(Pro)collagenase (matrix metalloproteinase-1) is present in rodent osteoclasts and in the underlying bone-resorbing compartment

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SUMMARY

Osteoclasts resorb the extracellular matrix of bone by secreting enzymes and acid into a sealed-off compartment that they form upon attachment to the bone surface. Although the lysosomal cysteine proteinases can degrade collagen after the demineralization of bone at low pH, several lines of evidence suggest that collagenase (matrix metalloproteinase-1, EC 3.4.24.7) may also be involved in this process. The question of whether collagenase is present in the osteoclast and/or in the bone-resorbing compartment has however not been resolved.

We have prepared an anti-mouse collagenase antiserum and affinity-purified an IgG fraction that specifically immunoblots and immunoprecipitates (pro)collagenase. Using these antibodies, we demonstrate by immunolocalization the presence of (pro)collagenase both in the osteoclasts and in the extracellular subosteoclastic bone-resorbing compartment. These specific localizations were observed not only in mice but also in rat and rabbit osteoclasts and using not only the antibody we have prepared but also antibodies raised in other laboratories against rat (Jeffrey et al., J. Cell. Physiol. 143, 396-403, 1990) and rabbit (Brinckerhoff et al., J. Biol. Chem. 265, 22262-22269, 1990) collagenase. Intracellular collagenase was observed in the osteoclasts whether the cells were plated on bone or cultured on glass coverslips. It is proposed that osteoclastic collagenase is secreted in the resorbing compartment where it may cooperate with the lysosomal cysteine proteinases in the degradation of the collagen component of the matrix during the resorption of bone.

Key words: (pro)collagenase, matrix metalloproteinase, osteoclast, bone resorption, extracellular proteolysis, immunolocalization

INTRODUCTION

The resorption of bone requires the removal of both the mineral and the organic (collagenous and non-collagenous) matrix components. This degradative process occurs in an extracellular compartment acidified by proton transport at the osteoclast ruffled border membrane (Baron et al., 1985; Blair et al., 1989a; Chatterjee et al., 1992). The low pH allows dissolution of the mineral phase, exposes the organic matrix and favors the degradative action of lysosomal enzymes (Vaes, 1968, 1988; Baron, 1989). In this acid environment, the lysosomal cysteine proteinases (i.e. non-specific collagenolytic acid proteases) secreted by the osteoclast in the bone-resorbing compartment are not only involved in the degradation of collagen (Delaissé et al., 1980, 1984, 1987; Everts et al., 1988; Rifkin et al., 1991) but could also be sufficient for its complete degradation without requiring the participation of collagenase (i.e. matrix metalloproteinase-1 or MMP-1, EC 3.4.24.7, a member of a distinct class of enzymes characterized by its specific ability to cleave collagen within the triple-helical body of its native molecule).

Collagenase may nevertheless be implicated in the bone-resorption process. Despite early reports that collagenase could not be detected in osteoclasts (Sakamoto and Sakamoto, 1984; Blair et al., 1986a, 1989b), agents that stimulate bone resorption in organ culture have been reported to enhance the accumulation of collagenase in bone (Delaissé et al., 1988); inhibitors of matrix metalloproteinases prevent the resorption of cultured bone explants (Delaissé et al., 1985, 1988) and interfere with the processes leading to collagen degradation in the subosteoclastic resorption compartment (Everts et al., 1992). On the other hand, the secretion of collagenase by osteoblasts is also regulated in vitro (Heath et al., 1984; Otsuka et al., 1984; Sakamoto and Sakamoto, 1984; Partridge et al., 1987) and complete extraction of the collagenase present in bone requires demineralization (Eeckhout et al., 1986; Delaissé et al., 1988), which led to the hypothesis that it could be localized within the mineralized matrix, where it may have been deposited during osteogenesis (Gillet et al., 1977; Mechanic et al., 1982). Hence, although collagenase may be involved in the resorption of bone, its localization relative to osteoclasts and resorbing areas remains undetermined.
The present study was therefore designed to obtain collagenase-specific antibodies and further determine by immunolocalization the relationship between collagenase and bone-resorbing cells. Our results demonstrate the presence of this enzyme in the osteoclasts and in the subosteoclastic bone-resorbing compartment, thereby suggesting that osteoclastic collagenase plays a direct role in the degradation of the bone matrix.

