bone metastasis is associated with bone pain, hypercalcaemia, pathological fracture and it also leads to premature death. Despite its importance, its mechanism is not well elucidated. This may be partly due to the lack of an adequate experimental model. This paper reviews morphological studies of bone metastasis using an animal experimental model and human surgical specimens. In addition, the effects of bisphosphonate on bone metastasis are also described.

MORPHOLOGICAL STUDIES ON BONE METASTASIS

Histopathological classification of bone metastasis

Histologically, bone metastasis is classified into osteolytic, osteoblastic, mixed and intertrabecular types [1, 2]. The osteolytic-type characterised by a marked destruction of the bony trabeculae is the most common and represents a much greater clinical problem than the other types. Bone resorption is an important process for the establishment and progress of this type of bone metastasis. The osteoblastic-type shows extensive new bone formation, with the possible involvement of soluble factors produced by tumour cells, such as transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), plasminogen activator sequence and bone morphogenetic proteins (BMP) [3]. In addition, the extent of reactive bone formation appears to be inversely proportional to the rate of tumour growth [4]. The mixed-type shows transitional or mixed features of the osteoblastic and osteolytic types. Therefore, they are thought to be closely related. The intertrabecular-type is characterised by infiltration of tumour cells in the bone marrow space without significant alteration of the trabecular bone. Because it is difficult to detect this type of metastasis on a radiograph, it presents a major problem for diagnosis [1].

Animal experimental model

In order to clarify the mechanism of bone resorption induced by a tumour *in vivo*, it is necessary to have a reliable animal experimental model of bone metastasis. Several models for inducing experimental bone metastasis have been reported (Table 1). Initially, tumour cells were directly injected close to bone or into bone marrow to establish tumour growth in the bone, but this is obviously different from the natural course of bone metastasis. In this respect, an intravascular injection is much better. The method of intracardiac injection using B16 melanoma cells was introduced by Arguello and associates in 1988 [13]. In our investigations, the process of bone metastasis was studied by injecting tumour cells into the left cardiac ventricle of mice because this induces bone metastasis at a high frequency. The method is also useful in evaluating the effects of drugs such as bisphosphonates on bone metastasis.

Of the four types of bone metastasis, the osteolytic-type is the most common in animal experimental models. Osteoblastic bone metastasis occurs most frequently in prostate cancer. Human prostate cancer cells, however, rarely grow in culture without changing their phenotype. Thus, bone formation is usually not induced by their injection in *in vivo* experiments. The intertrabecular-type bone metastasis is also rarely reported, but we found that B16/F1 mouse melanoma cells form intertrabecular-type bone metastasis. To study the mechanism underlying these different reactions of bone cells to metastasis, reliable animal models for the respective types of bone metastasis are needed.

Osteoclastgenesis induced by tumour

Although it is currently accepted that osteoclasts play a major role in tumour-associated bone resorption, the mechanism of osteoclastgenesis induced by a tumour remains to be clarified. In our study, three kinds of human tumour cell lines, A375 (amelanotic melanoma) [18], HARA

Table 1. *Animal experimental models of bone metastasis*

Method of tumour inoculation	Reference
Intramedullary injection	5, 6
Injection close to bone	7, 8
Intra-arterial injection	9, 10
Intravenous injection	11, 12
Intracardiac injection into the left ventricle	13–16

(squamous cell carcinoma of the lung) and MDA-231 (adenocarcinoma of the breast), were used. A375 cells are known to produce transforming growth factor-α (TGF-α) and granulocyte-macrophage colony stimulating factor (GM-CSF). The latter induces tumour necrosis factor (TNF) production by host cells. However, the cells do not produce parathyroid hormone-related protein (PTHrP). With injection of A375, bone metastasis was not detected by radiography at 1 week, but very small tumour nests with well vascularised stroma and resorption of trabecular bone were observed microscopically (Figure 1a). When stained with tartrate-resistant acid phosphatase (TRAPase), a marker enzyme of osteoclastic cell lineage, many red-stained TRAPase-positive cells were observed surrounding the tumour (Figure 1b). At 5 weeks, bone marrow was replaced by growth of tumour cells at metastatic sites. An osteogenic response was often observed around them, and this was one of the characteristics of bone metastasis by A375 cells. Many TRAPase-positive multinucleated osteoclasts were seen on the bone surface with formation of lacunae and a TRAPase-positive cement line. The number of osteoclasts was abnormally high as compared to that of non-metastatic regions. Between tumour nests and bone surfaces, a well-vascularised stromal cell layer was observed and TRAPase-positive mononuclear cells were present among stromal cells (Figure 2a). Single TRAPase-positive mononuclear cells were also present in the tumour nests away from the bone surface. They were juxtaposed to tumour cells (Figure 2b). The findings suggested that tumour cells promote differentiation and activation of osteoclasts.

Autoradiographic studies using ^{125}I-calcitonin clarified the specific binding of calcitonin on the TRAPase-positive mononuclear cells away from bone (Figure 3). This is a characteristic feature of osteoclastic cell lineage [19, 20]. In addition, the ultrastructures of these cells were consistent with those of osteoclast precursor cells.

Ultrastructurally, osteoclast precursor cells were directly opposed to tumour cells in some instances, which seemed to indicate that tumour cells might participate directly in the differentiation of osteoclasts. Moreover, stromal cells or extracellular matrix usually existed between the two cells. Here, it has been reported that direct contact of osteoblasts or stromal cells with haematopoietic stem cells is needed for osteoclast differentiation [21, 22]. Therefore, the findings of our study [18] were consistent with the interpretation that the interaction between stromal cells and osteoclast precursor cells is important in the process of osteoclast formation.

