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Human Skin Collagenase. The Role of Serum Alpha-Globulins in the Control of Activity *In Vivo* and *In Vitro*

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ABSTRACT **An antibody against human skin collagenase obtained from tissue culture has been used to demonstrate the presence of immunoreactive collagenase in human skin extracts that have no detectable enzyme activity. Gel filtration of these skin extracts permits the separation of collagenase in its active form from the other proteins in the crude mixture. The recovery of enzymatically active collagenase appears to be due to the chromatographic separation of the enzyme from the serum antiproteases, alpha$_1$-antitrypsin and alpha$_2$-macroglobulin, suggesting that collagenase activity in fresh tissue extracts is masked by these known collagenase inhibitors. These findings are supported by *in vitro* studies utilizing human skin explants in tissue culture.**

The demonstration of collagenase *in vivo* in human skin indicates that the enzyme is present at concentrations that are of physiologic significance in collagen remodeling. Evidence is also presented that these findings are not unique to human skin.

A specific human skin collagenase, active at neutral pH, has been isolated and characterized from culture medium in which living skin explants were grown (1). Extracts of skin, however, have consistently failed to yield an enzyme with collagenase activity. This is also true for practically all other human (2–4) and animal (5, 6) collagenases so far identified, except for the collagenases from the crustacean hepatopancreas (7) and human granulocytes (8) which are extractable.

In addition, a puzzling and as yet unexplained observation is the delay of about 24–48 hr in the appearance of detectable collagenase in the culture media of human skin, and other human and animal tissues (see review, Eisen *et al.*, 1970) (9). The absence of detectable collagenase activity in human skin extracts, and the delayed appearance of enzyme activity in tissue culture, raise the question as to whether collagenase isolated from such cultures is of biological significance in the remodeling of connective tissue *in vivo*.

A possible explanation for these observations, based on the fact that human serum—and in particular the alpha-globulin component—is an effective inhibitor of human skin collagenase (10), is that collagenase activity in extracts of fresh tissue may be masked by the inhibitory action of serum alpha-globulins present together with the enzyme in such crude tissue extracts. Similarly, the lag in the appearance of collagenase activity *in vitro* could be due to the fact that the enzyme is inhibited initially by the presence of serum proteins bound to the tissue explants when they are placed in serum-free culture medium. Enzyme activity, then, would not be detectable in the medium until these serum proteins are degraded or diluted out of the culture.

The availability of a specific antibody to human skin collagenase (11) offers an approach to answering these questions. We report here the use of anti-collagenase antibodies produced against a purified human skin collagenase to demonstrate the presence of collagenase in extracts of whole human skin. In addition, evidence is presented that collagenase can be obtained in its active form from these extracts and that the presence of inactive collagenase both *in vivo* and during the first day of tissue culture is related to the inhibition of this enzyme by serum alpha-globulins.

METHODS

Normal human skin was cultured in Dulbecco's Modified Eagle's Medium as described (1). Culture medium was changed daily for 10 days and media having collagenase activity were pooled, dialyzed at 4°C against several changes of distilled water, and lyophilized; the crude enzyme powder was stored at −20°C. Medium obtained during the first 24–48 hr of culture, during which no collagenase activity was detected, was handled in a manner identical to medium that contained active enzyme.

Fast-moving human skin collagenase was purified as described by Bauer *et al.* (11). The fast-moving enzyme, which appears to be a pure protein by electrophoretic criteria (11), was used as the immunogen for the experiments presented in this study. Antisera were prepared and gel diffusion performed as described (11).

Antiserum to human alpha$_1$-antitrypsin (alpha$_1$-at) was obtained from Hoechst (Beringwerke) Pharmaceutical Co.; specific antisera prepared to human alpha$_2$-macroglobulin (alpha$_2$-M globulin) and whole human serum were obtained from Hyland Laboratories.

Preparation of tissue extracts

About 8–10 g of normal human skin, obtained at surgery, was trimmed of fat, minced with scissors, and homogenized in an all-glass homogenizer in 4.0 ml of 0.05 M Tris·HCl (pH 7.5) containing 0.005 M $CaCl_2$. The homogenates were centrifuged at 10,000 × *g* for 15 min at 4°C to remove particulate material and the supernates assayed for collagenase activity and for immunoreactive collagenase.