MATERIALS AND METHODS

Materials

Chemicals for electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). CNBr-activated Sepharose, Protein A-Sepharose, Protein G-Sepharose and chromatography media were from Pharmacia LKB Biotechnology (Uppsala, Sweden). Biotinylated anti-goat IgG and streptavidin-biotinylated peroxidase were from Amersham (UK). All other reagents used were of analytical grade.

Source, purification and assay of procollagenase

Conditioned medium of newborn mouse calvaria cultured in the presence of 0.3 mg/ml heparin (Vaes, 1972) was the source of procollagenase. The following successive purification steps were done in the presence of 50 mM cacodylate buffer, pH 7.5 mM CaCl₂, 0.01% Triton X-100 and 0.02% NaCl: (1) affinity chromatography on heparin-Sepharose; elution with a linear (0.1 to 1.3 M) NaCl gradient; (2) ion-exchange chromatography on DEAE-Sepharose; elution with a linear (0.1 to 0.6 M) NaCl gradient; (3) gel filtration on Ultrogel ACA 54 in the presence of 1 M NaCl; (4) dialysis of purified procollagenase (3,000 to 4,000 units/mg protein) against 0.15 M NaCl. Procollagenase was activated by trypsin and assayed as previously described (Eeckhout et al., 1986).

Preparation of antibodies to (pro)collagenase and immunoaffinity columns

A 1 ml (80 µg) sample of purified (partially autoactivated) procollagenase was emulsified with an equal volume of complete Freund’s adjuvant and injected into three intradermal sites of an adult goat. Two further injections were given on days 14 and 28. A bleed of 400 ml, taken on day 56, served as a source of anti-(pro)collagenase serum and IgG. The IgG from preimmune (IgG/PR) and immune (IgG/AC) sera were prepared by ammonium sulfate precipitation (40%, pH 6.8) of the whole sera, purified with DEAE-Sepharose; elution with a linear (0.1 to 0.6 M) NaCl gradient; (3) gel filtration on Ultrogel AcA 54 in the presence of 1 M NaCl; (4) dialysis of purified procollagenase (3,000 to 4,000 units/mg protein) against 0.15 M NaCl. Procollagenase was activated using A (1%; 1 cm, 280 nm)=14.0.

IgG/PR and IgG/AC were coupled to CNBr-activated Sepharose 4B® (5 mg IgG/ml drained gel) as described by the manufacturer. The IgG (PR or AC)-Sepharose columns were equilibrated with CCTN buffer (0.05 M cacodylate, pH 7.0, 5 mM CaCl₂, 0.05% (w/v) Triton X-100 and 0.03 M NaCl) supplemented with 0.15 M NaCl. The antigens (conditioned medium of cultured mouse calvaria containing 18 units (pro)collagenase) were preincubated in the column, for 1 hour at room temperature, with 4 ml drained gel. The columns were sequentially eluted with 8 ml 0.15 M NaCl in CCTN buffer (fraction 1), 8 ml 1 M NaCl in CCTN buffer (fraction 2) and 8 ml 3 M KSCN in CCTN buffer (fraction 3).

Immunocytochemistry on bone tissue

Four-day-old Wistar rat pups, 5-day-old C57 mouse pups or 10-day-old New Zealand rabbits were perfused via the femoral artery with PBS for 1 minute followed by parafomaldehyde (2%)/lysine (0.75 M)/sodium periodate (0.01 M) (PLP) for 5 minutes. The proximal tibiae were dissected out and slices cut out of the primary spongiosa area under the growth plate. The slices were fixed in PBS-Tween containing 10% DMSO as a cryoprotectant. Some sections were returned to PLP fix containing 4% EDTA and 5% PVP for 2 hours at 4°C with agitation. The remaining sections were rapidly decalcified by incubation either (routinely) in 4% EDTA, 0.44% NaOH, 5% PVP or (when indicated)

Affinity purification of anti-(pro)collagenase antibodies from immunoblots

Electrophoretic immunoblotting was performed essentially as described by Towbin et al. (1979) using Tween-20 as a blocking agent (Batteiger et al., 1982). (Pro)collagenase-enriched culture media were dissolved in 1% (w/v) SDS non-reducing sample buffer and resolved by electrophoresis (Laemmli, 1970) in a 11.3% polyacrylamide slab gel (13 cm × 18 cm × 0.15 cm) with two molecular mass markers slots and a single sample slot extending most of the width of the 4.6% polyacrylamide stacking gel. Electrophoresis was at 55 V until the tracking dye was within 1 cm of the bottom of the separating gel.

