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DIRECT MEASUREMENT OF NEUTRAL COLLAGENASE ACTIVITY IN HOMOGENATES FROM BABOON AND HUMAN LIVER

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Summary

Neutral collagenase (EC 3.4.24.7) has been detected in human liver biopsies, baboon liver biopsies and human autopsy liver for the first time. The reaction mixtures were composed of collagen in solution and total liver homogenate in 0.5 M Tris-HCl, pH 7.5 at 25°C/0.2 M NaCl/10 mM $CaCl_2$/3 mM *p*-chloromercuribenzoic acid. Collagenase activity was found by directly subjecting the reaction mixtures to viscometric assay and the activity was confirmed to be due to neutral collagenase by identifying the products using disc gel electrophoresis. It proved necessary to use *p*-chloromercuribenzoic acid in order to reveal collagenase activity in total liver homogenates from these species. The *p*-chloromercuribenzoic acid served to inhibit thiol proteinases and all other signs of nonspecific collagenolysis on disc gel electrophoresis, and it was able to activate latent collagenase which trypsin could not.

Introduction

Since Gross and Lapiere [1] first used tissue explants to demonstrate the activity of neutral collagenase (EC 3.4.24.7), which is characterized by the

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ability to degrade native collagen fibrils under physiological conditions of temperature and pH, there have been a number of reports of this enzyme activity in various tissues [2—6]. In most of them, collagenolytic activity had to be demonstrated in the culture medium of growing tissue explants. Experimenters have reported collagenase activity in sundry tissues without culturing explants [6], but almost all of them had to employ an extraction and/or purification procedure to show the presence of the enzyme. One group, which reported collagenase activity directly in the reaction mixtures (composed of collagen in solution and total tissue homogenate), studied tumor rather than normal tissue [7,8].

Liver possesses very weak collagenase activity and also contains various other proteinases and enzyme inhibitors which interfere with the assay of collagenase. Therefore, the evidence for the presence of collagenase had been found only after using purification [9—11] or tissue culture techniques [12—14] with rat and mouse livers. It may be expected that collagenase is present in several states and that its activity is as complexly regulated in hepatic tissue as it is known to be in other, more extensively studied, tissues. The total process of collagen degradation due to neutral collagenase must be affected by procollagenase production, collagenase activation, circulating inhibitors and collagenase destruction, to list some of the known influences. Therefore, it is important to develop measurements of collagenase activity in whole liver homogenates to complement and extend the appreciation of hepatic collagenase, which is furnished by immunohistochemical, tissue explant and extraction techniques. In order to measure the collagenolytic activity in liver homogenate using a collagen solution assay, we have modified the method of Nagai et al. [15] to exclude other proteinases present in liver. Collagenase activity was measured at neutral pH to exclude acid proteinases and in the presence of *p*-chloromercuribenzoic acid to inhibit thiol proteinases. Furthermore, we examined the role of *p*-chloromercuribenzoic acid as an activator of latent collagenases. We used disc-gel electrophoresis to compare the reaction products produced by collagenase after trypsin or *p*-chloromercuribenzoic acid pretreatment.

Materials and Methods

Reagents. Reagent-grade Tris, EDTA (trisodium salt), *p*-chloromercuribenzoic acid, soybean trypsin inhibitor and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin (treated with L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone) was obtained from Worthington Biochemical Corp., (Freehold, NJ). Acrylamide and methylenebisacrylamide were purchased from Eastman Organic Chemicals (Rochester, NY). 1,10-Phenanthroline (monohydrate) was obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Preparation of collagen substrate. Acid soluble rabbit skin collagen was extracted and purified by the method of Glimcher et al. [16]. Type I collagen was extracted and purified by the method of Timpl et al. [17] and was used for the present collagenase assay. The purity of this collagen substrate was confirmed by disc gel electrophoresis using the method of Nagai et al. [18].