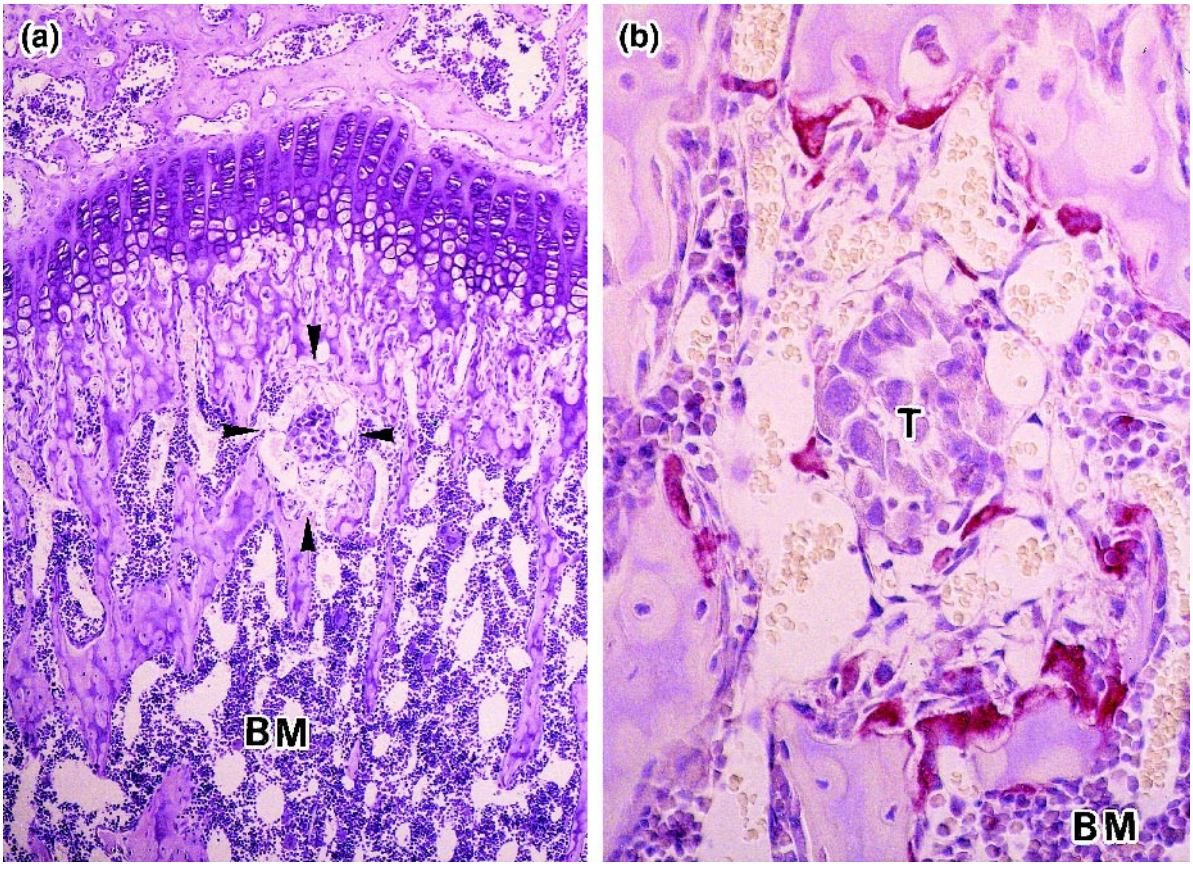

Figure 1. Metastatic lesions of A375 at 1 week. (a) A small tumour nest (arrow heads) showing trabecular bone resorption. (BM, bone marrow; haematoxylin and eosin staining, magnification ×25). (b) Higher magnification of the metastatic lesion. Well-vascularised stroma was present between tumour (T) and bone surface. Many red-stained TRAPase-positive cells were observed surrounding the tumour. (BM, bone marrow; TRAPase staining, magnification ×100.)

Because the character of the stromal cells was unknown, the localisation of alkaline phosphatase (ALPase) activity in these cells was studied. Most of the stromal cells between tumour cells and bone surface were shown to be positive for ALPase (Figure 4a). These ALPase-positive cells were in contact with TRAPase-positive cells away from the bone surface (Figure 4b). Thus, the presence of ALPase may be a characteristic feature of stromal cells supporting osteoclast formation.

To study the role of extracellular matrix for osteoclastgenesis, the distributions of heparan-sulphate proteoglycan (HSPG) and fibronectin, which are representative extracellular components, were examined immunohistochemically [18]. HSPG was observed in the stroma around tumour nests, but not in tumour cells. TRAPase-positive cells were embedded in an HSPG positive stroma. HSPG was also positive in the stroma away from bone surface. HSPG is a ubiquitous component of stroma, which retains heparin-binding growth factors such as FGF and GM-CSF [23, 24] and protects them from degradation and inactivation [25, 26]. It has also been reported that HSPG binds a bone resorption activating factor released from osteoblasts [27].

