Osteoclasts form an acidic compartment at their attachment site in which bone mineralization and matrix degradation occur. Although both the cysteine proteinases and neutral collagensases participate in bone resorption, their roles have remained unclear. Here we show that interstitial collagenase has an essential role in initiating bone resorption, distinct from that of the cysteine proteinases. Treatment of osteoclasts with cysteine proteinase inhibitors did not affect the number of resorption lacunae (“pits”) formed on the surface of dentine slices, but it generated abnormal pits that were demineralized but filled with undegraded matrix. Treatment with metalloproteinase inhibitors did not alter the qualitative features of lacunae, but it greatly reduced the number of pits and surface area resorbed. Treatment of bone cells with an inhibitory anti-rat interstitial collagenase antiserum reduced bone resorption markedly. In the presence of collagenase inhibitors, resorption was restored by pretreatment of dentine slices with rat interstitial collagenase or by precoating the dentine slices with collagenase-derived gelatin peptides or heat-gelatinized collagen. Immunostaining revealed that interstitial collagenase is produced at high levels by stromal cells and osteoblasts adjacent to osteoclasts. These results indicate that interstitial collagenase can function as a “coupling factor,” allowing osteoclasts to initiate bone resorption by generating collagen fragments that activate osteoclasts.

Normal bone turnover is highly regulated. Osteoclasts, the cells that degrade bone, require activation to trigger their bone-resorptive capacity. Once activated, osteoclasts secrete both protons and proteinases at their attachment site, resulting in dissolution of bone mineral and degradation of the matrix (1). Osteoclasts produce several cysteine proteinases (2–4), enzymes with acidic pH optima, of which cathepsin K appears to be essential for normal bone resorption (5, 6). Studies indicate that cathepsin K plays a role in initiating bone resorption, distinct from that of cysteine proteinases and neutral collagenases participate in bone matrix degradation occur. Although both the cysteine proteinases and neutral collagensases contribute to osteoclast matrix degradation (11), recent evidence indicates that osteoclasts do not produce collagenase (12). Collagenase is produced by cells of osteoblastic lineage and may be required for resorption of intact bone tissue (13–16). Observations that isolated osteoclasts that had no detectable collagenase activity were able to resorb bone prompted the suggestion that collagenase promotes resorption by removing unmineralized matrix from the bone surface, facilitating osteoclast attachment (8, 17, 18).

In this study we have examined the qualitative and quantitative roles of acid cysteine proteinases and interstitial collagenase in bone resorption by mouse marrow osteoclasts on dentine wafers. We demonstrate distinct roles for the two classes of enzymes and show that collagen degradation by interstitial collagenase produces collagen fragments that activate osteoclast bone resorption.

**Experimental Procedures**

**Materials**—Reagents were supplied from Sigma unless noted otherwise. SC44463 is supplied by the Monsanto Corporation (St. Louis). 1,25-Dihydroxyvitamin D3, a generous gift from Dr. M. Uskoković (Hoffman-LaRoche; Nutley, NJ). Sperm whale teeth were obtained from the U.S. Department of Marine Fisheries.

**Assays of Resorption Pit Formation**—Marrow was flushed from mouse long bones and cultured on tissue culture plates (5 × 10⁷ cells/plate) in αMEM D10 (αMEM with 10% FBS) with 10⁻⁶ 1,25-dihydroxyvitamin D₃ (OC medium) as described (19). On day 6 adherent cells, which included many osteoclasts, as well as other cells, were scraped and replated in OC medium on 1-cm² sperm whale dentine slices in 24-well plates. Cultures were incubated on dentine slices from day 6 to day 10 with or without proteinase inhibitors (25 μM E64, 10 μM SC44463, 10 μM eglin C). Cells were then removed with 2% SDS and the bone slices prepared for scanning electron microscopy (scanning EM) as described (19). Surface area resorbed was quantified from a grid of 50-μm squares placed over photographs of three random fields taken with a tilt angle from at least three bone slices; grid intersections over pits were counted and expressed as a percentage of total intersections.