Preparation of liver homogenate. For the collagenase enzyme source, human

or baboon liver biopsies or human autopsy liver was used. Baboon liver was taken by surgical biopsy and human liver was obtained by Menghini needle biopsy or at autopsy. Immediately after samples were obtained they were cut into small pieces and put into a beaker which contained 100 ml cold buffer (0.05 M Tris-HCl, pH 7.6 at 5°C/0.15 M NaCl) and washed six times (each time for 5 min), with changes of the same buffer. They were then collected on filter paper, weighed and homogenized ice cold in 10 vol. of a second buffer (0.5 M Tris-HCl, pH 7.6 at 5°C/0.2 M NaCl/5 mM $CaCl_2$/0.1% Triton X-100) using a Teflon homogenizer with motor-driven pestles for 30-s intervals. When processing human liver biopsies, a microglass hand homogenizer was employed to handle the small amount of liver tissue (approx. 10 mg). A part of the liver homogenate was used for determination of protein by the method of Lowry et al. [19] with bovine serum albumin in Tris homogenate buffer as the standard. Another part of the liver homogenate was used for histological examination.

Viscometric assays. Collagenolytic activity was assayed by the decrease in viscosity of undenatured collagen in solution at pH 7.5, 25°C. Only baboon liver and human autopsy liver were used for this assay, because a separate sample tube is required for each incubation period and relatively large amounts of liver tissue are needed. The reaction mixture (0.9 ml) comprised total liver homogenate (3 mg protein) and 600 μg collagen substrate in 0.05 M Tris-HCl, pH 7.5 at 25°C, for appropriate times in a water bath with constant shaking. Assay blanks did not contain liver homogenate. After each incubation period, tubes were centrifuged at 12 000 × *g* for 5 min at room temperature, and 0.7 ml supernatant was transferred into a Cannon-Manning Semi-Micro viscometer, size 150, with flow times for water of 28—30 s at 25°C. Each flow time was measured three times as soon as possible, and the mean value was expressed as a percentage of the initial specific viscosity, η_{sp}. The viscosity changes after using different amounts of human liver protein were also measured after 24 h incubation.

Disc gel electrophoresis. Disc gel electrophoresis of collagen reaction products was performed as described by Nagai et al. [18]. After examining the viscosity, 300 μl reaction mixture was immediately taken and 12 μl glacial acetic acid was added to it to stop the reaction (final pH 3.4). After spinning at 12 000 × *G* for 5 min, the supernatant was dialyzed repeatedly against cold acetate buffer, pH 4.8. Acid soluble supernatant was collected after centrifugation and applied to 7.5% acrylamide gels after thermal denaturation of the collagen. Gels were stained in 0.1% Coomassie brilliant blue R-250 in methanol/acetic acid/water (5 : 1 : 4, v/v) for 3 h and destained in 7.5% acetic acid/5% methanol. Some samples were examined immediately (without viscometry) by disc gel electrophoresis to detect the reaction products and compare them under various assay conditions. For this purpose, the first tube, our routine assay, was composed of collagen substrate and liver homogenate with *p*-chloromercuribenzoic acid. A second tube had the same contents as the routine assay but without *p*-chloromercuribenzoic acid. Third and fourth tubes contained trypsin-treated liver homogenate with and without *p*-chloromercuribenzoic acid, respectively. Trypsin treatment was as follows: 10 or 100 μg/mg liver homogenate protein were added to total liver homogenate and incubated for

5 min at 37°C, after which 10-fold (w/w) of soybean trypsin inhibitor was added and incubation continued for 10 min more at 37°C. The homogenate so treated was used for collagenase assay. Each assay tube had its own blank of the same composition as the reaction tube with 10 mM EDTA or 1 mM phenanthroline instead of $CaCl_2$. Liver homogenate without collagen substrate and collagen substrate without liver homogenate were also examined. The effect of soybean trypsin inhibitor and/or phenylmethylsulfonyl fluoride (a partial inhibitor of serine proteinases) upon the reaction in each tube was also tested. To compare the amount of degradation products of collagen in each reaction mixture, stained gels were measured with a densitometer (Beckman, Model R-112).

Results

The purity of Type I collagen prepared for use as substrate in the collagenase assay was confirmed by disc gel electrophoresis.

The baboon liver used in the viscometric assay was histologically normal, but the human autopsy liver showed mild central and sinusoidal congestion. Human liver obtained by biopsy and used only to demonstrate reaction products by disc gel electrophoresis had moderate fatty infiltration.