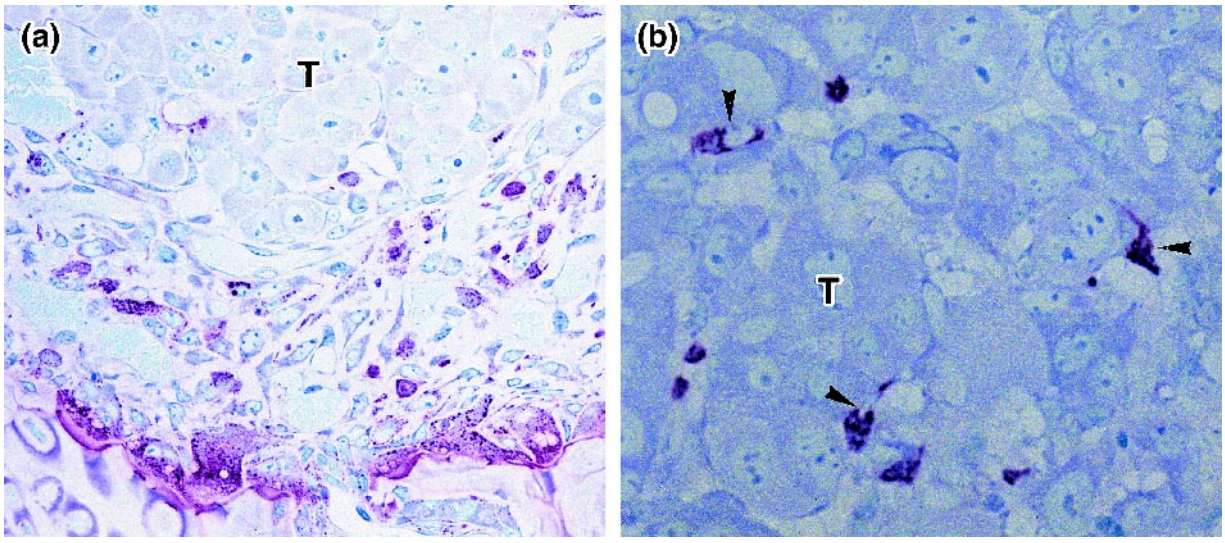

Figure 2. Metastatic lesions of A375 at 5 weeks. (a) Many TRAPase-positive multinucleated osteoclasts were seen on bone surface with formation of lacunae and a TRAPase-positive cement line. Between the tumour nest (T) and bone surface, a well-vascularised stromal cell layer was observed. TRAPase-positive mononuclear cells were present among stromal cells. (TRAPase staining, magnification ×100). (b) Single TRAPase-positive mononuclear cells (arrow heads) were present in a tumour nest (T) away from bone surface. (TRAPase staining, magnification ×250.)

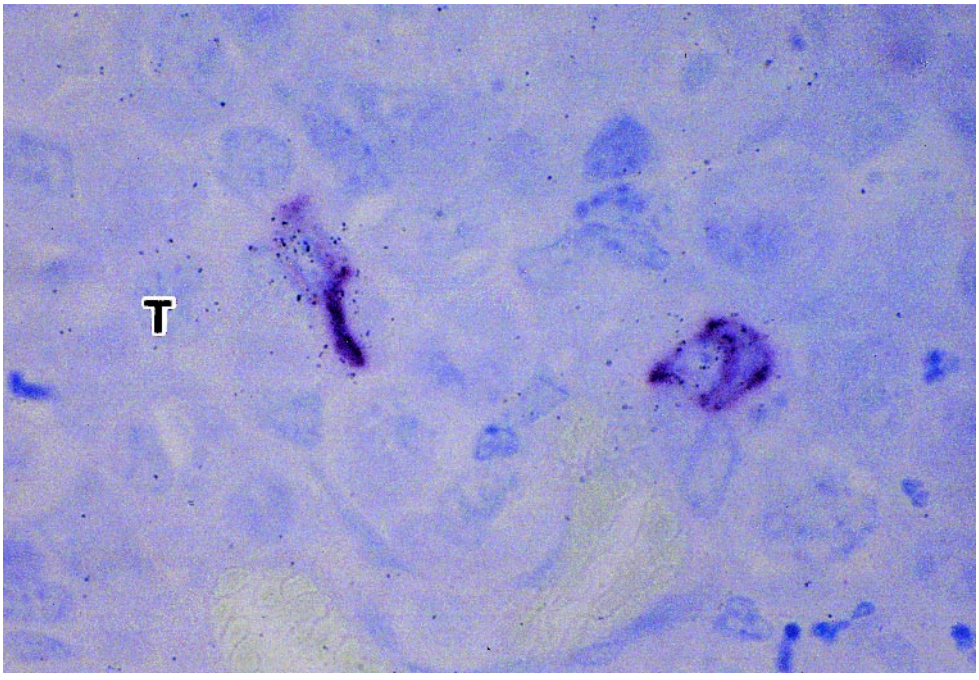

Figure 3. An autoradiograph of TRAPase-positive mononuclear cells apart from bone using ^{125}I-calcitonin. Dense deposition of grains on TRAPase-positive mononuclear cells was observed. (T, tumour cells; TRAPase staining, magnification ×250.)

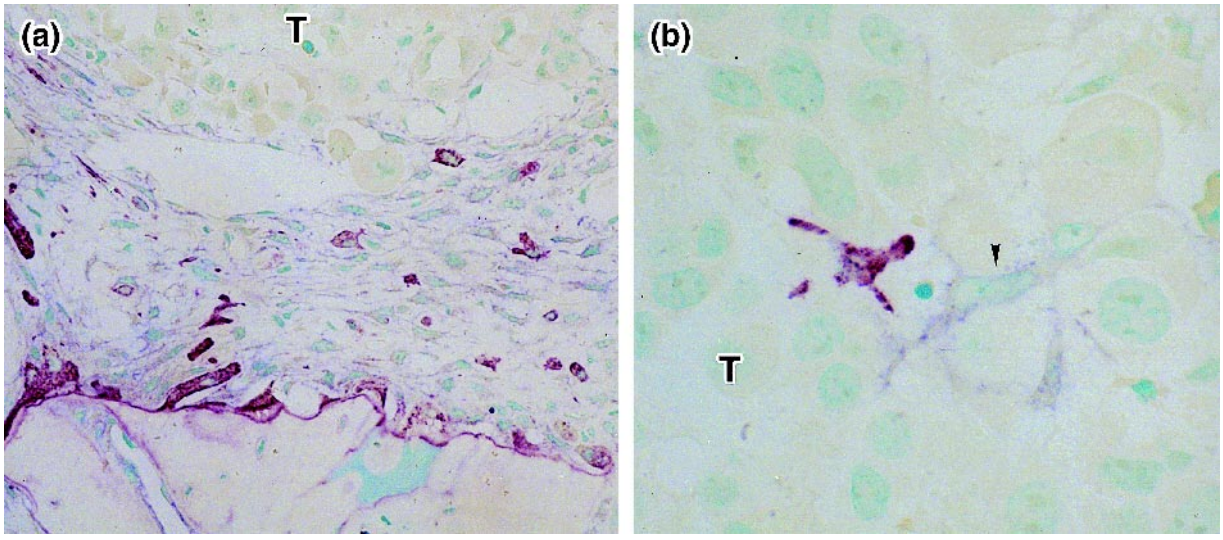

Figure 4. ALPase-positive cells in a metastatic lesion of A375. (a) Most of the stromal cells between tumour cells (T) and bone surface were positive for ALPase, stained blue. (ALPase and TRAPase double staining, magnification ×100). (b) Single TRAPase-positive mononuclear cells away from bone surface were in contact with ALPase-positive cells (arrow head). (T, tumour cells; ALPase and TRAPase double staining, magnification ×250.)

