Human Skin Fibroblast Collagenase Inhibitor

COMPARATIVE STUDIES IN HUMAN CONNECTIVE TISSUES, SERUM, AND AMNIOTIC FLUID*

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In order to gain insight into the biological significance of a collagenase inhibitor secreted by human skin fibroblasts, we examined various human connective tissues and body fluids for such a protein. The inhibitors found in these tissues were compared immunologically to skin fibroblast inhibitor by Ouchterlony analysis and by the development of a highly specific enzyme-linked immunosorbent assay (ELISA). Using this ELISA, cell cultures of human skin fibroblasts, corneal fibroblasts, gingival fibroblasts, and adult and fetal lung fibroblasts secreted similar amounts of immunoreactive inhibitor protein. Each culture medium displayed a reaction of immunologic identity with skin fibroblast inhibitor when examined in Ouchterlony gel diffusion. In testing for functional inhibitory activity, the same 1:1 stoichiometry of collagenase inhibition was observed in each culture medium that characterizes the human skin inhibitor. Other mesodermally derived human cell types, including human fetal osteoblasts, uterine smooth muscle cells, fibrosarcoma cells, and explants of tendon and articular cartilage behaved in the same manner as the fibroblast cultures.

Because collagenase inhibitors with biochemical similarities to skin fibroblast inhibitor have been described in serum and in amniotic fluid, we also examined these sources of inhibitory proteins. The data indicate that both serum and amniotic fluid contain collagenase inhibitors which are immunologically and functionally identical with the skin fibroblast inhibitor. The concentration of inhibitor in serum, as measured by ELISA assay, is 1.03 ± 0.27 μg/ml.

The results suggest that collagenase inhibitors which are functionally equivalent and immunologically identical with human skin fibroblast collagenase inhibitor are synthesized by many, if not all, fetal and adult mesodermal tissues in the human organism. The inhibitor apparently gains access to certain body fluids such as serum and amniotic fluid. This inhibitor protein may, therefore, function in the regulation of collagen degradation in most human connective tissues.

Since the vertebrate collagenases initiate the specific degradation of native collagen (1, 2), the activity of these enzymes is critical to the maintenance and turnover of connective tissues. In normal tissue remodeling, collagen degradation requires precise regulation while, in certain pathologic states,

elevated levels of collagenase have been associated with excessive tissue destruction (3–5).

Modulation of collagenolytic activity may also occur through the intervention of naturally occurring collagenase inhibitors which have been identified in both serum and connective tissues. Human serum is a potent inhibitor of collagenase (6); α_2-macroglobulin, a nonspecific antiprotease, accounts for more than 95% of this inhibitory activity (7). α_2-Macroglobulin inhibits all vertebrate collagenases investigated to date, but its large size (780,000 D), lack of specificity, and irreversible mechanism of inhibition (8) probably confine its role to the inactivation of protease that has gained access into the bloodstream, rather than to the regulation of collagenolysis within the interstices of an organized connective tissue. Tissue-derived inhibitors of collagenase, on the other hand, are more likely to be of biologic significance in the control of collagen degradation in the extracellular milieu. Using either cell or explant culture techniques, such inhibitors have been identified in several tissues including skin (9, 10), tendon (11), bone (12, 13), uterus (13), and aorta (14, 15) from various animal species. These inhibitors may constitute a distinct biologic class of compounds since they appear to share certain common features: all are glycoproteins of $M_r \sim$ 25,000–30,000, they are very stable to changes in temperature and pH, and they specifically inhibit connective tissue metalloproteases. Interestingly, a β_1-serum protein (7, 16) and a glycoprotein in amniotic fluid (17, 18) have also been identified which display the same properties as the tissue inhibitors. Another class of biologically active collagenase inhibitors appears to be cationic proteins of $M_r \sim$ 10,000 which have been reported from cartilage (19, 20), aorta (19), teeth (21), and would also include platelet factor 4 (22). The cartilage-derived collagenase inhibitor may be related to substances extracted from this tissue which inhibit tumor-induced vascular proliferation (23) and bone resorption in vitro (24).

Human skin fibroblasts in monolayer culture synthesize both procollagenase (9, 25, 26) and a specific inhibitor of the active form of this enzyme (9, 10). The inhibitor is a glycoprotein of $M_r = 28,500$ that inhibits collagenase on a 1:1 stoichiometric basis (10, 27). The preceding paper describes the purification to homogeneity of human skin fibroblast collagenase inhibitor as well as its biochemical characterization and the development of a monospecific antibody (27). In this report, we demonstrate the immunologic and functional identity of the skin fibroblast inhibitor with collagenase inhibitors elaborated by human cell cultures of lung fibroblasts, corneal fibroblasts, gingival fibroblasts, fibrosarcoma cells, uterine smooth muscle cells, osteoblasts, tendon, and cartilage explants, and suggest that fibroblast collagenase inhibitor is important in the regulation of collagenolysis in most human tissues. Furthermore, the presence of this inhibitor in certain human body fluids, such as serum and amniotic fluid, is also documented.

