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PROCOLLAGENASE FROM BOVINE GINGIVA

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Summary

1. Collagenase (EC 3.4.24.3) is released from bovine gingival explants in vitro as a zymogen. The zymogen does not hydrolyze collagen and does not form a complex with α_2-macroglobulin (α_2-M). It elutes in gel filtration with an apparent molecular weight of approx. 80 000.

2. Incubation of the zymogen with trypsin results in a 15 000—20 000 dalton decrease in molecular weight and imparts to the enzyme the ability to hydrolyze collagen and to form a complex with α_2-M.

3. The zymogen can be completely separated from the active enzyme by gel filtration, after binding of the active enzyme to α_2-M. Likewise, the zymogen can be harvested from cultures supplemented with serum.

Introduction

Collagenases (EC 3.4.24.3) are believed to initiate a physiological and pathological breakdown of collagen by extracellular cleavage of the helical portion of native collagen molecules. It has been suggested that collagenase is released from the cells as a zymogen, procollagenase, which is later activated by extracellular proteolysis [1,2]. In support of this, it has been demonstrated that collagenase from a number of cells and tissues either is inactive until exposed briefly to trypsin, or is substantially activated by this treatment [1,3—6]. Moreover, Vaes [1] reported that trypsin-activation of mouse bone collagenase resulted in a 20 000-dalton decrease in molecular weight. A similar inactive collagenase was harvested from culture fluids of skin explants and from skin homogenates of metamorphosing tadpoles [2]. That enzyme was activated by factors present in the culture medium, but apparently not by trypsin [2,7].

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We have previously demonstrated and partially characterized a collagenase from bovine gingiva [8]. The enzyme had a molecular weight of 63 000 and was active against collagen in solution and as fibrils. It was later shown [9] that media from the first days of culture also contained substantial amounts of latent collagenase, which could be activated by trypsin. Part of this was tentatively characterized as a collagenase-α_2-M complex; the remainder eluted slightly ahead of active collagenase on gel columns, which suggested its occurrence as a collagenase zymogen. The present study indicates that collagenase is indeed released from bovine gingiva in vitro as a proenzyme.

Materials and Methods

Preparation of collagenase

Bovine gingiva was obtained from newly slaughtered 1—2 year old cattle. Tissue excised from the incisal region was processed and cultured in serum-free Tyrode-antibiotic solution as previously described [8]. The culture fluid was harvested every second day, and the media from day 6—13 were either concentrated by osmotically-forced dialysis (Aquacide II, Calbiochem) or fractionated by ammonium sulfate precipitation [10]. The pellet formed between 20% and either 50 or 60% saturated ammonium sulfate was redissolved in a volume of Tris/NaCl/$CaCl_2$ buffer (0.03 M Tris · HCl, 0.2 M NaCl, 5 mM $CaCl_2$, pH 7.4) equivalent to 1—10% of that of the original culture fluid. All preparations were finally dialyzed against the same buffer and stored frozen. Other cultures were supplemented with 10% fetal calf serum (Grand Island Biological Company) for 12 days. The medium was changed every second day and culture fluids from day 3—12 were pooled and fractionated by ammonium sulfate precipitation (20—50% satd.). In experiments which required active collagenase exclusively, preparations were used which either (a) contained manifest collagenase activity only when harvested from the cultures ("initially active collagenase"), or (b) which were activated with trypsin (see below) and chromatographed on a Sephadex G-150 column to remove excess soybean trypsin inhibitor ("activated collagenase"). Although the distinction between these two preparations later proved to be academic, it was important for the argumentation to maintain the distinction until their identity was established.