Chromatography of skin extracts and culture medium

Gel filtration was performed at 4°C using reverse flow on a column of Sephadex G-150 equilibrated with 0.05 M Tris·

Abbreviations: Alpha$_1$-at, alpha$_1$-antitrypsin; alpha$_2$-M globulin, alpha$_2$-macroglobulin.

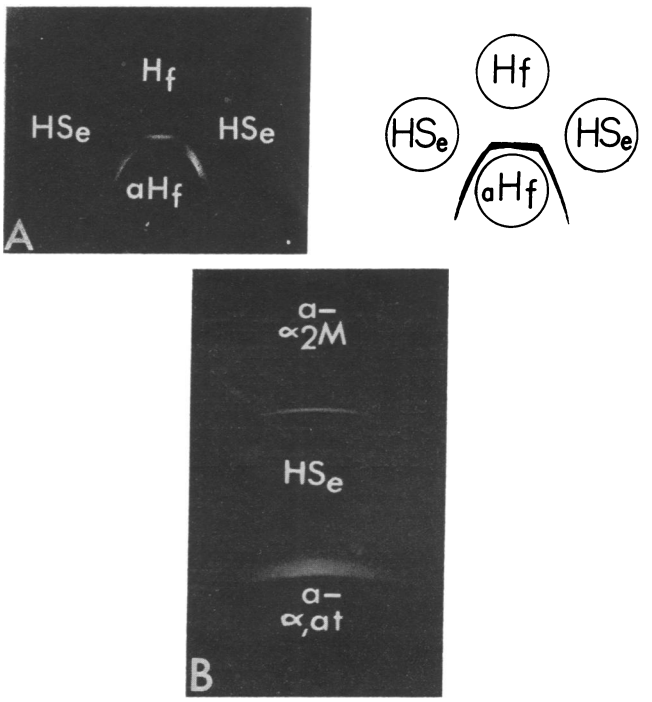

FIG. 1. (*A*) Gel diffusion analysis of crude human skin extracts and cultured human skin collagenase against anti-human skin collagenase antiserum. HS_e, human skin extract; H_f, fast-moving cultured human skin collagenase; aH_f, anti-fast-moving human skin collagenase antibody. (*B*) Gel diffusion analysis of human skin extract, HS_e, against antisera to human alpha$_1$-antitrypsin (a-α_1-at) and human alpha$_2$-macroglobulin (a-α_2-M).

HCl (pH 7.5) containing 0.005 M $CaCl_2$ and 0.2 M NaCl. Gel filtration was also performed using Sephadex G-200, equilibrated with the same buffer.

Crude powder from day one culture medium was dissolved in the original column buffer to a concentration of approximately 25 mg/ml. Chromatography was performed as described above. Individual fractions were assayed for collagenase activity and examined for immunologic evidence of collagenase, alpha$_1$-at, and alpha$_2$-M globulin.

Assays

Collagenase activity was determined by the enzymatic release of soluble, [^{14}C]glycine containing, peptides from native, reconstituted, guinea-pig skin collagen fibrils (1, 12). Protein was determined by the method of Lowry *et al.* (13).

RESULTS

In vivo studies

Crude extracts of human skin show no evidence of collagenase activity since the extract is unable to degrade native collagen fibers. On immunodiffusion, however, these skin extracts give a single precipitin band when reacted with anti-human skin collagenase antibody (Fig. 1*A*). Furthermore, the crude skin extracts and an enzymatically active human-skin collagenase, obtained from the medium of skin cultures, react in a completely identical manner with anti-human skin collagenase antibody (Fig. 1*A*). Since it has been shown that alpha-globulins are potent inhibitors of human skin collagenase (10), we examined the tissue extracts, using specific antisera, for the presence of both alpha$_1$-at and alpha$_2$-M globulin. Precipitin

