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IMMUNOREACTIVE COLLAGENASE AND BONE RESORPTION

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Summary

1. Active mouse bone collagenase is excluded from its inhibitory antibody by preincubation of that antibody with various forms of inactive enzyme, e.g. 'procollagenase', some collagenase-inhibitor complexes or partially denatured or degraded collagenase. This property allows the detection of several enzymatically inactive forms of collagenase.

2. The accumulation of immunoreactive collagenase in the culture fluid of mouse bones occurred only in the presence of heparin and was not correlated with bone resorption induced by parathyroid hormone. These experiments provide further (see Lenaers-Claeys, G. and Vaes, G., Biochim. Biophys. Acta (1979) 584, 375—388), more conclusive evidence that the critical role in the resorption of the organic matrix of these explants may be due to another enzyme system than collagenase.

Introduction

Mouse bone explants in culture release collagenase (EC 3.4.24.3) into the surrounding medium (for review, see Ref. 1). In our previous studies, the enzyme was recovered almost exclusively under a latent, trypsin-activatable form ('procollagenase') and only when the cultures were done in the presence of heparin [2]. We could establish no correlations between the release of either latent or active collagenase by the explants and the resorption of their collagen induced by parathyroid hormone [3]. This suggested that the bone-resorbing cells might use another enzyme system, possibly lysosomal [1,4,5], for the degradation of collagen.

However, the possibility remained that active collagenase rapidly loses its catalytic activity by partial denaturation when it is diluted in the culture medium after its extracellular action on collagen. We therefore developed a method that allows the immunochemical detection of inactive mouse bone collagenase, e.g. 'procollagenase', some collagenase-inhibitor complexes and partially denatured collagenase and we searched for the presence of such an immunoreactive enzyme in the culture medium of resorbing mouse bones.

Experimental

Preparations and assays of procollagenase and collagenase

Calvaria from 5-day-old NMRI mice were cultured [2] to supply procollagenase-containing culture fluids; these fluids were handled and stored as described [2]. Procollagenase was purified from these fluids by affinity chromatography on Sepharose-collagen [6]. Assays and units of activity of procollagenase and collagenase were as described [3,7].

Preparation of antibodies to mouse bone procollagenase

Rabbits were injected intramuscularly three times at 1-week intervals with 50 μg (1—2 ml) of highly purified procollagenase (3000—4000 U/mg) emulsified with 1 vol. of Freund's complete adjuvant. They were bled 2 weeks after the last injection. Immunoglobulins G (IgG) were purified from serum [8] by chromatography on a DEAE-Sephadex A-50 column, equilibrated and eluted with 0.08 M Tris-HCl buffer (pH 7.4), followed by precipitation of the immunoglobulins by the addition of 1 vol. saturated $(NH_4)_2SO_4$ (pH 6.8). The precipitate was separated by centrifugation for 10 min at 43 500 $\times g$, resuspended in a volume of water equal to that of the initial volume of serum and dialysed against 0.05 M Tris-HCl buffer (pH 7.6)/0.15 M NaCl/5 mM $CaCl_2$/0.2 mg NaN_3/ml. IgG solutions containing 15—25 mg protein/ml were obtained.

Immunoinhibition of collagenase

To assay the capacity of the antibodies to inhibit collagenase, increasing amounts (up to 200 μl) of IgG solution were added to 125 μl of a culture fluid containing active (completely activated, usually by trypsin) collagenase. Appropriate amounts of IgG purified from the serum of non-immunized rabbits were added so as to achieve the same protein concentrations in all the test-tubes. After 20 min incubation at 25°C, collagenase was then assayed, with similar results, either in the mixture or in the supernatant obtained after 15-min centrifugation at 13 000 $\times g$.

Assay of immunoreactive collagenase

The assay was developed following the method proposed by McGee and Udenfriend [9] for measuring enzyme precursors by excluding active enzyme from its antibody, the unbound enzyme being measured by an enzymatic assay. An advantage of the method is that it is unnecessary to have available a monospecific antiserum to the enzyme since only those antibodies reacting with enzyme are measured in such enzyme-immunoassay. Tadpole procollagenase was assayed following this principle [10].