Collagenolytic activity was observed by viscometric assay in the reaction mixtures which contained collagen substrate and homogenate of baboon or human liver. The specific viscosity of the reaction mixture with human autopsy liver decreased at fairly constant velocity until it reached a 65% decrease from the initial specific viscosity ($\eta_{sp} = 3.28$) at 24 h of incubation. Using baboon liver, the rate of decrease in viscosity slowed after 6 h of incubation and resulted finally in only a 33% decrease from initial specific viscosity ($\eta_{sp} = 3.41$) at 24 h. Blank incubations (without liver homogenate) remained within 8% of the initial viscosity ($\eta_{sp} = 3.04$) after 24 h of incubation. Disc gel electrophoresis of the reaction mixture containing human autopsy liver, at lengthening intervals of incubation, showed the degradation products of the collagen molecule due to collagenase (α^A, β^A and α^B) to be increasing commensurate with the cumulative decrease in viscosity (Fig. 1). Even after only 2 h of incubation similar specific products were obtained (data not shown). Acrylamide gels of reaction mixtures of baboon liver enzyme plus substrate showed the same findings as those using human autopsy liver (data not shown).

A linear correlation between the change of initial viscosity after 24 h of incubation at 25°C and various amounts of human autopsy liver present in the reaction mixture, persisted up to 3 mg liver homogenate protein.

The acrylamide gel electrophoretic patterns of products of various incubation mixtures after 24 h of incubation are compared in Fig. 2. Collagen which was incubated for 24 h without liver homogenate showed only monomer (α), dimer (β) and higher molecular weight species (Fig. 2a), indicating absence of cleavage of the substrate. Liver homogenate incubated without collagen substrate revealed only a band at the front (Fig. 2g). Addition of trypsin and soybean trypsin inhibitor to liver homogenate, without substrate, yielded essentially the same frontal band (data not shown). Our routine assay, which contained collagen and liver homogenate with *p*-chloromercuribenzoic acid,

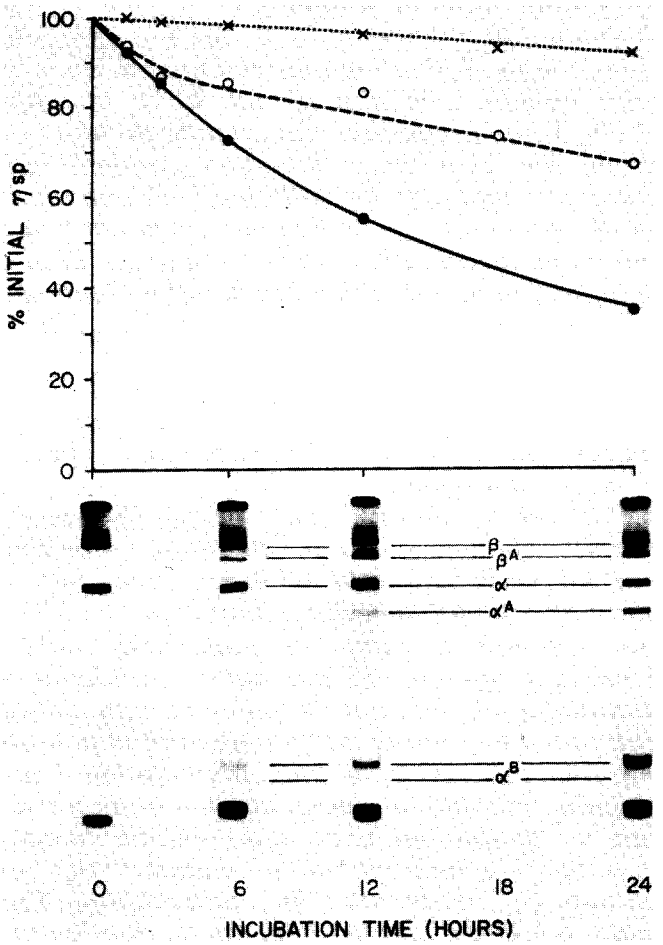

Fig. 1. Viscometric assay of the collagenase from human liver at autopsy (●), and biopsy of baboon liver (○) using collagen in solution at 25° C. The control reaction mixture did not contain liver homogenate (x). The results are expressed as a percentage of the initial specific viscosity of the relevant reaction mixture ●———●, η_{sp} = 3.28; ○ — — — ○, η_{sp} = 3.41; x - - - - - - x, η_{sp} = 3.04.