In experiments testing whether acid cysteine proteinase activity was required to remove matrix proteins from resorption lacunae, dentine slices were biotinated with 1:8 ml sulfosuccinimidyl 6-(biotinamido) hexanoate (Pierce) for 1 h at 22 °C in phosphate-buffered saline, pH 7.0; the slice was then washed thoroughly with αMEM D10 (αMEM with 10% FBS) with 10⁻⁶ 1,25-dihydroxyvitamin D₃ (OC medium) as described (19). On day 6 adherent cells, which included many osteoclasts, as well as other cells, were scraped and replated in OC medium on 1-cm² sperm whale dentine slices in 24-well plates. Cultures were incubated on dentine slices from day 6 to day 10 with or without proteinase inhibitors (25 μM E64, 10 μM SC44463, 10 μM eglin C). Cells were then removed with 2% SDS and the bone slices prepared for scanning electron microscopy (scanning EM) as described (19). Surface area resorbed was quantified from a grid of 50-μm squares placed over photographs of three random fields taken with a tilt angle from at least three bone slices; grid intersections over pits were counted and expressed as a percentage of total intersections.

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E64-treated slices were resuspended in 30 µM E64, 10 µM SC44463, 10 µM eglin C. Slices were processed for scanning EM and analyzed as described. Left panel, effect of inhibitors on indices of bone resorption. The average area of dentine analyzed was 840,000 ± m²; for controls, mean values for area analyzed were 43.2 pits, 1,880 ± m²/pit, and 8.9% of surface area resorbed. *p < 0.05 versus control. Right panel, representative fields of dentine slices incubated with mouse marrow cultures under conditions described above. A, control; B, E64; C, SC44463; D, eglin C. Scale bar = 50 µm.

For testing the effect of precoating dentine slices with collagen fragments or heat-denatured collagen on bone resorption, type I collagen from rat tendon (21) was gelatinized by incubation at 1.6 mg/ml in 0.4 M NaCl for 15 min at 60 °C. 1.6 mg/ml of the denatured collagen was incubated with 7.5 µg/ml rIC for 90 min at 37 °C; slices were then incubated with 1.0 mg/ml of the either the heat-denatured gelatin or proteolyzed gelatin for 3 h at 37 °C. The slices were washed and incubated overnight with αMEM D10 at 37 °C. Mouse marrow cultures for 5 days on plates in OC medium were scraped and applied to the slices ± SC44463 (25 µM) as indicated, and resorption pit formation was assayed after 5 days.

Determination of Collagen Fragments Associated with Latex Beads—Mouse marrow was cultured for 5 days on plates, scraped, and applied to biotinylated dentine slices ± 25 µM E64 (6 slices/treatment). After 5 days, slices were stripped of cells, labeled with streptavidin-coated latex beads, and digested with cathepsin B as described above. Beads released by proteolysis were collected by washing slices with a stream of phosphate-buffered saline (10 ml/slice) and centrifuging the bead-containing solution for 10 min at 10,000 × g. Beads from the control and E64-treated slices were resuspended in 30 µl of SDS-polyacrylamide gel electrophoresis sample buffer (22) and boiled for 10 min; the supernatant was separated by SDS-polyacrylamide gel electrophoresis on 15% gels, transferred electrochemically to nitrocellulose (Schleicher & Schuell), and probed with an anti-collagen(I) antibody (Monsanto).