Fibronectin showed a pattern of localisation essentially similar to that of HSPG. In addition, fibronectin, which has heparin binding sites, may also be bound to heparan sulphate chains of HSPG. The findings that many osteoclast precursor cells were observed in a stroma rich in these extracellular matrix components may suggest that they play an important role in providing a microenvironment favourable for osteoclast differentiation and activation. Although the origin of HSPG and fibronectin is unknown, their dense distribution around tumour nests in our study leads us to believe that tumour cells may contribute to the deposition of these extracellular matrix components. The above relationships of tumour cells, osteoclast precursor cells, stromal cells and extracellular matrix are illustrated in Figure 5.

Some cytokines are known to be mediators of osteoclastic bone resorption. At the sites of bone metastases, tumour cells are a powerful producer of these cytokines. In an immunohistochemical study on localisation [18] of interleukin-6 (IL-6), prostaglandin E2 (PGE2) and TGF-α, which promote osteoclast differentiation and activation, IL-6 and PGE2 were recognized in stroma with TRAPase-positive cells around tumour cells, but not within tumour cells. TGF-α was weakly positive only within tumour cells. Thus, these cytokines are possible candidates for causing osteolysis at metastatic sites of A375.

In metastatic lesions of HARA [28], which is known to produce parathyroid hormone related peptide (PTHrP), there were many TRAPase-positive cells on the bone surface as well as away from bone. Also present were the formation of a stromal cell layer between tumour and bone surface and an osteogenic response around tumour nests (Figure 6). The findings were substantially similar to those of A375.

In the metastases of MDA-231 [16], which is also capable of producing PTHrP, the histological features were somewhat different from those of the previous two cell lines (Figure 7). An osteogenic response around the tumour was not observed in most lesions and tumour cells were in direct contact with bone marrow cells. Trabecular bones involved in metastatic tumours were resorbed. TRAPase-positive cells were observed on the residual bone surface in the close proximity of tumour nests, but their number was somewhat fewer and induction of stromal cells also seemed to be poorer compared with A375.

PTHrP has been reported to be important in causing bone resorption induced by human breast cancer [29]. However, its role in the HARA and MDA-231 models is unknown because its expression in the metastatic lesions of these cells has not been studied. Because A375, which does not produce PTHrP, induced bone resorption, PTHrP may play a role in some tumours, but is not always essential in tumour-induced bone resorption.

Direct bone resorption by tumour

Direct bone resorption by tumour cells is still controversial. In addition to osteoclasts, some cells have been reported to resorb bone directly (Table 2). According to Galasko [39], there are at least two main mechanisms for bone destruction by tumours. One, which is responsible for the early phase, is

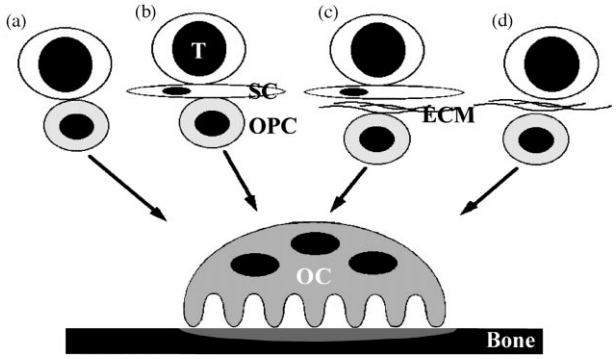

Figure 5. Schematic drawing of relationships of tumour cells, osteoclast precursor cells, stromal cells and extracellular matrix. Osteoclast precursor cells were occasionally apposed to tumour cells in some instances (a), but stromal cells and/or extracellular matrix usually existed between the two cells (b), (c), (d). (T, tumour cells; SC, stromal cells; ECM, extracellular matrix; OPC, osteoclast precursor cells.)

mediated by osteoclasts and the other is late bone destruction by a mechanism not involving osteoclasts. Eilon and colleagues [37] reported direct bone resorption by human breast cancer cells in an *in vitro* study. Recently, Orr and associates [40] and Sanchez-Sweatman and colleagues [38] also showed evidence of tumour-mediated osteolysis by B16/F1 melanoma cells. In bone metastasis by B16/F1, tumour cells were in direct contact with resorbing bone surfaces and the number of osteoclasts and osteoblasts was reduced dramatically in the affected bones.

In our animal study [18], many osteoclasts were usually observed at the sites of osteolysis suggesting that they are involved in tumour-induced bone resorption. At the tumour and bone interface, some cells, such as osteoclasts, bone lining cells and stromal cells, were usually present and tumour cells were rarely in direct contact with bone. Even if tumour cells were juxtaposed to bone surface with a scalloped appearance and osteoclasts could not be identified on H & E sections, TRAPase staining revealed the presence of osteoclasts. These osteoclasts were small and flat and TRAPase activity was weaker compared with that of typical osteoclasts. Obviously, they were not actively resorbing bone. The presence of these cells, however, may indicate that they had once been involved in bone resorption.

When studied with our model, B16/F1 cells formed the intertrabecular-type bone metastasis (Figure 8). Tumour cells grew and invaded into intertrabecular spaces without causing trabecular bone resorption. TRAPase-positive cells were observed on bone surface, but their number was not increased. We could not obtain evidence that would indicate direct bone resorption by B16/F1 cells, ultrastructurally (data not shown).