Preparation of α_2-M and antiserum to α_2-M

α_2-M was prepared from bovine serum (Microbiological Associates) by ammonium sulfate fractionation, ion exchange chromatography on DEAE-cellulose, and gel chromatography on Sepharose 6B and Sephadex G-150 columns as previously described [9]. Antiserum against the purified bovine α_2-M was raised in 3—4 kg white New Zealand rabbits [9]. The animals were injected subcutaneously and intramuscularly with 3 mg α_2-M in 1 ml Freund's complete adjuvant. The injection was repeated at 3 and 6 weeks, and the antiserum was harvested 10 days after the last injection. The gamma-globulin rich fraction was prepared by precipitation with 45% satd. ammonium sulfate. Radial immunodiffusion was performed on Ouchterlony immunodiffusion plates (Meloy) in 1% agarose in phosphate buffer, pH 8.0.

Assay of collagenase activity

Collagenase activity was determined using the [^{14}C]glycine radiofibril assay described by Robertson et al. [11]. [^{14}C]glycine labelled collagen produced by chick calvaria in culture was mixed with a 5 mg/ml solution of acid-soluble rat skin collagen. The mixture was dialyzed exhaustively against Tris/NaCl/$CaCl_2$ buffer, and 200 µl aliquots were gelled for 15—30 min at 37°C. Reaction mixtures usually contained from 0—5 units of collagenase activity (see below) made up to a total volume of 500 µl with Tris/NaCl/$CaCl_2$ buffer. This mixture was incubated with the substrate gel at 36.5°C for 2—16 h, depending on the level of activity. The assay was terminated before substrate concentration became rate-limiting by filtering the reaction mixtures through 0.9 µ Versapor filters (Gelman). Aliquots of the filtrate were mixed with Ready-Solv Solution VI (Beckman instruments) and ^{14}C-activity measured in a liquid scintillation counter. The limits of the assay (background and total counts) were determined by substituting Tris/NaCl/$CaCl_2$ buffer or 20 µg clostridial collagenase (Sigma, Type III, Fraction "A") for the gingival enzyme. The substrate was resistant to non-specific proteolytic degradation: only 7—10% of the collagen gel was solubilized during 16 h incubation with 0.01% trypsin. Latent collagenase was quantitated as the activity difference between trypsin-activated and unactivated aliquots. Activation was achieved essentially according to the method of Vaes [1] by 10 min incubation at room temperature (22—24°C) with 2 µg L-1-tosyl-amido-2-phenylethylchloromethyl ketone-trypsin (Worthington) per 1—5 units of latent collagenase, followed by addition of a 5—15 fold molar excess of soybean trypsin inhibitor (Sigma). The inhibitor was allowed to react for 10 min with the trypsin before the substrate gel was added. In this assay system, one unit of collagenase was defined as the activity which solubilized 1 µg collagen gel/min at 36.5°C. One unit of latent collagenase (procollagenase) was defined as the amount of enzyme which gave rise to one unit of active collagenase when activated as described.

Assay of esterase activity

The presence of esesterases in serum-free culture fluid was detected essentially by the method of Hummel [12]. Hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (Tos-Arg-Ome) and benzoyl-L-tyrosine-ethyl ester (Bz-Tyr-OEt) was recorded spectrophotometrically at 247 nm and 256 nm, respectively. Incubation was at room temperature and pH 7.4 in Tris/NaCl/$CaCl_2$ buffer, which in the case of Bz-Tyr-OEt also contained methanol at 23% w/w.

Characterization of reaction products from hydrolysis of collagen in solution

Reaction products resulting from digestion of collagen in solution were obtained by incubating 100 µl of acid-soluble rat skin collagen (5 mg/ml in Tris/NaCl/$CaCl_2$ buffer) with 50 µl of either trypsin-activated or unactivated enzyme at room temperature for 1—8 h. After termination of the reaction by addition of 50 µl 0.5 M acetic acid and denaturation of the collagen by heating to 56°C for 10 min, 100 µl of the reaction mixture was analysed by disc gel electrophoresis. The conditions included 7.5% acrylamide gels at pH 4.3 with a constant current of 5 mA/tube, essentially as described by Sakai and Gross [13]. The gels were fixed and stained for 1 h in 0.5% Amido black in 7% acetic