Electrophoretic transfer of the resolved proteins was done at 180 mA for 4 to 5 hours in a Trans-Blot® Cell (Bio-Rad). The transfer efficiency was checked by staining the polyacrylamide gel with Coomassie brilliant blue. The nitrocellulose membrane (NCM) was blocked with PBS containing 0.05% (v/v) Tween-20 (PBS-Tween). The two lateral strips bearing the molecular mass markers were excised and stained with Ponceau S solution or Indian ink. The remaining central NCM part was divided into strips (0.5 to 1.0 cm), either vertical ones for staining or horizontal ones for affinity purification of antibodies.

Antigenic proteins bound to nitrocellulose were detected by incubating the strips with the IgG diluted in PBS-Tween under slight agitation, either for 3 hours at 35°C or for 16 hours at 4°C and 1 hour at 35°C. The strips were washed in PBS-Tween (3 changes during 15 to 30 minutes, total), incubated for 2 hours at room temperature with the second antibody (biotinylated anti-goat IgG) diluted 400-fold in PBS-Tween and washed again as before. The biotinylated antibodies were revealed by an incubation of 30 minutes at room temperature with peroxidase-streptavidin diluted 400-fold in PBS-Tween, a 10 minute wash with PBS-Tween followed by a similar wash with 20 mM Tris-HCl, 0.5 M NaCl, pH 7.5, and a final incubation with 4-chloronaphthol and H₂O₂.

A horizontal strip corresponding to the antigenic bands of 59-65 kDa was excised from unstained blots and used for the affinity purification of antibodies (Olmdsted, 1981). The strip was incubated for 3 hours at 35°C with antibody (20 to 100 µg/ml) diluted in PBS-Tween and washed during 5 to 10 minutes (3 changes) in PBS-Tween. The specific antibodies were eluted by incubating the strip for 2 minutes at room temperature in 3 ml 0.2 M glycine-HCl, pH 2.8, containing 0.01 mg/ml gelatin. The eluted material was immediately neutralized (final pH was 7.0 to 7.7) with a titrated volume (200 µl) of 1 M Tris-base containing 1.6 mg/ml sodium azide and stored at 4°C. The NCM strip was rinsed with PBS-Tween containing 0.1 mg/ml sodium azide and reused for several absorption cycles. Concentration of affinity-purified antibodies was accomplished by dialysis under vacuum. The technique was used to elute antibodies binding to other antigenic bands present on our immunoblots (see below).
in 40% formic acid, 8% sodium formate (Vermeulen et al., 1989) for 2 to 16 hours at 4°C with agitation. In the experiments designed to detect intracellular signals, the specimens were treated (90 minutes at 37°C) with chondroitinase ABC at a concentration of 0.24 unit/ml PBS in order to enhance the immunoreactivity of the antigen (Pelletier et al., 1990). All sections were washed (2 hours) in PBS and incubated overnight in the respective primary antibodies diluted at about 40 µg/ml PBS + 0.1% BSA.

After washing (2 hours) in PBS + 0.1% BSA, the sections were incubated with Fab fragments of peroxidase-labeled rabbit anti-goat IgG (Biosys, France) for the mouse anti-collagenase primary, goat anti-rabbit IgG for the rat anti-collagenase primary and goat anti-rabbit for the anti-collagen primary. Sections were incubated at a dilution of 1:100 in PBS + 1% BSA for 2 hours at 20°C. After washing, the sections were reacted in DAB (1 mg/ml in 0.05 M Tris buffer, pH 7.4, Polysciences) in the presence of 0.1% H2O2 and post-fixed in ferrocyanide-reduced OsO4. After embedding in Epon (Polybed 812, Polysciences, Inc., Warrington, PA) 1 µm thick sections were cut with a glass knife and counterstained with methylene blue-azure II for identification of areas of interest. Selected areas were then sectioned with a diamond knife and stained with lead citrate. Grids were viewed on a JEOL-CX 100 electron microscope.