The assay was done in 0.05 M sodium cacodylate/HCl buffer (pH 7)/0.15 M NaCl/5 mM $CaCl_2$/0.2 mg NaN_3/ml in a total volume of 0.6 ml. Four sets of test tubes were prepared containing respectively, under equal volumes, (1) buffer alone; (2) an amount of IgG which is able to inhibit 50—75% of a given collagenase activity; (3) that same amount of IgG supplemented with increasing amounts of the solution in which immunoreactive collagenase is to be assayed; (4) the same increasing amounts of the solution to be assayed for immunoreactive collagenase, without added IgG (this set of tubes is needed only to determine the presence, within that solution, of possible non-specific collagenase inhibitors which may interfere with the assay). The tubes were preincubated for 20 min at 25°C. Trypsin-activated collagenase was then added to all tubes in an amount that is inhibited by 50—75% by the IgG present in tubes (2) or (3). The tubes were further incubated for 20 min at 25°C before being centrifuged for 15 min at 13 000 × *g*, and collagenase was then assayed in the supernatant, A standard curve was run with each series of assays, using as standards, dilutions of a culture fluid containing known activities of trypsin-activatable procollagenase and free of non specific collagenase inhibitors.

Bone resorption in tissue culture

Calvaria from 19-day-old NMRI mouse embryos were cultured in medium 199 (Hanks' base) with or without 300 μg heparin/ml and with 1 U.S.P. unit purified parathyroid hormone/ml, under an atmosphere of air [3]. Bone resorption was evaluated [3] by following the loss of hydroxyproline and of calcium from the explants and by scoring 0—5 the extent of the resorption lacunae observed in the tissues under a dissecting microscope.

Materials

The culture fluids and the other special chemicals were from the same suppliers as in Ref. 3.

Results and Discussion

Immunological detection of latent collagenase

The IgG of the anti-mouse bone procollagenase sera completely inhibited trypsin-activated or 'autoactivated' [11] mouse bone collagenase (Fig. 1a); IgG from non-immunized rabbits had no effect. Purified collagenase was inhibited as well. Active collagenase was excluded from its antibody by catalytically inactive procollagenase (Fig. 1b), allowing thus the recovery of the collagenase activity after its addition to the preformed mixture of antibody and procollagenase. The initial slope of the curve relating the excluded collagenase activity to the volume of fluid assayed for immunoreactive collagenase (here procollagenase), was linearly related to the amount (units) of procollagenase that was detected in that solution after trypsin-activation (Fig. 1c). Also, in that initial part of the exclusion curve, the amount of collagenase excluded was usually about equal to the amount of procollagenase that had been preincubated with the antibody indicating that under these assay conditions, the complexes formed between antibody and procollagenase did not dissociate appreciably when active collagenase was added. A good estimation of procollagenase was

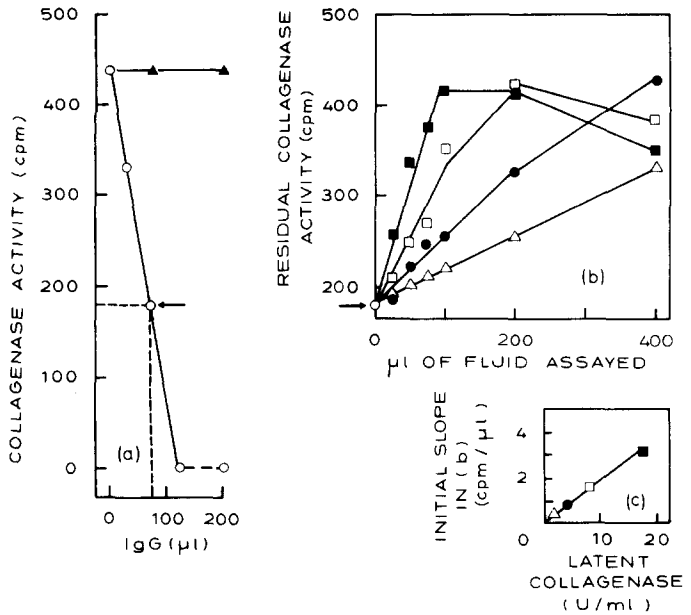

Fig. 1. Immunoinhibition of collagenase and immunochemical assay of latent procollagenase. (a) Inhibition of collagenase activity (○) by IgG from rabbit antiserum against mouse bone procollagenase. IgG was added to 2.6 U of trypsin-activated collagenase, and, 20 min later, collagenase was assayed in the mixtures by a 57 min incubation with [^{14}C]collagen (1726 cpm/tube). IgG from non-immunized rabbits (▲) was used as a control. (b) Loss of the collagenase-inhibitory capacity of antibody by preincubation, before the addition of collagenase (2.6 U), of 75 µl of anti-mouse bone procollagenase IgG with increasing amounts of procollagenase-containing culture fluids (18 U/ml) used either as such (■) or after being diluted 1 : 2 (□), 1 : 4 (●) or 1 : 8 (△) with buffer. This culture fluid and its dilutions (not shown) had no inhibitory effects on collagenase when they were incubated with collagenase without added IgG. (c) Relation between the initial slopes of the antibody inactivation curves drawn above, under (b), and the amount of procollagenase present in the fluid assayed for immunoreactive collagenase.

thus obtained by this method, with a sensitivity equal to that of the catalytic enzyme assay, providing however that non-specific collagenase inhibitors were not present in excessive amounts in the solutions to be tested. Low concentrations of such inhibitors manifested themselves by a progressive inhibition of collagenase subsequent to its exclusion from antibody.