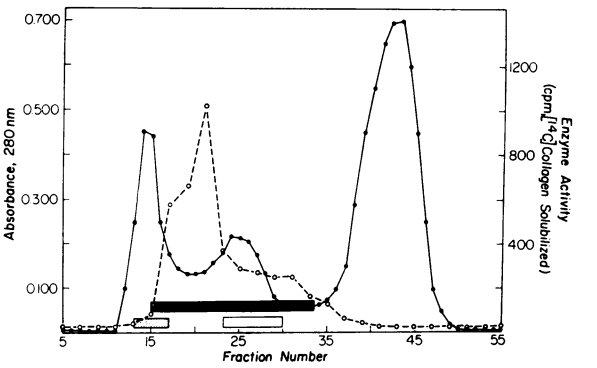

FIG. 2. Gel filtration of a fresh extract of human skin, containing 30 mg of protein, on a column (1.2 cm × 100 cm) of Sephadex G-150 with 0.05 M Tris·HCl (pH 7.5) containing 0.2 M NaCl and 0.005 M $CaCl_2$ as eluent. Effluent fractions of 3.5 ml were collected at a rate of 10.5 ml/hr. ●——●, absorbance at 280 nm, O- - -O, enzyme activity. ■, immunoreactive collagenase; □, alpha$_1$-anti-trypsin; ▨, alpha$_2$-macroglobulin.

bands indicating the presence of both alpha$_1$- at and alpha$_2$-M globulin are readily demonstrated in the crude skin extracts (Fig. 1*B*).

In an effort to separate the serum alpha-globulins from the enzyme, crude skin extracts were chromatographed on Sephadex G-150. Fig. 2 shows that enzymatically active collagenase can be separated from other proteins present in the crude mixture, and that this enzyme activity corresponds to fractions that are immunoreactive with anti-human skin collagenase antibody. In general, more enzyme activity is present after chromatography of extracts of skin obtained from young, growing individuals.

Alpha$_2$-M globulin is present principally in the excluded peak, although there is some overlap of this protein into the area of collagenase activity. Alpha$_1$-at is found primarily beyond the area of maximal enzyme specific activity, but it also overlaps with the collagenase peak (Fig. 2). It is clear that much of the collagenase activity is not associated with either alpha$_2$-M globulin or alpha$_1$-at.

The enzyme taken from, for example, fraction 20 cleaves the collagen molecule in a manner identical to that originally described for the collagenase isolated from the medium of human skin cultures (1).

Lyophilization of the total peak (fractions 15–35, Fig. 2) of immunoreactive and enzymatically active collagenase virtually abolishes all enzyme activity even though collagenase immunoreactivity persists. The concentrated material gives a precipitin reaction on immunodiffusion for both alpha$_1$-at and alpha$_2$-M globulin. Rechromatography of this material on Sephadex G-200 again separates the active collagenase from both of these alpha-globulins.

Tissue culture

Measurements of collagenase activity in the culture media from explants of human skin analyzed daily indicates a delay in the appearance of enzyme activity of about 24–48 hr (Fig. 3). Collagenase activity first appears at a time when the total protein concentration of the culture medium has decreased to about 10–15% of that found after 6 hr of culture. However, immunoreactive collagenase is present within 6 hr (the first time period examined) after the cultures are initiated (Fig. 4).

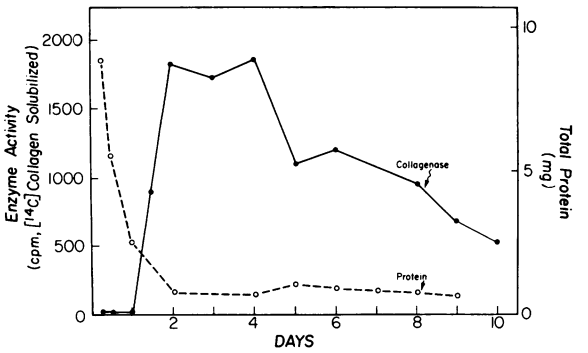

FIG. 3. The appearance of collagenase activity in the culture medium of human skin explants as a function of time. The medium from 12 flasks was harvested at 6, 12, and 24 hr, and daily thereafter. The media for each time period we e pooled and handled as described in *Methods*. Reaction mixtures consisted of 50 μl of 0.4% [^{1} C]glycine-labeled collagen as a substrate gel and 25 μ of concentrated culture medium (enzyme source) in a total volume of 200 μl. Incubation was for 2 hr at 37°C in a shaken water bath. Substrate gel contained 4208 cpm.