Because tumours develop from a variety of cells, it is difficult to deny completely the possibility of direct bone resorption by tumour cells, but bone resorption was always associated with the occurrence of osteoclasts in all tumour cells studied in our animal model. To prove direct bone resorption by tumour cells *in vivo*, at least ultrastructural evidence is needed.

Table 2. Cells that have been reported to resorb bone directly

	Reference
Osteoclast	
Nonosteoclastic cell	
Osteocyte	30, 31
Monocyte	32–34
Macrophage	35, 36
Tumour cells	
Human breast cancer cell	37
Mouse melanoma cell	38
VX2 carcinoma cell	39

Human specimens of bone metastasis

Because of the difficulty of performing a well controlled study, well documented reports on the morphology of bone metastasis in human specimens are relatively scarce. For instance, TRAPase staining is an easy method for detecting osteoclastic cell lineage [41], but its activity is lost by routine decalcification using formic acid. Therefore, though time consuming, we studied human specimens after decalcification with ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) to avoid inactivation of TRAPase activity.

Human specimens of bone metastasis from primary tumours of various organs, including colon, salivary gland, thyroid, breast, lung and stomach were examined. Histologically, bone marrow was replaced by tumour cells with a

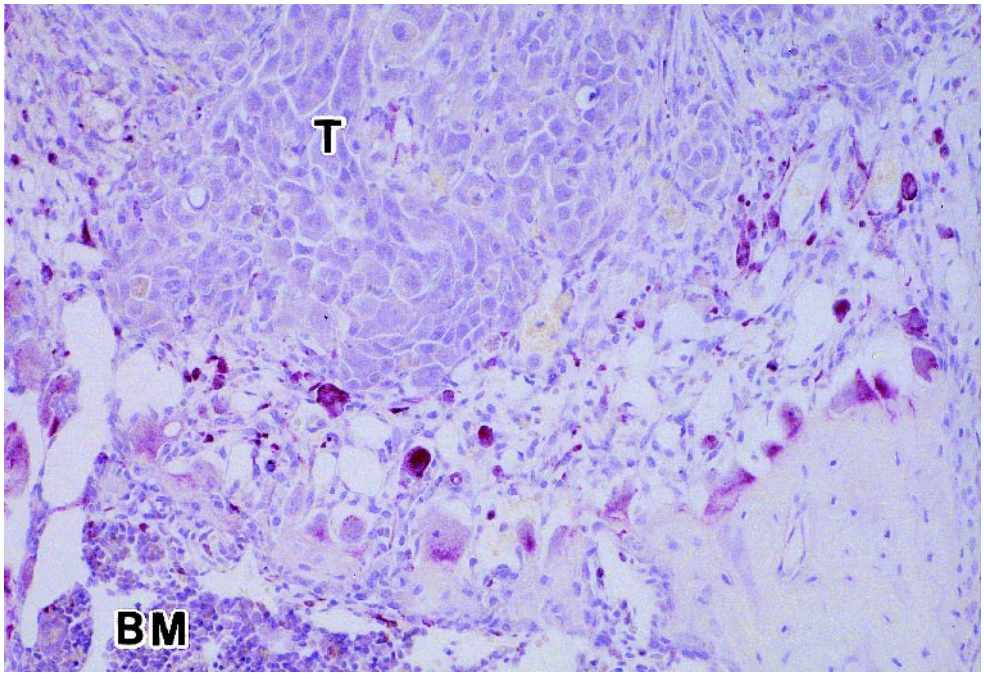

Figure 6. A metastatic lesion of HARA. There were many TRAPase-positive cells on bone surface as well as away from bone, formation of a stromal cell layer between tumour (T) and bone surface and an osteogenic response around tumour nests. (BM, bone marrow; TRAPase staining, magnification ×50.)

fibrous and well-vascularised stroma and most of the original bony architecture had been destroyed (Figure 9). Residual bones surrounding or in the metastatic lesions had scalloped surfaces. Tumour cells were rarely in direct contact with the bone surface. Histochemically, irrespective of the histology of the tumours, many TRAPase-positive cells were observed on the residual bone surface in all cases. TRAPase-positive small mononuclear cells were also observed in most lesions. There was no indication of direct bone resorption by tumour cells.

EFFECTS OF BISPHOSPHONATE ON BONE METASTASIS

Bisphosphonates are pyrophosphate analogues in which the oxygen bridge has been replaced by a carbon (P-C-P) with various side chains [42]. They tightly bind to calcified bone matrix, hydroxyapatite and inhibit bone resorption. A variety of bisphosphonates have been used in the treatment of hyperresorptive bone disease, such as hypercalcaemia, osteoporosis and bone metastasis. They are reported to reduce osteoclastic