Detection of partially denatured, inactive collagenase

Several experiments were done to determine to what extent a denaturation or a partial degradation of collagenase, causing the loss of its catalytic activity, would provide products that still exclude active collagenase from its antibody and thus react as immunoreactive collagenase.

When bone culture fluids are treated with trypsin, their latent collagenase is first activated but, with excess trypsin action, the collagenase activity may subsequently be lost [2]. However, a trypsin-treatment of a culture fluid that resulted in the loss of 90% of its collagenase activity, did not modify the capacity of that fluid to exclude active collagenase from its antibody (Table I, Expt. A). Active (autoactivated) collagenase, added to bone culture fluid and incubated at 37°C under the conditions of the bone cultures, is also rapidly

TABLE I

IMMUNOCHEMICAL ASSAY OF INACTIVATED OR INHIBITED COLLAGENASE

Culture fluids containing latent collagenase (Expts. A and D), autoactivated collagenase (Expts. B and C) or trypsin-activated collagenase (Expt. E) were treated as indicated so as to inactivate or inhibit part or all of their enzyme activity. The residual activity (expressed in U/ml), and immunoreactivity of collagenase (measured as in Fig. 1, b and expressed as in Fig. 1, c by the initial slopes of the antibody inactivation curves, i.e. in cpm/μl) were then assayed in these fluids. In Expt. A, trypsin-treatment was followed by the addition of soybean trypsin inhibitor, 160 μg/ml, before the collagenase assays. In Expt. B, 50 μl of a 22-times concentrated (by ultrafiltration on an Amicon UM-10 membrane) culture fluid containing autoactivated collagenase was added to 1 ml of a culture fluid taken after 2 days of culture of bone explants with parathyroid hormone (1 U/ml) in the absence of heparin. The mixture was then incubated for the time indicated at 37°C in the usual conditions of the cultures. In Expt. C, a sample of autoactivated collagenase was purified by chromatography on Sepharose-collagen followed by concentration of the fractions containing activity by ultrafiltration on Amicon UM-10 membranes. In Expt. D, a procollagenase-containing fluid was heated for the times indicated; the residual procollagenase was then activated by trypsin before the collagenase assays. In Expt. E, 1 vol. of active collagenase was preincubated before the collagenase assays with 3 vol. of either fresh non-cultured medium (to serve as control) or of heat-treated (5 min at 100°C, so as to completely inactivate both 'catalytic' collagenase and immunoreactive collagenase: see Expt. D) calvaria culture medium (obtained after 4 days culture) that contained an heat-stable collagenase inhibitor. Values in brackets are expressed in percentage of control.

Expt.	Treatment of the culture fluid	Collagenase activity (U/ml)	Immunoreactive collagenase (cpm/μl of fluid assayed)
A	Non-treated control	31.4 (100)	5.05 (100)
	Trypsin, 4 μg/ml, 4 h at 25°C	3.1 (10)	5.20 (103)
B	Non-treated control (0 h 'culture')	9.2 (100)	2.47 (100)
	4 h 'culture' at 37°C	5.8 (63)	2.56 (104)
	8 h 'culture' at 37°C	5.1 (55)	2.68 (109)
	24 h 'culture' at 37°C	2.2 (24)	2.68 (109)
C	Non-treated control	28.1 (100)	3.55 (100)
	Recovered after purification	3.7 (13)	3.43 (97)
D	Non-treated control	22.2 (100)	4.45 (100)
	30 min at 60°C	7.8 (35)	3.8 (85)
	5 min at 69°C	1.5 (7)	2.0 (45)
	15 min at 69°C	0	0
	2.5 min at 79°C	0	0
E	Non-treated control	5.0 (100)	1.11 (100)
	+ heated bone culture fluid	0.72 (14)	1.12 (101)

inactivated [3]. However, a 24 h incubation resulting in the loss of 76% of the collagenase activity, did not affect significantly its immunoreactivity (Table I, Expt. B). Similarly, a loss of 87% of the catalytic activity but only of 3% of the immunoreactivity of collagenase was observed in an experiment involving purification of active collagenase (Table I, Expt. C). Also upon heating of culture fluids, the catalytic activity of collagenase was lost more rapidly than its immunoreactivity (Table I, Expt. D), although, with excess heat-denaturation, both were lost.

