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Direct extraction and assay of bone tissue collagenase and its relation to parathyroid-hormone-induced bone resorption

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A method has been developed for the quantitative extraction of collagenase from as little as one 19-day-fetal-mouse calvarium. About 20–40 munits of collagenase are extracted per mg of tissue, all in a latent form that, after proper activation, shows the typical properties of mammalian collagenase. Culturing the calvaria for 2 days with parathyroid hormone (PTH) increases their procollagenase content up to 3-fold and induces bone resorption. Both PTH effects are prevented by cycloheximide, but not by indomethacin. Calcitonin inhibits resorption without affecting the PTH-induced procollagenase synthesis. The role of this synthesis is discussed in relation to the mechanisms of bone resorption.

INTRODUCTION

The demonstration that a synthetic inhibitor of mammalian collagenase (EC 3.4.24.3) inhibits the resorption of explanted mouse bones [1] led us to re-evaluate the role of this enzyme in bone resorption. Studies on the association of collagenase with bone resorption have been hitherto carried out in bone culture systems by assaying the enzyme activity released into the medium. They have not been conclusive, as this release does not parallel the progression of bone resorption [2, 3]. However, if collagenase participates in bone collagen degradation, it should be present inside the resorbing tissue matrix. So far, quantitative extraction of collagenase from resorbing bone tissue has not been reported, although minute collagenase activities have been detected in extracts from gram quantities of embryonic chick bone [4] and human dentine [5]. We therefore developed a procedure allowing the extraction and assay of collagenase from as little as one fetal-mouse calvarium, i.e. 4–5 mg of fresh tissue. It is based on the following assumptions: (i) omission of tissue homogenization, which might bring together collagenase and inhibitor(s) [6], (ii) dissociation of collagenase from insoluble collagen by high NaCl concentrations [1,7], (iii) use of cacodylate buffer at pH 6.0 for bone demineralization and for preserving collagenase activity [7] and (iv) addition of a non-ionic detergent to stabilize collagenase. Applying the method to calvaria in culture we observed that PTH induces a 3-fold dose-dependent increase of their collagenase content, thus supporting our hypothesis [1] that the enzyme participates in the PTH-induced bone resorption.

MATERIALS AND METHODS

Materials

[^{3}H]Acetic anhydride (500 mCi/mmol) was from Amersham (Brussels, Belgium); 4-aminophenylmercuric acetate was from Aldrich Chemie (Brussels, Belgium); PTH (trichloroacetic acid powder, 152 U.S. Pharmacopeia units/mg), calcitonin (synthetic salmon form; 3680 units/mg), indomethacin and cycloheximide were from Sigma (St. Louis, MO, U.S.A.). Purified human TIMP was kindly given by Dr. A. Galloway (G. D. Searle, High Wycombe, Bucks., U.K.). Other chemicals were from suppliers previously mentioned [1].

Assay of collagenase

Collagenase, activated or not (see the Results section), was assayed at 25 °C with collagen in solution as described in [8] but with incubation times of 18–20 h and with [^{3}H]acetylated [9] acid-soluble guinea-pig skin collagen [(1–4) × 10^6 d.p.m./mg]. All assays were done in triplicate; extracts were replaced by the extraction buffer in controls. Under these conditions, the collagenase activity (50–250 munits/ml) was directly proportional to the enzyme concentration up to 35% degradation of the collagen, and trypsin (10 μg/ml) did not degrade more than 6% of this substrate. One unit of collagenase corresponds to the amount of enzyme which degrades 1 μg of soluble collagen/min at 25 °C; we have established that this corresponds to the degradation of approx. 1 μg of reconstituted collagen fibrils/min at 37 °C.

Bone cultures

Calvaria from 19-day NMRI mouse embryos were cultured for 2 days (two calvaria in 2 ml of medium 199) with daily change of medium and monitored for resorption and for enzyme release as described in [1].

RESULTS

Direct extraction of collagenase from mouse calvaria

After several unsuccessful trials, measurable collagenase activities were extracted from newborn (1- or 5-day-old) or fetal (19-day) mouse calvaria by the following procedure. One to four calvaria, dissected free of surrounding tissues and of the occipitals, were rinsed in mammalian Tyrode solution, immersed in a cylindrical

Abbreviations used: PTH, parathyroid hormone; TIMP, tissue inhibitor of metalloproteinases.