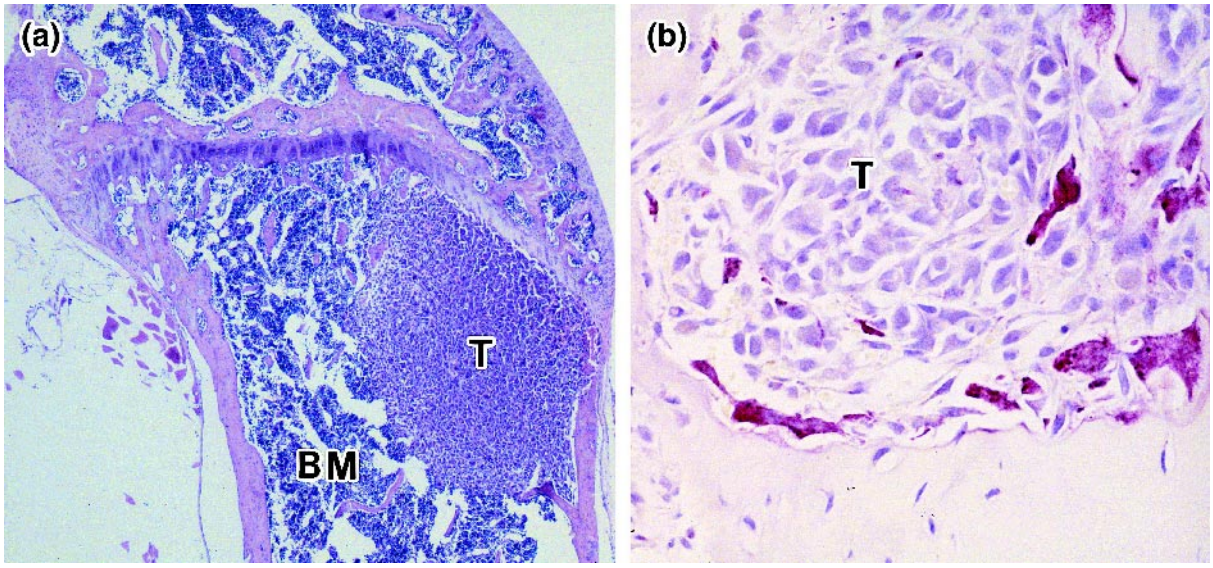

Figure 7. Metastatic lesions of MDA-231. (a) An osteogenic response around the tumour (T) was not observed, and tumour cells were in direct contact with bone marrow cells (BM). Trabecular bone involved in metastatic tumours was resorbed. (Haematoxylin and eosin staining, magnification ×5.) (b) TRAPase-positive cells were observed on residual bone surface in close proximity of tumour nests (T) of MDA-231, but their number was somewhat lower and induction of stromal cells was also poorer compared with A375 and HARA cells. (TRAPase staining, magnification ×100.)

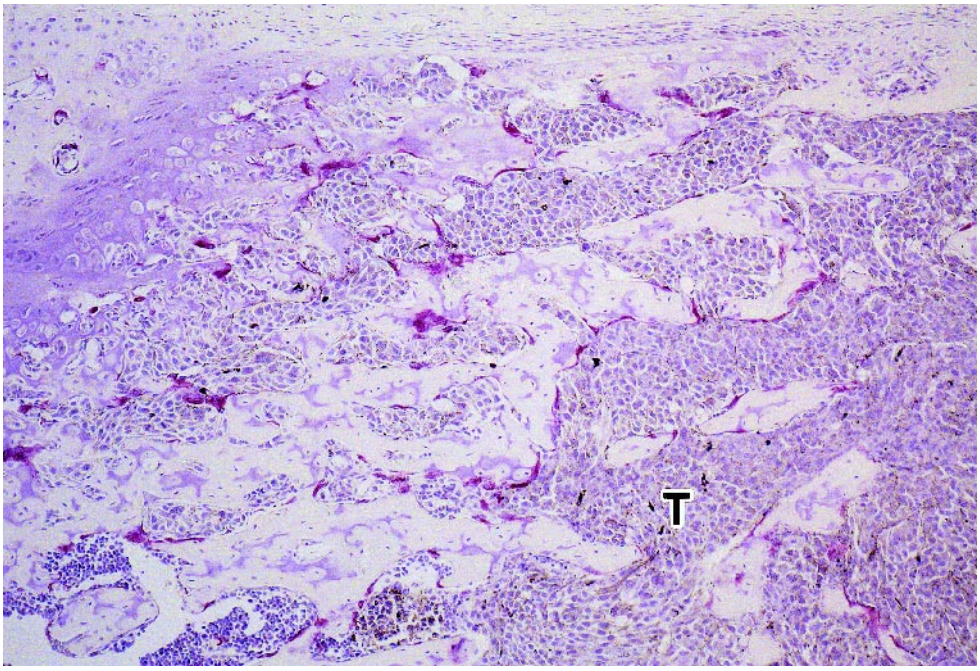

Figure 8. A metastatic lesion of B16/F1. Tumour cells (T) grew and invaded into intertrabecular spaces without causing trabecular bone resorption. TRAPase-positive cells were observed on bone surface, but their number was not increased. (TRAPase staining, magnification ×25.)

bone resorption through: inhibition of osteoclast recruitment to the bone surface; inhibition of osteoclast activity on the bone surface; and shortening of the osteoclast life span [42]. There is a unanimous agreement that the final target of bisphosphonate action is osteoclasts. At the molecular level, bisphosphonates are a potent inhibitor of the osteoclast vacuolar H^+-ATPase [43] and inhibit acid extrusion [44]. They also inhibit protein-tyrosine phosphatase (PTP) activity which regulates osteoclast formation and function [45, 46]. However, the detailed mechanism of action may differ among bisphosphonates and has not been fully elucidated. We studied the effects of bisphosphonates on bone metastasis morphologically using the animal model and human specimens.

Animal experiments

In the animal experiment with A375, a third-generation bisphosphonate (YM175; disodium dihydrogen (cycloheptylamino)-methylene-1,1-bisphosphonate) was used [47]. Four weeks after cell inoculation, YM175 (1 mg/kg) was administrated intravenously once and the animals were sacrificed 3 days later. The results were compared with those of animals given a saline injection.

There was no difference in the tumour size and the number of tumour cells between the experimental and control animals. There was also no sign of toxicity of YM175 on tumour cells. A layer of stromal cells between tumour cells and bone surface was extensively reduced in most areas and only a few osteoclasts were seen (Figure 10). The number of osteoclasts and the proportion (%) of bone surface with osteoclasts were significantly decreased at doses equal to or greater than 0.3 mg/kg (Figures 11 and 12) [48]. The results indicate that YM175 decreases the number of mature osteoclasts. Histochemically, most of the osteoclasts were only weakly stained with TRAPase and there was little evidence of cell polarity. Occasionally, they were vacuolated. Ultrastructurally, they were round and devoid of ruffled borders and clear zones. These morphological changes indicate a marked reduction in the activity of osteoclasts. Furthermore, some osteoclasts were undergoing necrosis or apoptosis, indicating that the bisphosphonate caused irreversible damage on osteoclasts. This was one of the reasons for the decrease of osteoclasts. In addition, there was an obvious decrease of osteoclast precursor cells and an extensive reduction of the stromal cell layer. However, the fine structures of osteoclast precursor cells, stromal cells and tumour cells were similar to those in the control group and their relationships were not substantially altered. Because stromal cells have been thought to play an important role for induction and differentiation of osteoclasts, the reduction of stromal cells may, in some way, be involved in the decrease of osteoclast precursor cells. This might also have been responsible for the decrease of osteoclasts.