acid, and destained electrophoretically in 7% acetic acid. Similar incubation mixtures were used for preparing segment-long-spacing aggregates. In this case, however, after acetic acid was added to terminate the reaction, the mixture was dialyzed against 0.05 M acetic acid for 24 h in the cold, and an equal volume of a 0.4% solution of ATP (free acid) in water was added. The cloudy suspension formed within 1 h in the cold was applied dropwise to carbon-coated grids, stained with 1% phosphotungstic acid (1 h) and supersaturated uranyl acetate in 50% ethanol (7 min) at room temperature. The grids were examined in a Philips 300 electron microscope.

Column chromatography

Gel filtration of the collagenase preparations was performed on Sephadex G-150 (1.6 cm × 95 cm) and Sephadex G-200 (1.6 cm × 92 cm) columns equilibrated with Tris/NaCl/$CaCl_2$ buffer. Elution was at 10—20 ml/h at room temperature with the same buffer. Fractions of 3—5 ml were collected at 4°C and assayed for latent and manifest collagenase activity. Elution of ultra-violet absorbing material was recorded continuously with a Beckman DB-G spectrophotometer equipped with a flow cell. The columns were calibrated for molecular weight determination by the method of Andrews [14], using blue dextran, aldolase (158 000), bovine serum albumin (69 000), ovalbumin (45 000), chymotrypsinogen A (25 000) and ribonuclease (13 700) as standards.

Inhibition of collagenase activity

The effect of various enzyme inhibitors on collagenase activity was tested using "activated collagenase" prepared as described. The inhibitors included whole bovine serum, fetal calf serum, α_2-M prepared as described, human α_1-antitrypsin (Worthington), soybean trypsin inhibitor, phenylmethylsulfonylfluoride (a DFP substitute), EDTA, 1,10-phenanthroline, and cysteine. Inhibitors were incubated for 30 min at room temperature with aliquots of the collagenase preparation, and resultant collagenase activity was measured in radiofibril assay.

Results

The crude culture fluid, as well as preparations concentrated by ammonium sulfate fractionation, usually were mixtures containing both latent and manifest collagenase activity. Both latent and active collagenase precipitated quantitatively between 20 and 60% saturated ammonium sulfate and usually more than 90% of both were precipitated even with 50% saturated ammonium sulfate. A survey of a large number of preparations revealed that the relative content of latent collagenase varied from 0 to 100%. Under the conditions employed in this study, the release of total (latent plus manifest) collagenase activity by the gingival explants averaged approximately 1 unit per ml culture fluid per day, or about 100 units per animal (3 g wet weight) per day.

Latent collagenase was activated by brief exposure to trypsin. Conditions employing 0.2—5 μg trypsin per unit of latent collagenase at room temperature gave a wide useful timespan: maximal activation was achieved with 1—5 min and proteolytic degradation of active collagenase was negligible even after 120

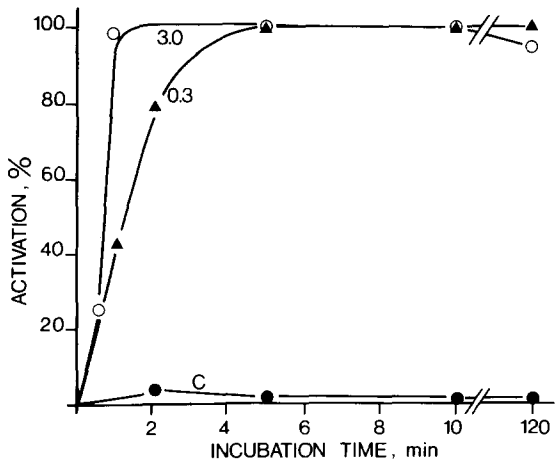

Fig. 1. Activation of latent fibroblast gingival with trypsin. Aliquots of 1 unit of latent collagenase were incubated at room temperature with 3.0 (○———○) and 0.3 μg trypsin (▲———▲). The activation was stopped by addition of a 10-fold molar excess of soybean trypsin inhibitor. Controls (●———●) were incubated with premixed trypsin (3.0 μg) and inhibitor (30 μg). Resultant collagenase activity was measured in radioassay (12 h) and expressed as per cent of maximal activity.