Immunocytochemistry and Histochemistry—Mouse marrow cultures on dentine slices were fixed with 2% formaldehyde in HENAC for 30 min, washed, permeabilized with 0.1% Triton X-100 in HENAC for 30 min, washed, and incubated overnight with HENAC plus 10% FBS and 5 mM sodium azide at 4 °C. Slices were then incubated with anti-H'-ATPase monoclonal antibody E11 (64 µg/ml) (23), and either rabbit interstitial collagenase serum or preimmune serum (both at 1:1,000 dilution) in HENAC with 10% FBS for 2 h at 22 °C. Slices were washed in HENAC with 10% FBS and incubated for 1 h with Texas Red-conjugated anti-mouse IgG and fluorescein-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Park, PA), both diluted 1:500 in HENAC with 10% FBS. After an overnight wash, the slices were photographed by phase-contrast and fluorescent microscopy; the location of each photograph was recorded, and the slices were then stained for tartrate-resistant acid phosphatase and alkaline phosphatase activity with commercial kits (Sigma), following the manufacturer’s protocol.

RESULTS

Mouse bone marrow cells cultured for 5 days in the presence of 1,25-dihydroxyvitamin D₃ generate osteoclasts in vitro (24) and form resorption lacunae on sperm whale dentine wafers in a reproducible manner that can be assessed qualitatively and quantitatively by scanning EM (19). The effect on bone resorption of several proteinase inhibitors was examined in this system (Fig. 1). The matrix metalloproteinases (MMPs) are inhibited by tissue-derived inhibitors (TIMPs) and specific peptidomimetic hydroxamates that bind to zinc complexes at the catalytic site of the enzyme (25). Treatment of the marrow cultures with the peptidomimetic hydroxamate inhibitor SC444632 markedly decreased the number of resorption pits formed and the surface area (Fig. 1). In one experiment, the addition of 3.6 µM TIMP-1 (Kᵢ ~10⁻⁹ M), purified as described

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2 An inhibitor of mammalian MMPs: Kᵢ versus human MMP-1, 1.45 nm; Kᵢ versus rIC (MMP-13), 0.65 nm (26, 27).
Mouse marrow cultures treated with cysteine proteinase inhibitors form resorption pits containing undegraded matrix. Dentine slices were biotinylated as described under "Experimental Procedures," and marrow cultures were plated on dentine slices as in Fig. 1. After a 5-day incubation the cells were removed with 0.1 M NaOH, and slices were incubated with streptavidin-coated latex beads. Panels A and B, control slices without E64. Small arrows in panel B point to beads (not easily visible in panel A); large arrow denotes the same spot on panels A and B. Panels C, D, E, and F, dentine slices from E64-treated cultures stripped of cells with 0.1 M NaOH, incubated with streptavidin-latex beads, and then treated with cathepsin B (250 μg/ml) at 37 °C for 12 h at pH 7.4 (panels C and D) or at pH 5.0 (panels E and F). Pits were visualized by scanning EM as in Fig. 1. Bars = 5 μm in B, D, and F, 10 μm in A; 20 μm in C and E. Panel G, E64 inhibits matrix degradation in resorption pits. Biotinylated dentine slices were incubated with marrow cultures ± 25 μM E64 and coated with streptavidin beads; bead density (beads/μm²) over pit and non-pit areas was quantified by scanning EM before and after treatment of slices with cathepsin B. The increase in bead density over demineralized pits following E64 likely reflects greater access of the beads to biotinylated matrix. *p < 0.05. Panel H, release of bead-adherent collagen fragments by cathepsin B treatment of dentine slices preincubated with marrow cultures ± E64. Biotinylated slices were treated as in panel G; after treatment of slices with cathepsin B, beads were collected and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with an anti-collagen(I) antibody. Collagen fragments were much more abundant on beads recovered from the E64-treated wafers, indicating that collagen was much more susceptible to digestion by cathepsin B if acid cysteine proteases were inhibited during osteoclast bone resorption.
(28), also diminished both the number of resorption pits formed and surface area resorbed (not shown). In contrast, the cysteine proteinase inhibitors (29) E64 and leupeptin (not shown) did not affect significantly the number or surface area of resorption pits formed on the dentine slices (Fig. 1), nor did the serine proteinase inhibitor eglin C (30); the inhibition of resorption pit number was observed only with the metalloproteinase inhibitors.