Human specimens

Bisphosphonates have been used clinically by many investigators, but morphological changes induced by the agents in

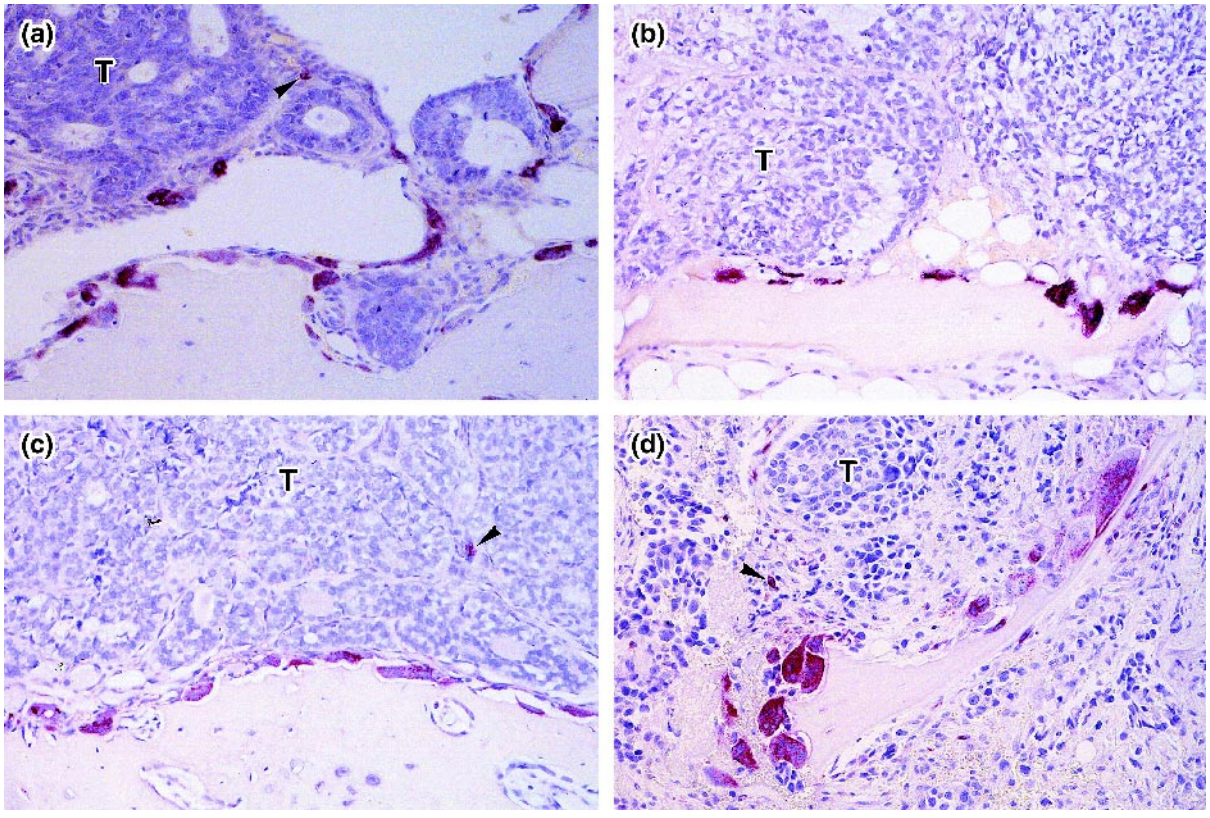

Figure 9. Metastatic lesions of human specimens. Bone marrow was replaced by growth of tumour cells (T) with fibrous and well-vascularised stroma and osteolytic response in most areas. Residual bones surrounding or in metastatic lesions had scalloped surfaces. Tumour cells were rarely in direct contact with bone surface. Many multinucleated TRAPase-positive cells were observed on residual bone surfaces in specimens of all cases. Small mononuclear TRAPase-positive cells (arrow heads) were also seen away from bone surface. (a) adenocarcinoma of colon; (b) pleomorphic adenocarcinoma of salivary gland; (c) adenocarcinoma of thyroid; (d) adenocarcinoma of breast. (TRAPase staining, magnification ×50.)

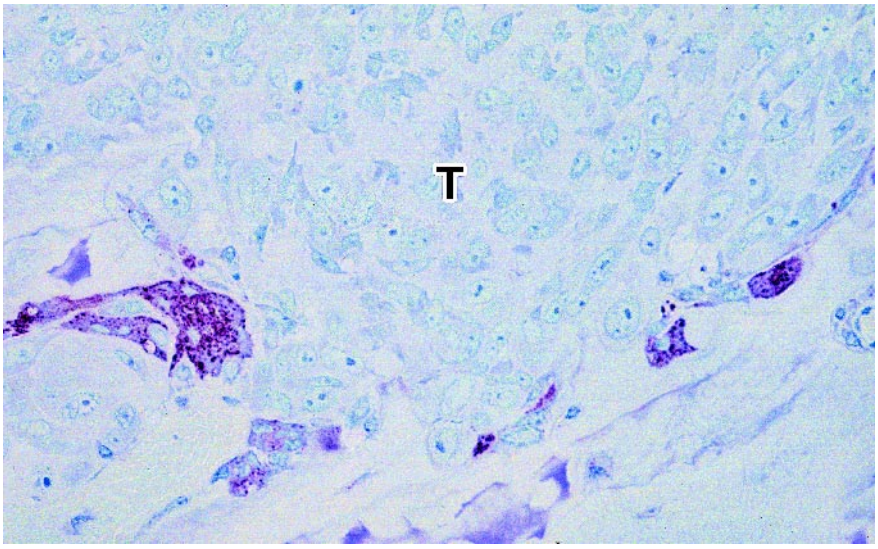

Figure 10. A metastatic lesion of A375 treated with YM175. There was no sign of toxicity of YM175 in tumour cells (T). The layer of stromal cells between tumour cells and bone layer was extensively reduced in most areas (compare with Figure 2(a) and only a few TRAPase-positive cells were seen. (TRAPase staining, magnification ×100.)