**Immunocytochemistry on isolated bone cells**

Cells were isolated from long bones of rat pups or 10-day-old rabbits as previously described (Ali et al., 1984) and allowed to settle on cortical bone slices or on glass coverslips. After 30 minutes non-adherent cells were discarded by shaking the slices vigorously. The remaining cells were cultured in fresh α-MEM medium supplemented with 10% heat-inactivated fetal calf serum. After 18 hours of culture, the specimens were processed for immunocytochemistry at room temperature. They were fixed for 10 minutes in 3.7% formaldehyde, washed in PBS, incubated for 30 minutes in PBS containing 0.5% BSA and 0.05% saponin (to permeabilize the cells), and thereafter for 90 minutes in the primary antibody diluted in the latter buffer at a concentration of about 40 µg/ml. This was followed by a washing in the same buffer (30 minutes) and an incubation (1 hour) in the same buffer containing rhodamine-labeled rabbit anti-goat IgG for the mouse collagenase primary antibody (Cappel-Organon Teknika Corp., West Chester, PA) and FITC-labeled goat anti-sheep IgG for the rabbit collagenase primary (Boehringer, Mannheim, Germany) at a dilution of 1:100. The specimens were washed, mounted and observed by epifluorescence, either on a Zeiss Axiophot microscope (with a 546 nm excitation filter and a 590 nm arrest filter) or on a Bio-Rad confocal microscope equipped with an argon-krypton laser beam. Confocal images were collected as the average of 9 to 15 scans of one optical section of 2 to 3 µm for cells viewed at ×40 magnification and 0.5 to 1 µm for cells viewed at ×63 magnification. The images are stored on optical disks and viewed on a Gateway 2000 or NEC MultiSync computer system. The images are optimized by subtracting the background and by normalizing to the maximum pixel intensity of that image.

**RESULTS**

**Characterization of the antibodies to mouse bone collagenase**

Immune IgG, but not preimmune ones, inhibited and precipitated bone collagenase from mouse (Table 1) and rat (not shown). Antibody specificity was checked by Ouchterlony double diffusion, crossed immunoelectrophoresis, western blot analysis and immunoaffinity chromatography.

**Table 1. Inhibition and precipitation of collagenase by anti-collagenase antibodies**

<table>
<thead>
<tr>
<th>Coprecipitating agent</th>
<th>Rabbit anti-(goat) IgG</th>
<th>Protein A-Sepharose</th>
<th>Protein G-Sepharose</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Total preimmune IgG†</td>
<td>90</td>
<td>89</td>
<td>105</td>
</tr>
<tr>
<td>Total immune IgG†</td>
<td>7</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>IgG eluted from blot</td>
<td>95</td>
<td>53</td>
<td>36</td>
</tr>
</tbody>
</table>

Total IgG or affinity-purified IgG were tested for their capacity to precipitate collagenase. A horizontal strip cut out at the level of the 59-65 kDa doublet bands (see Fig. 1A lanes T1 and T3) was used for 18 successive adsorptions and elutions of antibodies (see Materials and Methods). The eluates were pooled and concentrated 18-fold by dialysis under vacuum. A 10 µl sample of trypsin-activated collagenase (6 units/ml) was added to 200 µl IgG solution or solvent and preincubated for 150 minutes at 25°C. The indicated solutions were then supplemented with 10 µl of either rabbit anti-(goat) IgG (125 µg/ml) or solvent and further preincubated for 150 minutes at 25°C. When indicated, 20 µl of packed Protein A-Sepharose or Protein G-Sepharose was added and further preincubated for 150 minutes at 25°C. Collagenase activity was determined in the supernatants (130,000 g min) and expressed as a percentage of the corresponding controls where the IgG were replaced by the solvent.

*100% corresponds to 0.3 units/ml.
†145 µg/ml in the assay.

The IgG gave a single precipitation line on double diffusion with crude preparations of latent mouse procollagenase, but two lines with activated collagenase (not shown). Western blot analysis of crude latent procollagenase showed that immune IgG, but not preimmune ones, reacted with a characteristic doublet of bands (65 and 59 kDa) (Fig. 1A, lanes T1 and T3; Fig. 2A, lane +), corresponding to different glycoforms of procollagenase (see, e.g., Nagase et al., 1983). A doublet (50 and 45 kDa), corresponding to activated collagenase, and a few bands of lower molecular mass (26 and 20 kDa), corresponding presumably to fragments resulting from proteolytic cleavage (as reported in several other studies; see, e.g., Clark and Cawston, 1989), appeared upon activation or storage of (pro)collagenase and in heavily loaded blots (not shown). It was checked that the antibodies eluted from each band recognized all the others (not shown), thereby further establishing that these bands have at least one common epitope and may thus well correspond, respectively, to procollagenase, collagenase and degradation products. The IgG eluted from the 59-65 kDa bands did also precipitate collagenase (Table 1).