Resorption pits formed in the presence of the metalloproteinase inhibitor appeared normal but were decreased in number (Fig. 1C, right panel). Pits formed in the presence of E64, however, were abnormal, with a shallow and “fuzzy” appearance (Fig. 1B, right panel); identical results were obtained with 25 μM leupeptin (data not shown). To determine whether the abnormal appearance was the result of undegraded matrix remaining in the pit, we biotinylated dentine slices; analysis of the slices by fluorescent confocal microscopy indicated that the biotin label only penetrated about 1 μm into the dentine slice, whereas normal pits range in depth from 2 to 10 μm. The biotin-labeled dentine slices were incubated with marrow cells in the presence or absence of E64 for 5 days, and cells were removed with 0.1 M NaOH. 250 nm streptavidin-conjugated latex beads were allowed to bind to the slices, and the slices from the E64-treated cultures were then incubated with cathepsin B either at pH 7.4 or 5.0.

In controls (Fig. 2, A and B), streptavidin-coated beads were found on the surface of the dentine slice, but few were found within pits, whose depth exceeded the 1-μm thickness of the biotin labeling. In the E64-treated cultures, with cathepsin post-treatment at pH 7.4 (at which cathepsin B is inactive), beads were abundant both on the undisturbed bone surface and within pits (Fig. 2, C and D), indicating that in the presence of E64, even the surface protein matrix of the bone is not removed efficiently. Slices from E64-treated cultures that were post-treated with cathepsin at pH 5 also had shallow pits, but few beads were present within the pits (Fig. 2, E and F). Fig. 2G provides a quantitative analysis of these observations. The increase in bead density over pits in the E64-treated cultures likely reflects increased access of the beads to the bone moieties in the demineralized matrix. To confirm that the cathepsin was removing beads by proteolysis of the dentine matrix, we collected beads after proteolysis from six slices from E64-treated cultures versus six slices from control cultures and analyzed them for collagen fragments by immunoblotting with an anti-collagen antibody. Numerous collagen digestion products were detected associated with beads from the slices from the E64-treated cultures, compared with relatively low levels of collagen fragments obtained from the control slices (Fig. 2H).

In summary, the results from Figs. 1 and 2 indicate that the cysteine proteinases are required to degrade the matrix in resorption lacunae and that the neutral metalloproteinases, in contrast, function in the initiation of resorption lacuna formation.

Of the known neutral matrix metalloproteinases, interstitial collagenase has been reported to be produced by rodent osteoblasts under certain conditions (13, 15, 31–33) and has been found associated with bone matrix proteins and possibly osteoclasts (11). To determine if interstitial collagenase was the metalloproteinase involved in initiating bone resorption, we treated the marrow cultures with a rabbit antiserum specific for rodent collagenase (anti-rIC; 34), which inhibits its enzymatic activity (34, 35). Anti-rIC antiserum inhibited resorption by the marrow cultures in a concentration-dependent manner, with maximal inhibition comparable to that of peptidomimetic hydroxamat (Fig. 3A, inset). In cultures treated with anti-rIC, the initiation of bone resorption was restored by pretreatment of dentine slices with rIC (Fig. 3A). These results indicate that interstitial collagenase is the major metalloproteinase involved in initiating bone resorption in mouse marrow cultures.

It has been proposed that collagenase could promote bone resorption by enhancing osteoclast attachment to the bone surface (8, 17, 18). We found, however, that the number of attached osteoclasts in cultures treated with metalloproteinase inhibitors was no different from controls (Fig. 3B). Alternatively, collagenase could promote bone resorption by generating fragments of collagen which activate osteoclasts directly. To address this possibility, we examined the effect on resorption pit formation of treating dentine slices with gelatin predigested in vitro by rIC. As shown in Fig. 3C, treatment of marrow cultures with SC44463 inhibited resorption pit formation, as demonstrated above, but incubation of the bone slices with gelatin fragments generated by in vitro digestion with purified interstitial collagenase restored resorption activity to levels greater than or equal to control levels in the continued presence of SC44463. These results indicate that interstitial collagenase can initiate bone resorption by generating gelatin fragments that activate osteoclasts, rather than by clearing the bone surface of collagen.