resulted in discrete bands migrating in positions identical to the collagen products of lower molecular weight, α^A, β^A and α^B, which arise after the action of other known collagenases (Fig. 2c). Addition of 10 mM EDTA (Fig. 2b), or 1 mM phenanthroline (data not shown), to our routine assay prevented the appearance of α^A, β^A and α^B bands. The routine assay, except for the omission of *p*-chloromercuribenzoic acid showed α^A, β^A and α^B bands, whose appearance was not inhibited by the addition of soybean trypsin inhibitor, as well as additional small bands (Fig. 2f). Trypsin pretreatment without *p*-chloromercuribenzoic acid resulted in α^A and β^A bands among other bands (Fig. 2e), but the density of those specific bands was about the same as that due to untreated homogenate (Fig. 2f). The addition of *p*-chloromercuribenzoic acid to trypsin-treated liver homogenate seemed to make α^A and β^A bands

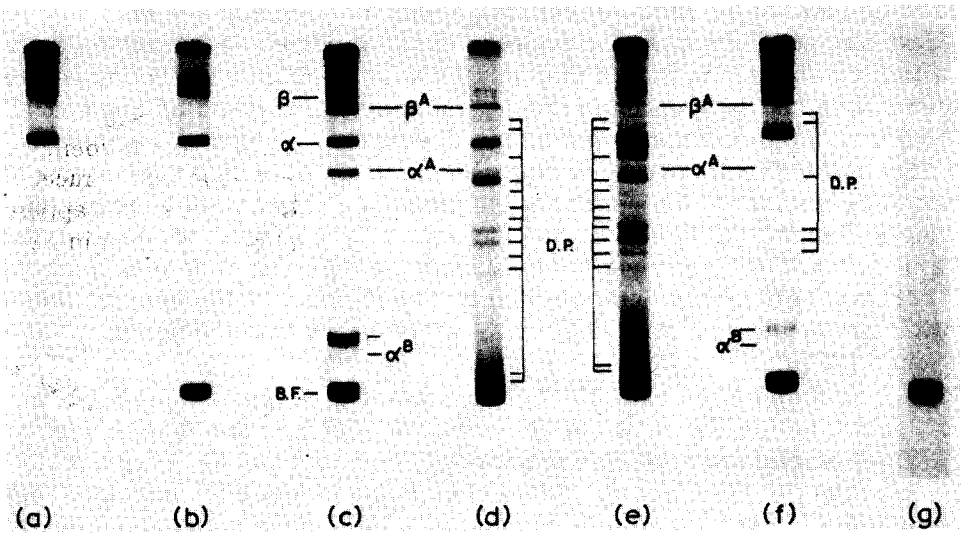

Fig. 2. Comparison of reaction products by disc gel electrophoresis of various incubation mixtures using human autopsy liver homogenate after 24 h incubation at 25°C. Each incubation mixture (0.03 ml) contained whole liver homogenate (1 mg protein) and 200 μg collagen in 0.05 M Tris-HCl, pH 7.5/0.2 M NaCl/10 mM $CaCl_2$/3 mM *p*-chloromercuribenzoic acid with the following modifications; (a) omission of liver homogenate; (b) 10 mM EDTA without $CaCl_2$; (c) no modification (our routine assay); (d) pretreatment of liver homogenate with 100 μg trypsin followed by 1 mg soybean trypsin inhibitor; (e) same as (d) but without *p*-chloromercuribenzoic acid; (f) omission of *p*-chloromercuribenzoic acid and (g) omission of collagen substrate. α, monomer; β, dimers; α^A, 3/4-length fragments of α-chain; α^B, 1/4 length fragments of α-chain; β^A 3/4-length fragments of β-chain; B.F., buffer front; D.P., nonspecific degradative products.

appear or become more prominent (Fig. 2d), compared to those with trypsin but without *p*-chloromercuribenzoic acid pretreatment (Fig. 2e). It also rendered the multiple nonspecific bands faint (Fig. 2d). EDTA added to this tube reduced the α^A and β^A bands, seen without the metal chelator, but did not affect other bands (data not shown). Soybean trypsin inhibitor and phenylmethylsulfonyl fluoride did not affect the findings which could be seen in our routine assay or the assay after trypsin pretreatment (data not shown).