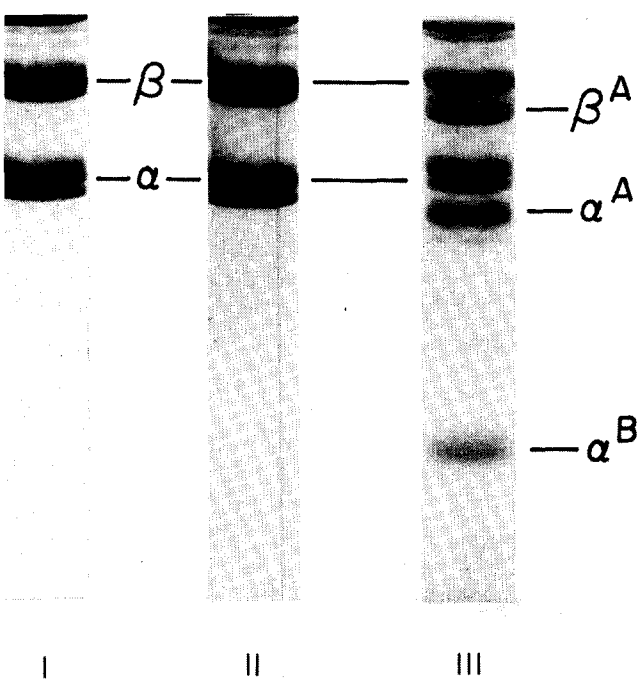

Fig. 2. Acrylamide gel electrophoresis of reaction products from enzymatic hydrolysis of acid soluble Type I collagen in solution. Incubation was for 4 h at 22°C and pH 7.4. (I) Buffer control; 250 μg collagen + Tris/NaCl/$CaCl_2$ buffer. (II) 250 μg collagen + 1 unit of latent collagenase. (III) 250 μg collagen + 1 unit of trypsin-activated collagenase. Incubation with activated collagenase gave rise to fragments α^A, β^A (3/4 pieces of monomer and dimer) and α^B (1/4 piece of monomer and non-crosslinked end of dimer). Latent collagenase did not hydrolyze collagen in solution.