human samples are lacking. Because patients with bone metastasis are usually treated with various drugs and/or irradiation, it is difficult to study only the effects of bisphosphonates in humans.

We studied the effects of bisphosphonate on bone metastasis in a patient with a squamous cell carcinoma of the tongue [49]. The primary carcinoma and metastatic lymph nodes were successfully treated, but multiple bone metastases associated with hypercalcaemia developed later. Thus, elcatonin was given. It was initially effective, but the serum calcium level started to increase within a week, probably due to an escape phenomenon. Therefore, pamidronate was administrated. Then, the calcium level decreased, but the patient died 6 days after the pamidronate injection. Because the serum calcium level was increasing even with the administration of elcatonin, a rapid shift from increase to decrease in the calcium level after injection of pamidronate would have been due to the effects of pamidronate. Thus, pamidronate was probably most responsible for the histological changes in metastatic bone. The bone marrow was replaced by tumour cells and fibrous tissue. Abundant fibroblast-like stromal cells were observed around tumour nests. Some of the tumour nests were located close to bone, whose surface showed a scalloped appearance. These findings were essentially similar to those of experimental and human specimens without bisphosphonate treatment, but multinucleated giant cells were rarely seen on the bone surface. Histochemically, only a few small TRAPase-positive cells were seen. Most were stained weakly and/or homogeneously with no evidence of cell polarity and some were detached from the bone surface. Ultrastructurally, some osteoclasts showed a morphology indicative of degeneration. The findings were consistent with the results of the animal experiment.

Tumour cells obtained from a metastatic submandibular lymph node of the patient were cultured and injected in the left ventricle of nude mice as described previously to estimate the bone resorbing activity of tumour cells before bisphosphonate treatment. A metastatic lesion which developed in a

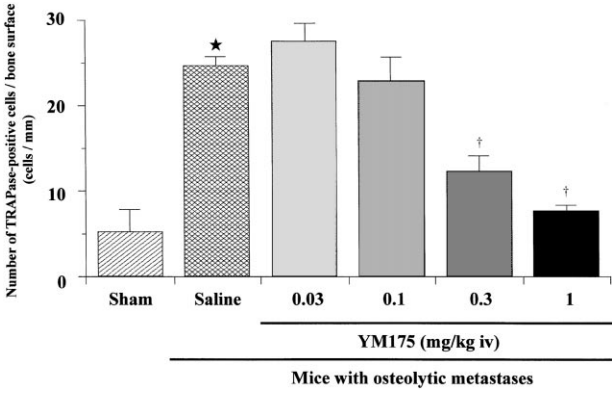

Figure 11. Effects of YM175 on the number of TRAPase-positive cells on bone surface in metastatic lesions of A375. Each column represents mean ± S.E. *$P < 0.001$ significantly different from sham-operated group (t-test). †$P < 0.01$ significantly different from saline group (Dunnett's multiple range test).

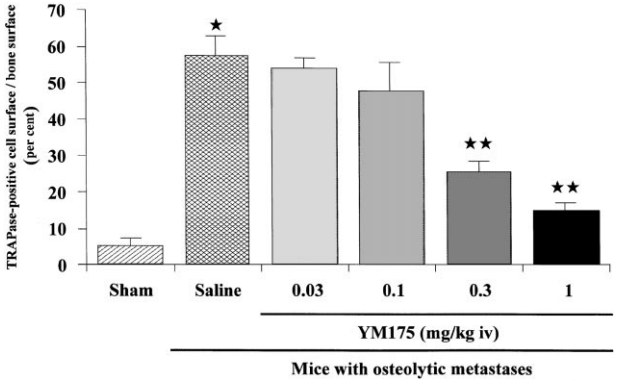

Figure 12. Effects of YM175 on the proportion (%) of bone surface with TRAPase-positive cells in metastatic lesions of A375. Each column represents mean ± S.E. *$P < 0.001$ significantly different from sham-operated group (t-test). †$P < 0.01$ significantly different from saline group (Dunnett's multiple range test).

vertebra consisted of nests of tumour cells with a stroma rich in stromal cells. Osteoclasts and their precursor cells were observed more frequently than in normal bone tissue. Most of the original bones had been replaced by the tumour and remaining bones were undergoing osteoclastic resorption. Because many TRAPase-positive cells were observed in the animal study, the tumour cells were shown to have a potent ability to induce osteoclast formation and activation. Therefore, pamidronate appeared to have been responsible for the decrease of TRAPase-positive cells in the autopsy specimens and the animal model proved to be useful for evaluating the bone resorbing activity of tumour cells.

CONCLUSION

In conclusion, bone resorption at the sites of metastases is mediated via osteoclasts and tumour cells may be involved in the differentiation and activation of osteoclasts in association with stromal cells, extracellular matrices and osteotropic cytokines. Bisphosphonates inhibit bone resorption induced by tumour cells by decreasing the number and activity of mature osteoclasts and possibly by affecting the production of osteoclast precursor cells. Because complicated cell-cell interaction is essential in inducing these reactions, further studies with more sophisticated methods are required to clarify the mechanism underlying bone resorption at the sites of tumour metastasis.

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