The specificity of the antibodies towards (pro)collagenase was further examined by immunoaffinity chromatography. Fig. 1 shows that the immobilized immune IgG, contrary to the preimmune ones, retain the characteristic doublets of procollagenase and of collagenase (A-C) and that they do not retain the 72-65 kDa gelatinase A (MMP-2), the 96 kDa gelatinase B (MMP-9) (see B), or the caseinolytic stromelysin (MMP-3) (approximately 53 kDa, see Fig. 1C). Biochemical assays confirmed that under these conditions 90 to 100% of the (pro)collagenase activity was retained by the immobilized immune IgG but not by the preimmune ones (not shown). The selective binding of the
antibodies to (pro)collagenase was also checked (Fig. 2) by comparing conditioned media that contained either high amounts (lanes +) or 100-fold lower amounts (lanes −) of (pro)collagenase but similar (MMP-2 and MMP-9; B) or less different (MMP-3; C) activities of other matrix metalloproteinases. The absence of signal in lane (−) of the immunoblot (A) demonstrates that the anti-(pro)collagenase IgG do not bind to these other metalloproteinases.

Immunolocalization of collagenase in the extracellular matrix

These results therefore demonstrate that the antiserum raised against mouse bone collagenase recognizes the enzyme but does not recognize closely related metalloproteinases. It could therefore be used to immunolocalize collagenase in the growth plate areas of tibiae from neonatal rats and mice. In the first series of experiments, the sections were incubated with the antibodies without prior digestion with chondroitinase. Using peroxidase-conjugated secondary antibodies, we found the reaction product to be localized and restricted mainly to the interface between osteoclasts and the bone matrix, in the bone-resorbing compartment (Fig. 3). No labeling was found along other bone surfaces, which are mostly lined with osteoblasts in these growing animals (Fig. 3). The purified IgG fraction from pre-immune serum failed to show any reaction product in tissues processed in the same manner (Figs 3, 5).

To check that this apparently specific distribution was not due to better access of the antibodies to the antigen in these areas, we performed control experiments with antibodies to collagen type I, a molecule that is both abundant and ubiquitous in bone matrix. The results clearly showed (Fig. 3) that our procedures allow antibodies to reach all of the interface between the bone matrix and the cells, although the diffusion of the antibodies in the matrix itself is limited (Fig. 3). In addition, these patterns of localization were altered neither by decreasing the decalcification time to 2 hours nor by including fixative solution in the decalcification buffer, thereby making it unlikely that selective extraction of the enzyme led to the observed localization. An even stronger labeling of the subosteoclastic resorption zones was found when substituting formic acid for EDTA as decalcifying agent (Vermeulen et al., 1989). We interpreted these results to indicate that the antigen recognized by the antiserum was indeed restricted, along bone surfaces, to the subosteoclastic bone-resorbing compartment.

Despite the apparent specificity of the whole antiserum, we further verified the specificity of our localization experiments in two different ways. Firstly, we used an IgG frac-
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puriﬁed by repeated adsorption and elution cycles on western blot strips corresponding to the 59-65 kDa antigenic bands, as explained in Materials and Methods. When these afﬁnity-puriﬁed antibodies were used for immunolocalization under the same conditions as before, we found that they localized to exactly the same regions as the whole antiserum: namely, the bone matrix in the subosteoclastic bone-resorbing compartment (Figs 4, 5, 6). Interestingly, and both with the whole IgG fraction and with the afﬁnity-puriﬁed antibodies, collagenase was found in close association with collagen ﬁbers, whether of type I in bone or type II in cartilage, but only when osteoclasts were in the process of resoring these matrices (Fig. 5). Second, immunolocalizations were performed with two other well-characterized antisera, directed against rat and rabbit collagenase, respectively, and prepared using other experimen-tal protocols (Blair et al. 1986b; Brinckerhoff et al., 1990). No difference in localization of the enzyme was found between mouse, rat (Fig. 7A) and rabbit bone (not shown). We concluded from these experiments that the antigen localized in the bone-resorbing compartment is indeed collagenase and that it is present in the bone-resorbing compart-ment of several animal species.