Both alpha$_1$-at and alpha$_2$-M globulin, as measured immunologically, are present in the culture medium only during the first day of culture. By the time collagenase activity first appears, these proteins have disappeared from the medium, even though other serum proteins can be demonstrated (immunologically) in the medium for at least 5 days. Thus, in the early stages of culture, collagenase is present in the medium together with potent inhibitors of its activity.

When day one medium from human skin cultures is chromatographed on Sephadex G-150, some of the collagenase activity is almost completely separated from alpha$_1$-at and alpha$_2$-M globulin (Fig. 5). A small amount of alpha$_1$-at overlaps the beginning of the enzymatically-active collagenase peak. The effectiveness of these alpha-globulins as collagenase inhibitors is demonstrated by the presence of immunoreactive, but enzymatically inactive, collagenase in effluent fractions (34–57, Fig. 5) containing alpha$_2$-M globulin and alpha$_1$-at. In contrast, those fractions with both enzyme activity and immunoreactivity are also virtually free of these alpha-globulins and retain activity upon concentration.

DISCUSSION

In these studies it has been possible, for the first time, to demonstrate conclusively the presence *in vivo* of collagenase in human skin. The enzyme can be detected immunologically in crude skin extracts that show no evidence of collagenase activity. After chromatography of these crude extracts, the enzyme can be obtained in active form. The collagenase present *in vivo* is identical to the enzyme obtained from the

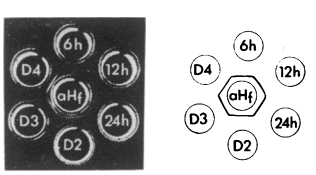

FIG. 4. Gel diffusion analysis of human-skin culture medium against antiserum to human skin collagenase. *6h, 12h, 24h* represent 6, 12, and 24 hr culture media; *D2, D3, D4:* culture media from days 2, 3, and 4; aH_f, anti-fast-moving human skin collagenase antibody.

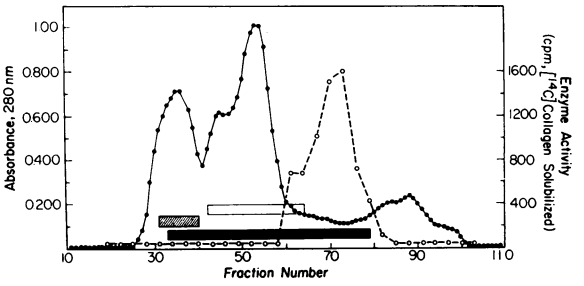

FIG. 5. Gel filtration of day one medium on Sephadex G-150 using the same buffer as in Fig. 3. A sample containing 125 mg of protein was applied to a column, 2.5 cm × 100 cm, and effluent fractions of 4.7 ml were collected at a rate of 14 ml/hr. ●——●, absorbance at 280 nm; O- - -O, enzyme activity. ■, immunoreactive collagenase; □, alpha$_1$-anti-trypsin; ▨, alpha$_2$-macroglobulin.

medium used to grow human skin cultures, both on an immunologic basis (11) and in its mechanism of attack on the collagen molecule (1).

Previous studies (10) have demonstrated that the serum alpha-globulin components, alpha$_1$-at and alpha$_2$-M globulin, are capable of effectively inhibiting human skin collagenase. The ability to obtain collagenase in its active form, from enzymatically inactive skin extracts, by chromatographically separating the enzyme from both alpha$_1$-at and alpha$_2$-M globulin suggests that collagenase activity in fresh tissue extracts is indeed masked by the inhibitory action of these alpha-globulins.

The concept that serum antiproteases may be involved in the control of collagenase activity is further supported by the *in vitro* studies. In the medium of human skin cultures immunoreactive, but enzymatically inactive, collagenase is present shortly after the cultures are initiated. During this lag period of about 24 hr before enzymatic activity is present, the culture medium contains both alpha$_1$-at and alpha$_2$-M globulin. At the time that collagenase activity first appears, these alpha-globulins can no longer be detected in the culture medium. It is likely then that the lag in the appearance of collagenase activity in skin cultures, in the presence of immunoreactive enzyme, is due to the inhibition of collagenase by these serum alpha-globulins, which are apparently bound to the tissue explants when they are initially placed in culture. In addition, collagenase activity can be demonstrated after chromatography of enzymatically inactive, day one medium. Thus, the chromatographic results from the early culture medium are similar to those obtained from human skin extracts.