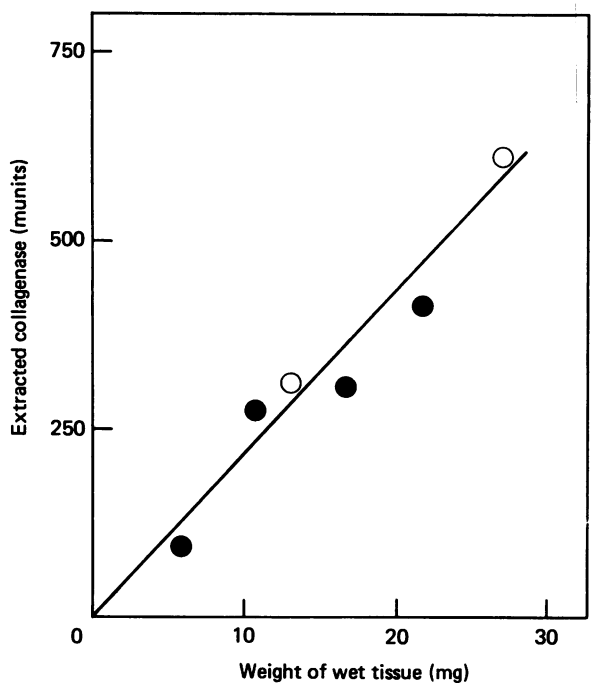

Fig. 1. Collagenase activity recovered as a function of the amount of extracted tissue

Calvaria from either 19-day-fetal (●; about 5 mg/calvarium) or 5-day-old (○; about 13 mg/calvarium) mice were extracted with respectively 0.5 ml or 1 ml of CNTN buffer. Collagenase was assayed after trypsin activation.

flat-bottomed polypropylene tube (40 mm × 10 mm) containing, as a rule, 0.5 ml of CNTN buffer [10 mM-cacodylate/HCl (pH 6.0)/1 M-NaCl/Triton X-100 (0.1 mg/ml)/1 μM-$ZnCl_2$/NaN_3 (0.1 mg/ml)] per fetal calvarium (or 1 ml per newborn calvarium) and agitated by gentle oscillation (12/min) at 4 °C for 24 h. The calvaria were then transferred to new tubes while the extract was supplemented with 10 μl of 0.2 M-$CaCl_2$/ml and assayed for collagenase. The extraction of the calvaria was repeated three or four times in the same manner until collagenase could no longer be detected in the extract (lower detection limit: 10 munits/ml). Much lower, or even undetectable, collagenase activities were observed if the extraction was performed with 0.15 M-NaCl or in the absence of detergent or with 50 mM-Tris/HCl buffer, pH 7.5, or if it was conducted at 25 °C or in the presence of 20 mM-$CaCl_2$ or, as previously described [2], on homogenized calvaria. Heating calvarial suspensions or homogenates for 30 min at 50 °C [10] did not lead to the recovery of collagenase activity.

Properties of collagenase extracted from mouse calvaria

The extracted collagenase is entirely latent and can be activated optimally either by a 10 min preincubation at 25 °C with trypsin (10 μg/ml) followed by the addition of a 10-fold excess of soybean inhibitor [8], or by the addition of 0.5 mM-4-aminophenylmercuric acetate [11] in the assay mixture. When stored for several weeks in CNTN buffer at 4 °C, the extracted pro-collagenase activates spontaneously, but its activity remains stable for several months.

When incubated at 25 °C with soluble Type I collagen, the bone collagenase produces the typical '¾' and '¼' fragments, as revealed by SDS/polyacrylamide-gel electrophoresis (results not shown). Inhibitors of metalloproteinases (7.5 mM-EDTA or 1 mM-*o*-phenanthroline) and more specific inhibitors of collagenase, such as 0.5 unit of TIMP/ml [6], the synthetic inhibitor CI-1 (13 μM) [1] or polyclonal antibodies raised against purified mouse bone collagenase [3], inhibit at least 90% of the enzyme activity, whereas inhibitors of serine proteinases (2 mM-phenylmethanesulphonyl fluoride, 12 μM-leupeptin), cysteine proteinases (5 mM-iodoacetamide, 0.2 mM-4-hydroxymercuribenzoate, 12 μM-leupeptin) or carboxyl proteinases (7 μM-pepstatin) have no significant effect.