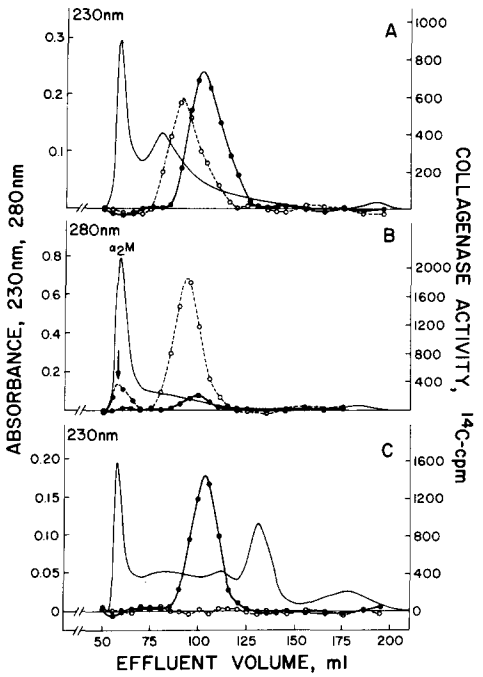

Fig. 3. Gel filtration of latent and active bovine gingival collagenase. The Sephadex G-150 column (1.6 cm X 95 cm) was eluted at 20 ml/h with Tris/NaCl/$CaCl_2$ buffer. Fractions of 5 ml were collected, and 200-μl aliquots were assayed for latent and manifest collagenase activity using the radiofibril assay (4 h). Total substrate lysis was 2150 cpm. Elution of ultra-violet absorbing material was recorded at 230 nm or 280 nm (———), and the presence of α_2-M was demonstrated by radial immunodiffusion. (A) 80 units of a 1 : 1 mixture of latent and active collagenase. The latent collagenase (○- - - - -○) eluted ahead of the active enzyme (●———●). (B) 240 units of the same preparation was incubated for 30 min at room temperature with 3 mg α_2-M and then chromatographed on the same column. Latent collagenase (○- - - - -○) eluted in the same position as before, but almost all of the active collagenase was removed (●———●). A small part of the activity which chromatographed with α_2-M was recovered in the void volume fractions (●- - - - -●, arrow) after incubation for 10 min at room temperature with 50 μg trypsin per 200 μl eluant, followed by addition of a 10-fold excess of soybean inhibitor. (C) 80 units of the stock preparation was activated with 30 μg trypsin for 10 min at room temperature, followed by addition of a 10-fold molar excess of soybean inhibitor and then chromatographed on the Sephadex G-150 column. All the latent enzyme was activated (○———○) and eluted at the position of initially active collagenase.

min (Fig. 1). Some preparations of latent collagenase, though never exposed to trypsin, eventually "autoactivated". Since esterase activity on Bz-Tyr-OEt, and to a lesser extent on Tos-Arg-Ome, was found in such preparations, it seems likely that contaminating proteases in the preparation were responsible for the slow "autoactivation". Latent collagenase had no activity on collagen in solution. After incubation with trypsin, however, it cleaved both collagen fibrils and collagen in solution in a manner similar to other mammalian collagenases. Reaction at 22°C gave rise to products which, when characterized by acrylamide disc gel electrophoresis, revealed only one cleavage of all three chains of native Type I collagen (Fig. 2). Electron micrographs of the segment-long-spacing aggregates demonstrated that the site of cleavage was 3/4 of the distance from the N-terminal end of the molecule. Activated collagenase also showed the same inhibition characteristics as "initially active collagenase". It

was inhibited by serum, α_2-M, cysteine, and by metal chelators such as EDTA and 1,10-phenanthroline, but not by α_1-antitrypsin (0.5 mg per unit collagenase), soybean trypsin inhibitor (0.5 mg per unit collagenase), or phenylmethylsulfonylfluoride (0.5 mM).

To characterize the latent enzyme further, a sample containing 80 units of collagenase (half latent and half active) was chromatographed on a calibrated Sephadex G-150 column. Fig. 3A shows that the active collagenase chromatographed at the position expected for "initially active" bovine gingival collagenase, corresponding to a molecular weight of approx. 63 000 [8]. The observation that latent collagenase clearly eluted ahead of the active enzyme indicates that it possessed a slightly higher molecular weight. Two separate experiments on calibrated Sephadex G-150 columns gave values of 82 000 and 78 000, some 15 000—20 000 daltons more than the active enzyme. A sample of 240 units of collagenase of the same preparation as used in Fig. 3A was then incubated for 1 h at room temperature with 3 mg purified α_2-M and chromatographed under identical conditions. It has previously been shown that α_2-M forms a tight complex with collagenase [15,16] and that this complex, despite reports to the contrary [17,18], cannot be chromatographically dissociated [15,16,19]. Fig. 3B shows that virtually all the active collagenase was removed from its own elution volume by the α_2-M. However, when the eluant fractions were incubated with a 10—20 fold molar excess of trypsin relative to α_2-M for 10 min at room temperature, 8—10% of the lost collagenase activity was recovered from the void volume fractions, as previously reported [9]. Fig. 3B also shows that the latent collagenase was not bound by the globulin. It eluted without any gain or loss of activity at the same position as before. To ascertain that the molecular weight of trypsin-activated collagenase does not differ from that of "initially active collagenase", a sample from the preparation used in Fig. 3A and B (80 units) was activated for 10 min at room temperature with 20 μg of trypsin, and the activation was stopped with a 10-fold molar excess of soybean inhibitor. The sample was then chromatographed on the Sephadex G-150 column under the same conditions as before, and each fraction was assayed for latent and active collagenase. Fig. 3C shows that the activation was complete. The latent collagenase had disappeared and there was a comparable, almost two-fold, increase of the amount of active collagenase eluting with an apparent molecular weight of approx. 60 000. When the peak of active collagenase from Fig. 3C was pooled, concentrated and incubated with 3 mg α_2-M for 1 h, all of the collagenase activity was bound to the globulin and consequently removed from its own elution volume on a Sephadex G-150 column, as previously reported [18]. Trypsin-activated collagenase thus shares all the characteristics of "initially active" collagenase with respect to molecular weight, inhibition, and collagen cleavage pattern.