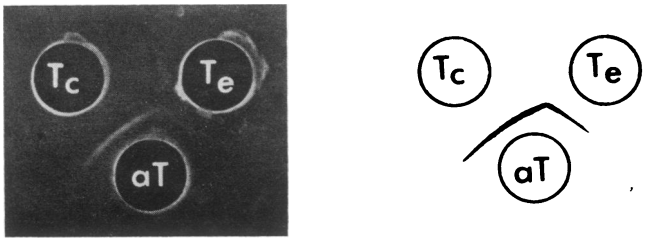

FIG. 6. Gel diffusion analysis of crude extract of tadpole tailfin, and tadpole tailfin collagenase obtained from tissue culture, against anti-tadpole collagenase antibody. T_e, tailfin extract; T_c, tailfin collagenase; aT, anti-tadpole collagenase antibody.

In the chromatographic preparations of day one medium, however, there is considerable overlap of immunoreactive material with the alpha-globulins, but no enzyme activity is present in this region (Fig. 5). This overlap area of immunologically detectable collagenase corresponds closely to the first peak, designated slow-moving human skin collagenase, of two enzymatically active peaks of collagenase separable by fractionation of medium obtained after the first 24 hr of culture (11). The area that contains both enzymatic and immunologic activity is similar to its chromatographic behavior to the second collagenase peak, referred to as fast-moving human skin collagenase (11). In contrast, in fresh tissue extracts the area containing both immunologic and enzymatic activity corresponds more closely to the slow-moving enzyme species.

We postulated (11) that the slow- and fast-moving collagenases may bear a polymer–monomer relationship to one another. If so, these studies indicate that enzyme obtained from tissue culture contains a higher proportion of low molecular weight subunits than that obtained by direct extraction of tissue. It is possible that tissue culture conditions, where there is a relatively low protein concentration in the medium after the first 24 hr (Fig. 3), favors dissociation of the higher molecular weight to the lower molecular weight species.

The demonstration that skin collagenase exists *in vivo* suggests that the enzyme is synthetized continuously, and is present in tissues at concentrations that are physiologically significant for the degradation of collagen. Although increased enzyme synthesis undoubtedly can occur following stimuli such as wound healing (14), and in certain disease processes involving the skin (15), significant amounts of collagenase appear to exist in normal skin at all times. Similarly, the *in vitro* studies of human skin show that immunoreactive collagenase is present in the medium within a very short time after the cultures are initiated.

Previous *in vitro* studies of both human skin (14) and tadpole tailfin (16) have shown that collagenase activity is inhibited by puromycin and freeze–thawing of the tissue, suggesting that the presence of collagenase in human skin extracts is the result of continuing synthesis and not the release of stored enzyme. The recovery of enzymatically active collagenase both from initially inactive human skin extracts and day one culture medium, by chromatographically separating the collagenase from serum anti-proteases, indicates that the enzyme is not stored either in an active form for later release or in an inactive form for subsequent activation.

Although the precise role of $alpha_1$-at and $alpha_2$-M globulin is unknown, these studies support the postulate (10) that they prevent the action of collagenase on substrate remote from the original site of enzyme production.

The presence of collagenase in tissue extracts does not appear to be unique to human skin. Crude extracts of tadpole tailfin, which show no collagenase activity, yield evidence of immunoreactive collagenase when reacted with an antibody prepared against highly purified amphibian collagenase obtained from the medium of cultured tadpole (*Rana catesbiana*) tailfin (Fig. 6). In addition, when crude tailfin extracts are chromatographed in a manner similar to that described for human skin, an enzymatically active collagenase can be separated from other proteins in the crude mixture (unpublished observations). Whether the inability to demonstrate collagenase activity in extracts of tadpole tailfin, in the presence of immunoreactive enzyme, is also related to the inhibition of collagenase by serum antiproteases will require further investigation with amphibian alpha-globulin components. It is clear, however, that tadpole collagenase can be inhibited by human serum alpha-globulins (10). These findings suggest that it may also be possible to demonstrate the *in vivo* presence of other animal and human collagenases which, to date, have been obtained only by tissue culture techniques (9).

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