Quantitative aspects of the extraction method

When less than 30 mg (wet weight) of calvarial tissue is submitted to four successive 24 h extractions in 0.5 or 1 ml of CNTN buffer as described above, the extracted

Table 1. Effect of PTH, calcitonin and indomethacin on the collagenase activity extracted from cultured calvaria

Fetal-mouse (19-day) calvaria were cultured with or without PTH (0.9 unit/ml), calcitonin (1.8 units/ml) and/or indomethacin (14 μM), as indicated. The results are means ± S.D. for four cultures of two calvaria each. The total collagenase extracted is presented, as well as the activity recovered in the first extract. Abbreviations used: C, calcitonin; I, indomethacin; *N*-A-β-gase, *N*-acetyl-β-glucosaminidase; calv, calvarium. Significance (*t* test) when compared with their respective controls (group 1 or 1′): $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Calvaria	Extracted collagenase (munits/calv)		Calcium release (μmol/calv)	Hydroxyproline in tissue (μg/calv)	*N*-A-β-gase released (munits/calv)
	Total	First extract			
Non-cultured	194±53	29±9	—	17.8±1.8	—
Cultured with:					
(1) No additive	247±60	50±40	0.20±0.06	20.3±2.0	0.49±0.09
(2) PTH	435±18***	204±7***	0.42±0.18**	15.7±1.7	0.78±0.24*
(3) PTH+C	461±51**	209±29***	0.20±0.02	17.0±2.4	0.47±0.04
(1′) I	221±22	31±8	0.11±0.02	17.7±1.1	0.32±0.06
(2′) I+PTH	473±88**	222±57***	0.41±0.06***	16.6±1.3	1.07±0.46*
(3′) I+PTH+C	393±27***	165±29***	0.21±0.03	17.6±2.5	0.42±0.05

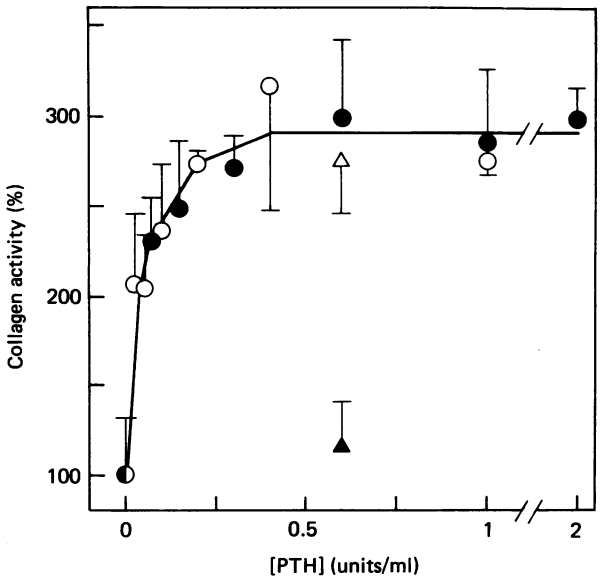

Fig. 2. Stimulation of tissue collagenase in cultured calvaria by PTH and its inhibition by cycloheximide

Fetal-mouse calvaria were cultured in the presence of 14 μM-indomethacin with the indicated concentrations of PTH, and, in ▲, with 1 μg of cycloheximide/ml. Collagenase was then extracted from the calvaria and assayed after trypsin activation. The results of three experiments are presented as means ± S.D. for four cultures for Expts. 1 (●) or 3 (△, ▲), and for three cultures for Expt. 2 (○); '100% collagenase activity' was respectively 140, 86 and 83 munits/calvarium in Expts. 1, 2 and 3.

procollagenase is directly proportional to the amount of tissue (Fig. 1); no difference is observed between fetal and 5-day-old mouse calvaria regarding the yield of enzyme per mg of tissue. Larger amounts of calvarial tissue extracted in the same volume give slower and incomplete release of collagenase. Increasing the individual extraction times to more than 24 h is less efficient than repeated extractions. It is noteworthy that at least 90% of the bone calcium is solubilized during the first extraction, but most of the procollagenase is recovered in the second and third extracts. Collagenase inhibitors are not detected in the extracts. Most of the extractable collagenase remains in the tissue after removal of the periosteum or of the fibrocartilaginous bone sutures before the extraction. Collagenase is also found in extracts from adult mouse tibial metaphysis (38 munits/mg of wet tissue) or 1-day-old-rat calvaria, but not in extracts from adult human or bovine bone, which were not demineralized by the extraction.