It may thus be concluded that the molecular conformation supporting the immunoreactivity of collagenase (or procollagenase) towards our antibody is usually more stable and has a greater resistance to denaturation than the structure responsible for its catalytic properties. It should however be emphasized

that this property is unlikely to be related to an intrinsic quality of the collagenase molecule but that it probably depends on our particular antibody preparation. Other antibody preparations, raised against antigen determinants located at different sites on the collagenase molecule, might well give a progressive loss of immunoreactivity parallel to the loss of catalytic activity.

Detection of collagenase-inhibitor complexes

In our previous work, a heat-stable trypsin-resistant inhibitor of collagenase has been found in the media surrounding cultured mouse calvaria [7]. Preincubation of a sample of active collagenase with these media (Table I, Expt. E) resulted in the loss of 86% of the collagenase activity without, however, changing its immunoreactivity. Collagenase-inhibitor complexes formed with α_2-macroglobulin [12] did, however, no longer react as immunoreactive collagenase. Indeed, when a sample of active (trypsin-activated) collagenase was partially inhibited (—84%) by serum before being gel-filtered on Sephadex G-200, the excluded peak, containing the α_2-macroglobulin-collagenase inactive complexes, did not display any immunoreactivity. It appears therefore that this macromolecular inhibitor prevents the access of the antibody to the antigenic determinants that are responsible for the immunoreactivity of collagenase.

Validity and limitations of the assay

Any enzyme solution, whatever its level of purity, may obviously be contaminated by a certain amount of inactivated enzyme molecules. Although the preparation of trypsin-activated collagenase used as the source of active collagenase for the assay was free of latent, trypsin-activatable procollagenase, it may contain unknown amounts of immunoreactive inactivated material that could be excluded from the antibody by the added test-immunoreactive collagenase. As the relative affinities of the anti-collagenase antibodies for the various possible forms of collagenase (latent, active, inhibited, denatured, or partially degraded enzyme) are also unknown, the excluded amount of trypsin-activated collagenase cannot be quantitatively equated to the amount of test-immunoreactive collagenase that was bound by the antibodies during the preincubation. The assay can thus not be considered as truly quantitative.

In practice, however, our data show that several inactive species of collagenase can be validly detected by the assay. Latent procollagenase can be estimated with enough precision and sensitivity by reference to a relative standard (Figs. 1 and 2). Moreover, under a variety of conditions (trypsin degradation, moderate heat-denaturation inactivation occurring in the cultures at 37°C or occurring during purification, inhibition by an inhibitor present in culture media), the collagenase activity may be to a large extent lost while its immunoreactivity is kept intact (Table I). Consequently, the present enzyme-immunoassay should be a useful complement to the catalytic assay of collagenase, not only in materials containing a latent precursor of the enzyme (procollagenase) but also in preparations where the enzyme could have been inactivated by partial denaturation or degradation or by association with some inhibitors. It has however a limitation in that excessively denatured collagenase (Table I, Expt. D) or collagenase complexed with some macromolecular inhibi-

tors (such as α_2-macroglobulin) may loose its immunoreactivity and thus escape detection. Moreover, the retention of immune recognition that forms the basis of our assay might well be related to our particular antibody preparations, as explained above.

Immunoreactive collagenase in bone culture media and extracts

No immunoreactive collagenase was found in culture medium taken after 1 or 4 days of culture of bones in heparin-free medium, even when heparin had been added to these media at the end of the cultures (Fig. 2). On the contrary, immunoreactive collagenase was detected in the media of cultures done in the presence of heparin; the amounts assayed were then closely similar to those evaluated for procollagenase by the enzyme assay. Addition of parathyroid hormone to the cultures induced a typical bone resorption. However, in the absence of heparin, no immunoreactive collagenase was detectable in the culture media (assays done in 8 pools media obtained from different series of cultures) although bone resorption was proceding with loss of calcium (resorbing bones: 87 ± 3.5 and control bones: 121 ± 29 μg/calvarium after 4 days culture, in the experiment presented in Fig. 2; mean ±S.D., $n = 4$) and hydroxyproline