Immunolocalization of collagenase in bone cells

The conﬁnement of collagenase to the resorption zones strongly suggests that the osteoclasts are at the source of this collagenase. However, this would imply its presence in the biosynthetic and secretory pathways of the osteoclasts, i.e. intracellularly. Since we did not ﬁnd intracellular collagenase with the experimental protocol used here above, we varied the experimental conditions in order to determine whether collagenase was also present within osteoclasts. These involved confocal immunomicroscopy of isolated osteoclasts cultured on bone slices or on glass coverslips and enzymatic digestion of tissue sections prior to incuba-tion with the anti-collagenase antibodies (Pelletier at al., 1990; Aeschlimann et al., 1993).

Bone cells were isolated from long bones of newborn rats, cultured for 18 hours on devitalized cortical bone slices, ﬁxed and incubated with anti-collagenase antibodies. Using rhodamine-conjugated secondary antibodies, we found a prominent ﬂuorescence in the osteoclasts (Fig. 8) and a weaker signal in many uncharacterized mononuclear cells (not shown). In the osteoclasts that were excavating bone, prominent ﬂuorescence was visible intracellularly, in perinuclear areas, towards the bone surface and even in regions located below the level of the surface of the sur-rounding bone (i.e. in the part of the cell present within the resorption pit) (Fig. 9). These observations thus demon-strated the presence of collagenase in resoring osteoclasts, from the perinuclear area towards the resorption compart-ment. In osteoclasts that were not associated with pits or that were plated on glass, and even after up to 24 hours, collagenase was also visible as intracellular punctate staining particularly prominent in perinuclear areas, in rabbits (Fig. 7B) as well as in rats. This localization was speciﬁc for collagenase, since it could be reproduced using afﬁnity-puriﬁed antibodies but not when using the IgG fraction from preimmune serum (Fig. 8).

Since these observations clearly established the presence of collagenase in isolated osteoclasts, we then modiﬁed our experimental procedures in order to determine whether we could detect intraosteoclastic collagenase in the tissues. When the sugar moieties were digested by treating the sec-tions with chondroitinase ABC prior to incubation with the anti-collagenase antibodies (Pelletier et al., 1990; Aeschli-mann et al., 1993), there was some decrease in the quality of the pictures, but under these conditions, intracellular
labeling became apparent not only in the osteoclasts, but also, and as described by others (Blair et al., 1989b), in osteoblasts, chondroblasts and other uncharacterized mononuclear cells (Fig. 6). The intracellular distribution of the label was consistent with that found in the resorbing isolated osteoclasts, with the highest accumulation in the area between the nuclei and the ruffled border. Furthermore, the labeling of the extracellular resorption zone appeared still stronger and staining was still restricted to these areas of the bone matrix.

**DISCUSSION**

This study demonstrates that collagenase is present in the bone-resorbing compartment underlying the osteoclasts as well as in the osteoclast itself, independently of it being in the process of resorbing bone or not. This specific localization was observed in three different species and using three different well-characterized antibodies against the enzyme. Since it has also been shown that bone resorption depends, at least in part, on the activity of collagenase in the bone-resorbing compartment (Everts et al., 1992) and that resorbing odontoclasts, a closely related cell-type, express mRNA for this enzyme (Okamura, 1992; Okamura et al., 1993), it is tempting to speculate that collagenase is secreted by the osteoclast and involved in the process of bone resorption.

The identification of the antigen present in the osteoclasts and in the bone-resorbing compartment as collagenase is strongly established by the extensive characterization of our antibodies, their inability to cross-react with three metalloproteinases that are closely related to collagenase (72 kDa gelatinase A (MMP-2), 96 kDa gelatinase B (MMP-9) and stromelysin (MMP-3)), and the use of IgGs that were affinity-purified by adsorption on 65-59 kDa procollagenase antigens, eliminating potential cross-reactions with matrilysin (MMP-7) or the tissue inhibitors of metalloproteinases, which all migrate between 28 and 22 kDa. The fact that the antigen is indeed collagenase is further, and in our eyes definitively, established by the observation that antibodies against rat collagenase or rabbit collagenase, raised and characterized independently (Jeffrey et al., 1990; Brinckerhoff et al., 1990), gave identical results in these two species.