In further support of the zymogen nature of latent collagenase was the observation that collagenase could be harvested also from serum-containing media after chromatographic separation from the α_2-M. Cultures of bovine gingiva were supplemented with 10% fetal calf serum and thus supplied with an α_2-M concentration of 200—300 μg/ml. To ascertain that the α_2-M was not inactivated, its capacity to inhibit active bovine gingival collagenase was tested before and after the culture period. Fetal calf serum at a 10% concentration

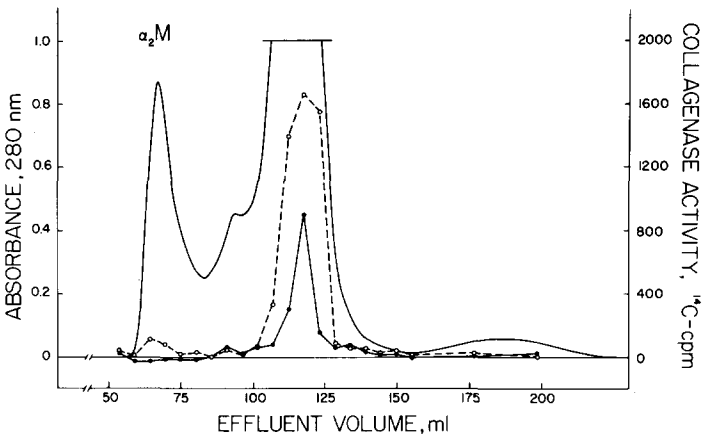

Fig. 4. Gel filtration on Sephadex G-200 of ammonium-sulfate fractionated culture fluid from serum supplemented cultures of bovine gingiva. The column (1.6 cm × 92 cm) was eluted at 10 ml/h with Tris/NaCl/$CaCl_2$ buffer. Fractions of 5 ml were collected, and 100-µl aliquots were assayed for latent and active collagenase using the radiofibril assay (16 h). Total substrate lysis was 2150 cpm. Activation of latent collagenase was with 20 µg trypsin for 10 min at room temperature, followed by addition of a 10-fold molar excess of soybean trypsin inhibitor. A major peak of latent collagenase (○- - - - -○) and a minor peak of active enzyme (●———●) were found at the same elution volume after separation from α_2-M. Absorbance at 280 nm (———).