Collagenase cleavage of collagen could be producing an activation signal either by generating a specific peptide as a cleavage product or by lowering the melting temperature of the collagen helix (9), allowing it to expose an activation signal concealed in the helical conformation. We found that heat-denatured gelatin was nearly as efficient as collagenase-cleaved gelatin at restoring bone resorption in collagenase-inhibited mouse marrow cultures (Fig. 3C), providing strong evidence in support of the latter mechanism.

Antiserum to interstitial collagenase was used to examine the immunocytochemical distribution of the enzyme in the marrow cultures on bone slices. Collagenase was not detected in osteoclasts but was found at high levels in small cells having a fibroblast-like appearance, some of which stained for alkaline phosphatase, a marker for osteoblasts (Fig. 4). Cells that stained with the collagenase antibody were often observed surrounding resorptive osteoclasts, suggesting the possibility that they might be engaged in initiating bone resorption. These findings do not eliminate the possibility that osteoclasts also produce interstitial collagenase; but if they do, it must be present at low levels compared with the surrounding stromal cells.

**DISCUSSION**

This study demonstrates that the initiation of bone resorption and formation of resorption lacunae are two independently controlled processes that require different classes of proteinases. Cysteine proteinases are required for degradation of matrix proteins in the resorption lacunae. Inhibition of cysteine proteinases produces abnormal pits but does not affect the surface area of bone resorbed. Interstitial collagenase, in contrast, is essential for the initiation of bone resorption in this system but not for degradation of mineralized matrix.

Several cysteine proteinases (cathepsins) have been investigated for their possible involvement in bone resorption (7, 36, 37). The recently discovered cathepsin K appears to be the most abundant cathepsin produced by osteoclasts (5, 38, 39), and human mutations in cathepsin K produce severe abnormalities in bone resorption (6). In this study we have provided direct experimental evidence that cysteine proteinases are required to degrade bone matrix proteins but are not required for osteoclast activation.

Several prior studies have indicated that interstitial colla-
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Genase is required for bone resorption (3, 8, 17, 18). The most widely held view has been that collagenase removes unmineralized osteoid, allowing osteoclasts to adhere to mineralized bone which triggers bone-resorptive activity (8, 17, 18). The present study provides evidence against this model, since the dentine slices have no unmineralized matrix layer, the presence or absence of interstitial collagenase activity had no effect on the number of adherent osteoclasts, and the addition of predigested gelatin to the bone slices stimulated resorption even in the presence of metalloproteinase inhibitor.

Our results indicate an alternative mechanism: that interstitial collagenase generates collagen degradation fragments that activate osteoclasts to resorb bone. What precisely does collagenase cleavage do to trigger bone resorption? Precoating dentine slices with heat-denatured but uncleaved gelatin restored normal bone resorption in the presence of collagenase inhibitors, suggesting that crucial key event is loss of the helical collagen structure. Collagen has binding sites for $\alpha_2\beta_1$ and $\alpha_V\beta_3$ integrins, both of which are found on the surface of osteoclasts. A recent study found that osteoclasts adhere to undenatured collagen through $\alpha_2\beta_1$ and to denatured collagen through $\alpha_V\beta_3$; adherence to the two different substrates coated on glass coverslips produced distinct physiologic changes in osteoclasts (40). Hence, collagenase cleavage of collagen, which results in a loss of helical collagen structure at 37 °C (41), could expose $\alpha_V\beta_3$ binding sites, an event that may be involved in osteoclast activation.