The fact that the collagenase specifically recognized by all three antibodies is present in the bone-resorbing compartment is also well established. All the preparations of anti-collagenase antibodies used in this study provided identical and specific staining of the bone-resorbing com-

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**Fig. 4.** Immunolocalization of affinity-purified anti-(mouse) collagenase IgG in the bone-resorbing compartment underlying mouse osteoclasts. The reaction product (A, arrows) is found exclusively in the area between the ruffled border of the cell (rb) and the bone matrix (bm); the attachment area (sealing zone, sz) shows no reaction product (curved open arrows); in (B), the area of the ruffled border and of the underlying bone matrix is shown at higher magnification; n, nuclei. Bars: (A) 1.5 µm; (B) 0.5 µm.
partment in mouse, rat and rabbit bone, leaving other bone surfaces unstained. This was in contrast with the results obtained with antibodies to collagen type I, which stained the bone matrix surface irrespective of its association with osteoclasts. Preimmune preparations were negative in all cases.

Finally, the fact that collagenase is found within the osteoclast is also well established by our study. Total anti-collagenase IgG as well as affinity-purified IgG, but not preimmune IgG, provided the same specific fluorescence of the intracellular areas of the osteoclasts and, here again with all three antibodies and in all three species. The intracellular distribution of the fluorescence observed with the confocal microscope in resorbing isolated osteoclasts was in good accord with that of the peroxidase reaction product observed with the electron microscope in chondroitinase-treated tissue sections. Since the intracellular signal appears strong in the perinuclear area of the osteoclasts and independently of whether the cells are resorbing (on bone) or not (on bone or on glass), it cannot be merely explained by the presence of extracellular collagenase in deep invaginations of the ruffled border or by the internalization of extra-
cellular collagenase. Furthermore, we have previously shown that most of the intracellular vesicles in the osteoclast are constituents of the secretory rather than endocytic pathways (Baron et al., 1985, 1988). Indeed, the intracellular distribution of collagenase observed here by confocal microscopy corresponds well to that of secretory enzymes in these cells (Baron et al., 1985, 1988). It is therefore tempting to speculate that newly synthesized collagenase is indeed within the biosynthetic pathway, transported towards the ruffled border and secreted into the bone-resorbing compartment. This hypothesis, i.e. that osteoclasts synthesize and secrete (pro)collagenase, is further supported by recent in situ hybridization data showing collagenase mRNA in resorbing bovine odontoclasts (Okamura, 1992; Okamura et al., 1993), a cell type involved in the resorption of the mineralized matrix of teeth and thought to be identical to osteoclasts. Furthermore, we (unpublished) and others (Case et al., 1989) have found that osteoclasts also contain stromelysin (MMP-3), a metalloproteinase that is closely related to collagenase (but not recognized by our antibodies) and that is implicated in the activation cascade of collagenase (Brinckerhoff et al., 1990; Nagase et al., 1991). Hence, given this array of independent observations, the conclusion that osteoclasts are capable of synthesizing and secreting metalloproteinases (both collagenase and stromelysin) seems warranted.

This conclusion, together with the results of Everts et al. (1992) showing that inhibition of collagenase affects collagen degradation in the subosteoclastic zone, suggests a role for metalloproteinases in bone resorption. As reviewed elsewhere (Vaes, 1988; Delaissé and Vaes, 1992), both lysosomal enzymes and collagenase are involved in this process. The participation of lysosomal enzymes, particularly the collagenolytic cysteine proteinases cathepsin L (EC 3.4.22.15) and cathepsin B (EC 3.4.22.1) (both present in resorbing bone; Delaissé et al., 1991a), is likely to occur at the level of the bone-resorbing compartment and their inhibition prevents the resorption of bone collagen by the osteoclast (Delaissé et al., 1987; Everts et al., 1988, 1992; Rifkin et al., 1991). Specific collagenase inhibitors also inhibit bone resorption, either in vitro, when acting in organ cultures of bone (Delaissé et al., 1985), or in vivo (Delaissé et al., 1991b). In view of the inability of others to detect collagenase in osteoclasts, it was proposed that collagenase was produced by osteoblasts and was responsible for the
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removal of non-mineralized collagen, which, when present, would prevent the adherence and activation of osteoclasts (Chambers et al., 1985). The presence of (pro)collagenase in the osteoclasts and in the underlying bone-resorbing compartment, established by the present study, suggests a much simpler explanation, i.e. that osteoclastic collagenase could also be active in the removal of the mineralized collagen during or after demineralization by the osteoclast.