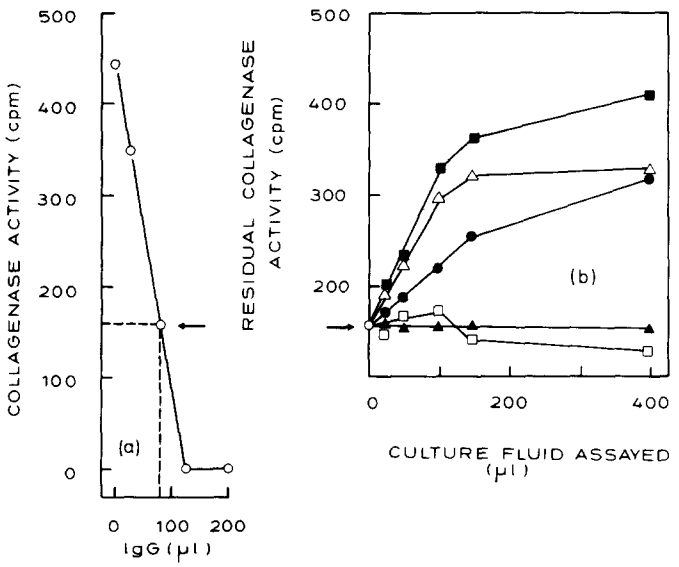

Fig. 2. Immunoreactive collagenase in bone culture fluids. (a) Inhibition of collagenase activity (○) by IgG raised against procollagenase. IgG was added to 2.4 U of trypsin-activated collagenase and, 20 min later, collagenase was assayed in the mixture by a 60 min incubation with [^{14}C]collagen (1718 cpm/tube). (b) Loss of the collagenase-inhibitory capacity of antibody by preincubation, before the addition of collagenase (2.4 U), of 75 μl of anti-mouse bone procollagenase IgG with increasing amounts of culture fluids containing the indicated amounts of catalytically assayed procollagenase (solid lines). These fluids were pools (from 4 cultures each) harvested after 4 days of culture of embryonic calvaria in media supplemented or not by various additives: (□), no additive (no significant procollagenase detectable); (▲), parathyroid hormone, 1 U/ml (no significant procollagenase detectable); (●), heparin, 300 μg/ml (3.4 U of procollagenase/ml); (△), parathyroid hormone, 1 U/ml, and heparin, 300 μg/ml (6.5 U of procollagenase/ml). A standard procollagenase-containing (6.9 U/ml) culture fluid was simultaneously assayed (■). None of these fluids was inhibitory for collagenase when it was incubated with it in the absence of added IgG.

(15.1 ± 1 and 21.1 ± 4.2 μg/calvarium respectively) and with development of resorption lacunae in the tissue (respective resorption indexes: 2.9 ± 0.6 and 0.6 ± 1). Extracts from resorbing bones (10 calvaria, taken after 1 or 2 days culture with parathyroid hormone in the absence of heparin, homogenized and extracted in 1.5 ml of 1 M NaCl [3] were also completely devoid of immunoreactive collagenase (data not shown).

These experiments thus stress the absence of significant amounts of immunoreactive precursors or remnants of collagenase in mouse bone tissue undergoing resorption or in heparin-free culture media of explanted bones. In view of the limitations of our assay discussed above, it is clear that the existence in these materials of inactive species of collagenase not reacting to our antibody or escaping our immuno-detection method, remains possible. Its probability seems however tenuous in view of the demonstration of the persistence of collagenase immunoreactivity after several kinds of treatments resulting in the loss of collagenase enzyme activity. Thus our experiments further provide more conclusive data indicating that the absence of enzymatically detectable collagenase previously noted in these materials [3] is unlikely to be due to the lack of activation of a precursor, to the inactivation of an unstable enzyme or to the inhibition of collagenase by the heat-stable inhibitor produced by the explanted bones [7]. They also confirm, with the immunoreactive assay, the positive action of heparin on the accumulation of collagenase in the culture media. The good agreement observed, in the presence of heparin, in the amounts of immunoreactive and of catalytic collagenase recovered from the culture media, is a further indication that procollagenase is stable in these media during the cultures, so that its level of accumulation is likely to represent its level of production by the explants. Finally, the present experiments again indicate that, in our cultures of mouse bones, bone resorption does not correlate with the accumulation of collagenase in the media. The source and the significance of that collagenase remain to be determined. On the contrary, excellent correlations exist between bone resorption in culture and the secretion of lysosomal enzymes [1,4,13,14] and we have recently demonstrated [5] that leupeptin and several other inhibitors of thiol-proteinases reversibly inhibit bone resorption without affecting collagenase production or activity. All this evidence are compatible with the view that lysosomal thiol-proteinases might have a critical role in the resorption of the organic matrix of bone.

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