Baboon liver was similar to human autopsy liver in that *p*-chloromercuribenzoic acid in the assay allowed specific reaction products (α^A, β^A, α^B) to appear without nonspecific bands, and in that trypsin pretreatment had no effect. Baboon liver differed in that it produced no specific bands without *p*-chloromercuribenzoic acid (i.e., it had no free activity).

Human biopsy liver (0.5 mg liver homogenate protein/300 μl) in our routine assay also resulted in the appearance of the specific degradation products of collagen due to collagenase.

Discussion

Although there have been reports attesting to the presence of collagenase in rat and mouse livers [9—14], there have been no reports prior to ours of such activity in human or other animal livers. Moreover, there have been no demonstrations, before this report, of the presence of collagenase activity directly in

the reaction mixtures using normal total tissue homogenate. We were able to get clear disc gel electrophoretic evidence of the products of neutral collagenase action on collagen in the reaction mixtures, by using total liver homogenate from baboons and humans and applying the modified method of Nagai et al. [15] to a collagen solution assay in the presence of *p*-chloromercuribenzoic acid. The difficulty in demonstrating the presence of collagenase in tissue homogenate may be attributed to: the existence of collagenase as a proenzyme-enzyme-inhibitor complex [20], the binding of collagenase to extracellular collagen [6] and the presence of nonspecific proteinases. It is not apparent why collagenase is readily demonstrated in tumor tissue [7,8], since in almost all other tissues the enzymes must first be extracted or an activation procedure is required [20].

Trypsin is commonly used [20] for the activation of latent collagenase, but thiol-blocking agents like *p*-chloromercuribenzoic acid have also been employed [20]. These reagents, except for 4-aminophenylmercuric acetate, were reported to be less effective than trypsin. Sellers et al. [21], for example, indicated that 0.7 mM *p*-chloromercuribenzoic acid gave only 13% of the activation given by trypsin. Our results showed that 3 mM *p*-chloromercuribenzoic acid was much better than trypsin in promoting collagenase activity in liver homogenate and was effective in excluding other proteinases. In our experiments, the addition of 3 mM *p*-chloromercuribenzoic acid to human or baboon liver homogenate was necessary to yield the typical pattern of collagen degradation products (α^A, β^A and α^B) on disc gel electrophoresis (Fig. 2c), expected of neutral collagenase action, and did not create other small bands. By contrast, trypsin treatment of baboon biopsy liver did not yield those typical products, but instead yielded multiple nonspecific bands. Also, in the case of human autopsy liver, trypsin could not be shown to activate latent collagenase (Fig. 2e) since the specific bands (α^A and β^A but not α^B) were already present (Fig. 2f) in the tube without the activation reagents, trypsin and *p*-chloromercuribenzoic acid. Our results showed that 3 mM *p*-chloromercuribenzoic acid was much better than trypsin in promoting collagenase activity in liver homogenate and was effective in excluding other proteinases.

The nonspecific bands which were seen in the reaction tube after trypsin pretreatment could not be prevented by the addition of 1 mM phenylmethylsulfonyl fluoride and 10 mM EDTA but were dimished by prior addition of 3 mM *p*-chloromercuribenzoic acid (Fig. 2d). Thus, the nonspecific bands are due to neutral proteinases which are not inhibited by soybean trypsin inhibitor, phenylmethylsulfonyl fluoride and EDTA but are partly inhibited by *p*-chloromercuribenzoic acid. Further investigation is necessary to clarify these points. In the case of human autopsy liver, we found ample free collagenase (Fig. 2f), which yielded α^A, β^A and α^B bands on disc gel electrophoresis without the necessity for any activation reagents and with the presence of soybean trypsin inhibitor during the incubation period [22]. The presence of free collagenase may be easily envisioned as due to activation of proenzyme by endogenous proteinases [6] released after death. However, there is a possibility that the free collagenase is also present in fresh human liver.

In the viscometric assay using human autopsy liver, correlations were seen between the change of viscosity and the incubation time up to 12 h, and

between the change of viscosity and different amounts of liver protein up to 3 mg. From these incubation mixtures, we obtained clear bands of α^A, β^A and α^B, the specific degradation products of collagen due to neutral collagenase, without any other nonspecific bands. Therefore, we believe that our routine viscometric assay with *p*-chloromercuribenzoic acid at neutral pH is very useful for the measurement of collagenase activity in total liver homogenate.

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