Effect of PTH on bone tissue collagenase

Culturing fetal-mouse calvaria for 2 days with PTH increases their collagenase content 2–3-fold and induces bone resorption as evaluated by their loss of calcium and hydroxyproline and their release of a lysosomal enzyme marker, *N*-acetyl-β-glucosaminidase (Table 1). The effect of PTH on collagenase accumulation is dose-dependent and already detectable with 0.025 unit of PTH/ml (Fig. 2); it is not inhibited by indomethacin nor by calcitonin, although the latter inhibits resorption and the release of lysosomal enzymes (Table 1). Cycloheximide suppresses both the PTH-induced increase of collagenase (Fig. 2) and bone resorption (results not shown). Interestingly most of the PTH-induced collagenase is found in the first extract (Table 1) and in a latent form. Significant collagenase inhibitory activity is observed in the culture media (where collagenase is not detected), but not in the extracts of cultured calvaria.

DISCUSSION

A method has been developed for the quantitative extraction and assay of collagenase from milligram quantities of mouse bone. The enzyme extracted from fetal- or newborn-mouse calvarium has the typical properties of mammalian collagenase: it cleaves Type I collagen into '$\frac{3}{4}$' and '$\frac{1}{4}$' fragments and is inhibited by inhibitors of collagenase and related metalloproteinases, but not by inhibitors of serine, cysteine or carboxyl proteinases. It is all recovered in a latent form, presumably a proenzyme [8], that is activated by limited proteolysis and by 4-aminophenylmercuric acetate. The absence of active collagenase in fresh extracts suggests either that only a minor, non-measurable, proportion of the enzyme is active in the tissue or that active collagenase is not extracted, is denatured or is irreversibly trapped by tissue inhibitors.

Wet calvarial bone tissue contains 20–40 munits of collagenase/mg, an activity that is able to degrade, in 1 h, 1.2–2.4 μg of either soluble collagen at 25 °C or reconstituted fibrillar collagen at 37 °C. Although we cannot rule out the possibility that some collagenase still remains in the tissue after our extraction procedure, it is worth noting that the extracted activity is 500–1000 times higher than the only reported activity of collagenase in bone [4], 10–20 times higher than that found in involuting rat uterus or human osteoarthritic cartilage and 3–6 times higher than in the growth plate of rachitic rats [12]. Comparatively, the high level of collagenase found in mouse bone might result from the greater efficiency of our extraction method or reflect a peculiarity of the tissue. The results show for the first time that remodelling bone contains enough procollagenase to degrade its own collagen in a few days. This enzyme appears to be bound to the bony part of the calvarial tissue, as indicated by its extraction from calvaria from which both the periosteum and the fibrocartilaginous sutures had been removed. This interpretation is supported also by indications that demineralization seems necessary for the extraction of procollagenase from fresh, non-cultured, bones; in this regard, procollagenase may be considered as a minor constituent of the non-collagenous bone proteins.

When fetal calvaria are cultured with PTH, their collagenase content increases 2–3-fold in a dose-related manner. PTH concentrations as low as 25 munits/ml induce a significant increase in collagenase. Indomethacin does not diminish the effect of PTH on calvarial collagenase, thus indicating that prostaglandin synthesis is not involved. The inhibitory effect of cycloheximide suggests that the PTH-induced increment of collagenase activity results from enzyme synthesis. This synthesis is likely to occur in osteoblasts. Indeed, these cells seem to be the target of PTH in bone [13] and, under cell-culture conditions, they can be stimulated to release collagenase by high doses of PTH [14,15]. Interestingly, calcitonin, whose target cells in bone seem to be the osteoclasts [16], inhibits bone resorption and the release of lysosomal

enzymes without affecting the PTH-induced synthesis of procollagenase. Thus the accumulation of procollagenase in bone tissue is not in itself a sufficient condition for the degradation of the bone matrix. Our previous work has shown, however, that both collagenase [1] and thiol proteinases [17] are required for bone resorption. It may be speculated that procollagenase, produced by osteoblasts and subsequently activated, may somehow prepare the bone surfaces for the action of the osteoclasts [18], allowing them to adhere to the mineralized bone matrix and to secrete the acid and the lysosomal cysteine proteinases necessary for its resorption [17,19]. We are still ignorant, however, as to whether the action of collagenase in bone resorption is limited to a degradation of non-mineralized collagen in the vicinity of the osteoblasts [18] or whether it also takes part in the sub-osteoclastic erosion of mineralized bone matrix.

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