was found to contain a 30—40 fold excess of the amount of collagenase inhibitor (α_2-M) necessary to inhibit completely the 2 units of collagenase per ml per harvest usually released in such cultures. Culture fluids from days 3—12 supplemented with 10% fetal calf serum were harvested every second day and, at that stage, found without detectable collagenase activity. After ammonium sulfate fractionation, the sample was chromatographed on a Sephadex G-200 column (Fig. 4). A peak of mainly latent collagenase eluted at a molecular weight of approx. 80 000, clearly separated from the inhibitor in the excluded material. Little or no collagenase activity was detected in the void volume fractions. Similar results were since obtained also with confluent fibroblast monolayer cultures (Birkedal-Hansen et al., unpublished). So the data indicate that collagenase was indeed released from the cells in latent form and thus protected from binding to the α_2-M of the calf serum. The eluants exhibited some manifest collagenase activity corresponding to the position of latent collagenase. This peak was seen occasionally and in varying proportions to that of latent collagenase, but usually representing only a minor fraction of the total activity. It was a general finding, even in preparations which showed only latent collagenase when assayed immediately after chromatography, that 'autoactivation' occurred more rapidly in samples prepared from serum-supplemented culture fluids than in similar preparations from serum-free media. It may be speculated that an explanation of this difference resides in the ability of at least two serum proteases, kallikrein and plasmin, to activate latent collagenase [20]. It is thus possible that following separation from the α_2-M, sufficient protease activity cochromatographed with the latent collagenase to activate this enzyme during the assay. The amount of latent collagenase harvested as just described compared favorably with that harvested from serum-free media under otherwise

identical conditions. Usually 60—90% of the activity exhibited by control samples was recovered from serum-containing samples after gel chromatography.

Discussion

The data show that serum-free media protein from bovine gingival explant cultures contains a latent collagenase which does not cleave collagen and does not bind α_2-M. Activation with trypsin lowers the molecular weight by 15 000—20 000 daltons and imparts to the enzyme the ability to hydrolyze collagen and to form a complex with α_2-M. Moreover, the observation that latent collagenase can be harvested in comparable yield from serum-supplemented cultures despite the high concentration of α_2-M (200—300 μg/ml), indicates that the enzyme is indeed released from the cells in latent form and, consequently, that it is a zymogen.

The conversion of zymogen to the active form in vivo probably requires the presence of other proteases. It can be assumed that such proteases occur abundantly in areas of tissue catabolism, and they have also been demonstrated as contaminating elements of collagenase preparations from tissue culture systems [21—23]. A specific activator of procollagenase has not yet been demonstrated. It thus remains unknown whether proteolytic activation of procollagenase in vivo is a result of a specific enzyme or is achieved by any of a multitude of proteolytic enzymes. So far, however, a series of proteases have been shown to activate procollagenase in in vitro experiments, including trypsin, chymotrypsin, cathepsin B_1, kallikrein and plasmin [1,20]. Furthermore, it is not clear whether these proteases act directly on the procollagenase molecule or whether their effect is mediated through a "proactivator" as suggested by Vaes [24].

The release of collagenase in the form of a zymogen constitutes an important mechanism for the regulation of collagen catabolism and adds to the number of biological means for controlling these processes. A series of such regulatory mechanisms, acting at different stages in the physiological life of the collagenase molecule, have been revealed during the last few years. There is accumulating evidence that the synthesis and release of collagenase from cells can be greatly increased both by general and by specific cellular stimulation [5,23,25—28]. It is likely that, once the zymogen is released from the cells, its conversion to active enzyme is the next link in the chain of regulatory measures. Further regulation of collagenase activity is exerted by enzyme inhibitors.

To date, unequivocal evidence for inhibition of collagenase has only been presented in the case of serum α_2-M, which forms a tight complex with the enzyme [15]. Under physiological conditions, this process appears irreversible. Even partial recovery of collagenase activity from the complex requires extreme conditions, such as denaturation of the globulin by NaSCN [15] or incubation with large excesses of trypsin [9]. Since the proenzyme is not bound by α_2-M, the release of an extracellular enzyme like collagenase as a zymogen is a feasible mechanism for both protecting the tissue against undesired collagen degradation and at the same time protecting the enzyme from inactivation by α_2-M.

This series of regulating mechanisms offers multiple possibilities for keeping collagen production and degradation in balance, but at the same time contains several links, which under pathologic conditions can be attacked, and will lead to a net imbalance between synthesis and breakdown. It is thus likely that change in collagen content, which is seen as a secondary response to many pathologic conditions, is a result of deterioration of one or more of the regulating mechanisms outlined above.

Acknowledgments

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