This hypothesis is indeed strongly supported by the fact that broad fringes of demineralized collagen are seen under osteoclasts after treatment with collagenase inhibitors (Everts et al., 1992), as they are also after the inhibition of lysosomal cysteine proteinases (Everts et al., 1988, 1992). This indicates that under these conditions demineralization of bone by osteoclasts proceeded up to a certain point whereas matrix degradation was inhibited. Thus collagenase is not only present in the subosteoclastic resorption zone, as shown in the present study, but is also active at that level, together with cysteine proteinases.

In trying to understand the respective roles of collagenase and cysteine proteinases in bone resorption, one could envision the following hypothesis. Besides their collagenolytic action, lysosomal cysteine proteinases could generate active collagenase from its zymogen, as cathepsin B is known to activate procollagenase (Eeckhout and Vaes, 1977). Collagenase could then co-operate with collagenolytic cysteine proteinases in the degradation of the demineralized collagen, a process that is likely to be rendered more efficient by the high concentration of Ca\(^{2+}\) present in the bone-resorbing compartment as a consequence of bone demineralization (Etherington and Birkedal-Hansen, 1987; Eeckhout, 1990). Because the optimal pH for these two classes of enzymes are very different (6.0 to 7.5 for collagenase (Vaes, 1972), but 4.5 for the cysteine proteases (Delaissé et al., 1991a)), it may be speculated that the combined action of these two classes of enzymes would broaden the pH spectrum at which matrix resorption can occur. Indeed the effective pH at any time and at any point of the resorption zone will depend both on the relative efficacy of the proton secretion and on the buffering capacity exerted by the solubilized bone salts. Thus for instance, the lysosomal cysteine proteinases might act predominantly in

Fig. 8. Immunolocalization of collagenase in isolated rat bone cells cultured on a bovine cortical bone slice. The specimens were analyzed in epifluorescence on a conventional microscope (A, B) or on a confocal microscope (C, D). Anti-(mouse) collagenase IgG (either affinity-purified IgG in (A) or total IgG in (C) and (D)) but not preimmune IgG (B), localize in the perinuclear area of osteoclasts (in B, small and large arrows indicate, respectively, the outline and the nuclei of an osteoclast). Intracellular localization is further demonstrated in confocal optical sections (C and D); in (C), the section is taken at the level of the bone slice surface and collagenase is found in several osteoclasts, most prominently the one at the bottom of the field; in (D), the same cell is shown at a higher magnification and at a higher level of optical sectioning than in (C), further above the bone surface, to demonstrate intracellular localization at the level of the nuclei. Bars: (A-C) 10 \(\mu\)m; (D) 2 \(\mu\)m.
the immediate vicinity of the ruffled border where protons are secreted, whereas the collagenolytic action of collagenase might be predominant in the presumably more neutral zone, localized at the interface between the mineralized and demineralized matrix. Also, the activation of collagenase by lysosomal enzymes would be favored in the latter zone, as this process is more efficient around pH 6 than at lower pH (Vaes, 1972; Eeckhout and Vaes, 1977). Moreover collagenase could be further required to degrade the fringe of yet undegraded but already demineralized collagen that is left behind by the osteoclast when it detaches to move along the bone surface, thereby rendering the pH neutral in the bone-resorbing lacuna. Such roles for collagenase and cysteine proteinases would explain both the accumulation of collagen under osteoclasts in the presence of collagenase and cysteine proteinase inhibitors (Everts et al., 1992) and the finding that the process of matrix resorption seems to go on even after the osteoclast has moved away (Gaillard, 1957).

In conclusion, and independently of speculation on its physiological role in bone resorption, our results support the concept that collagenase is present in the biosynthetic pathway of the osteoclast and secreted into the bone-resorbing compartment, where it participates in the bone-resorption process.

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