The predominant human collagenase in most human tissues, collagenase-1 (MMP-1), cleaves helical collagen but has little telopeptidase or gelatinolytic activity (21, 42). In transgenic mice mutated to disrupt the cleavage site in helical collagen, however, only modest abnormalities of bone remodeling were observed (43), suggesting that proteinases with substrate specificities different from human MMP-1 may be involved in bone remodeling. The substrate specificity of interstitial collagenase (MMP-13), the only collagenase in rodents, differs significantly from human MMP-1; it possesses telopeptidase and gelatinolytic activity in addition to cleaving helical collagen (43). An analogous human collagenase (collagenase-3; MMP-13) recently identified in human chondrocytes (44, 45), appears to be the human homolog of rodent interstitial collagenase. Our

![Fig. 3](image)

**Fig. 3.** Panel A, effect of anti-rat interstitial collagenase antibody or preincubation with collagenase on resorption pit formation. Mouse marrow was cultured and applied to dentine slices as in Fig. 1, but in the presence of a 1:50 dilution of preimmune antiserum (1:50) or anti-rIC antiserum (1:50). For the collagenase preincubation experiment, dentine slices were incubated as indicated under “Experimental Procedures” for 12 h with 250 μg/ml rat interstitial collagenase activated as described previously (34, 35). The average area of dentine analyzed was 762,300 μm²; for controls, mean values for area analyzed were 30.4 pits, 1,563 μm²/pit, and 6.4% of surface area resorbed. Inset, effect of anti-rIC antiserum dilution on resorption pit formation. *p < 0.05 versus controls. Panel B, effect of collagenase treatment on osteoclast adherence. Mouse marrow was cultured on dentine slices as in Fig. 1, in the presence of the metalloproteinase inhibitor SC44463 (25 μM). Slices were then fixed, stained for tartrate-resistant acid phosphatase, and the number of adherent tartrate-resistant acid phosphatase-positive mononuclear, multinucleated, and giant cells was counted (19). Data are mean from three slices/treatment ± S.E. Panel C, effect of precoating dentine slices with collagen fragments or heat-denatured gelatin on bone resorption in the presence of metalloproteinase inhibitor. Rat type I collagen was incubated with 7.5 μg/ml rIC for 90 min at 37 °C to produce small cleavage fragments (confirmed by SDS-polyacrylamide gel electrophoresis); dentine slices were incubated with 1.6 mg/ml of either the proteolyzed collagen or the heat-denatured (but uncleaved) gelatin. Mouse marrow cultures were incubated on the slices as in Fig. 1 ± SC44463 (25 μM) as indicated in the figure. The average area of dentine analyzed was 832,500 μm²; for controls, mean values for area analyzed were 30.0 pits, 3,126 μm²/pit, and 10.8% of surface area resorbed. *p < 0.05 versus controls.
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Fig. 4. Localization of interstitial collagenase in marrow cultures on bone. Mouse marrow cultures on dentine slices were fixed and labeled with antibodies and histochemical stains. Panel A, phase-contrast image; panel B, anti-H-1-ATPase antibody E11 (23) (64 μg/ml) detected with Texas Red-labeled anti-mouse IgG; panel C, anti-IC (1:10,000 dilution) detected with fluorescein isothiocyanate-labeled anti-rabbit IgG; panel D, tartrate-resistant acid phosphatase (red) and alkaline phosphatase (blue). Panel E, preimmune serum control for panel C showing no staining for collagenase. Panel F, tartrate-resistant acid phosphatase (red) and alkaline phosphatase (blue) in the same field as panel E, demonstrating that osteoblasts and osteoclasts are present. Panels A–D, two small arrows provide points of reference. Large open arrow indicates osteoclast (seen in panels B and D) located over a large resorption pit (seen in panel A). Panel C, collagenase staining is present in cells surrounding the pit, but not within the pit, and is most intense in areas with few alkaline phosphatase-positive cells (large filled arrow shows osteoblasts with little or no staining for collagenase). Scale bar = 20 μm.

studies suggest the possibility that the telopeptidase and gelatinolytic activities of MMP-13 may have an essential role in activating human bone resorption. As collagenase was detectable only in osteoblasts and other non-osteoclastic cells, the enzyme may function as a “coupling factor” allowing osteoblasts to exert control over osteoclast resorptive activity.