EXPERIMENTAL PROCEDURES

Materials—Polystyrene microtitration plates ("U" bottom) were purchased from Dynatech. Goat anti-rabbit IgG conjugated to alkaline phosphatase and *p*-nitrophenyl phosphate (disodium) were obtained from Sigma. Absorbance readings of the solutions in the microtitration plates were measured at 405 nm with an MR 580 Dynatech spectrophotometer.

PBS[1]-Tween-BSA, phosphate-buffered saline-Tween, and 10% diethanolamine buffer were prepared according to Voller *et al.* (28). Borate buffer was prepared as described by Kearney *et al.* (29).

Human Sera—10-ml venipuncture samples were obtained from seven healthy volunteers, ages 27–32. The specimens were allowed to clot overnight; the serum was removed and then centrifuged at 2000 × *g* for 20 min, followed by storage at −20 °C.

Human Amniotic Fluid—Samples of human amniotic fluid were obtained from the obstetrics ultrasound laboratory of the Washington University School of Medicine. The specimens represented excess fluid collected during amniocentesis to assess fetal lung maturity in women undergoing repeat cesarean section who were at least 36 weeks of gestation. All samples were free of blood and were centrifuged at 10,000 × *g* for 10 min prior to storage at −20 °C.

Urine Specimens—Urine was obtained from three healthy volunteers, ages 25–40. The samples were dialyzed overnight against 2.5 mM Tris, pH 7.5, then lyophilized and resuspended in water in order to achieve a 30-fold concentration prior to ELISA assay.

Cell Cultures—All cells were grown at 37 °C in 75-cm^2 flasks containing 10 ml of Dulbecco's modified Eagle's medium-high glucose + glutamine with 0.03 M Hepes buffer (pH 7.6), 20% fetal calf serum, and 200 units of penicillin and 200 μg of streptomycin/ml. At visual confluence, the cells were placed in serum-free medium for 24 h. The medium was harvested and stored at −20 °C.

Normal human skin fibroblasts were purchased from the American Type Culture Collection. Human corneal fibroblasts were obtained from J. Gordon, Washington University School of Medicine, and adult human lung fibroblasts were kindly provided by J. Clark, Washington University School of Medicine. Human uterine smooth muscle cells were obtained from J. Jeffrey, Washington University School of Medicine. Fibroblasts from a human fibrosarcoma were cultured following surgical excision of the tumor. Serum-free medium from confluent cell cultures was also kindly supplied by S. Wilhelm, University of South Carolina (human gingival fibroblasts), J. Clark, Washington University School of Medicine (human fetal lung fibroblasts, IMR-90), J. Wergedal, Loma Linda Veterans Administration Hospital (human fetal osteoblasts), and H. P. Baden, Harvard Medical School (human epidermal keratinocytes). Human skin explants were cultured as described previously (2). Serum-free medium from human tendon and human articular cartilage explants was supplied by C. A. Vater, Dartmouth-Hitchcock Medical Center.

Preparation of Collagenase Inhibitors—Human skin fibroblast collagenase inhibitor was purified to homogenity from monolayer cultures of normal human skin fibroblasts (27). Serum-free medium from adult and fetal lung fibroblasts, gingival fibroblasts, corneal fibroblasts, fetal osteoblasts, and fibrosarcoma cells was dialyzed overnight against 0.1 M acetic acid, lyophilized, resuspended in 0.05 M Tris, pH 7.5, to achieve a 10-fold concentration, and then centrifuged at 10,000 × *g* for 10 min. This concentrated medium, now free of any intrinsic collagenase activity, was used to test for functional inhibitory capacity and in Ouchterlony gel diffusion; unconcentrated medium was utilized to measure immunoreactive protein by the ELISA method. In the case of serum-free medium from tendon and cartilage explants, and amniotic fluid, a different method for concentration was employed because dialysis against acetic acid resulted in significant precipitation. These samples were instead dialyzed against 5 mM Tris, 10 mM EDTA, pH 7.5, then dialyzed three times against 5 mM Tris, pH 7.5, lyophilized to attain 10-fold concentration, and resuspended in water.

Collagenase Inhibitor Assay—Human skin fibroblast collagenase was purified to homogeneity as described previously (25, 26). The enzyme was activated proteolytically with trypsin, diluted to a concentration of 10 μg/ml, and stored at −20 °C. An inhibitor titration was then performed to obtain a standard curve using known concentrations of pure inhibitor ranging from 1 to 5 μg/ml. Inhibition of collagenolytic activity was measured at 37 °C using native reconsti-

tuted [^{14}C]glycine-labeled guinea pig skin collagen as substrate (30). 50 μl of a 0.4% collagen solution of specific activity 25,000 cpm/mg was allowed to form an insoluble substrate gel at 37 °C overnight. Following incubation at 37 °C with enzyme, inhibitor, or both, the reaction mixtures were centrifuged at 10,000 × *g* and the supernatant fraction was counted in a liquid scintillation spectrometer. Functional inhibitory activity of the concentrated test samples was measured by incubation with active collagenase (10 μg/ml), quantitation of the inhibition produced, and comparison to the standard inhibition titration curve that was developed using electrophoretically pure inhibitor. For each unknown, at least two inhibitor concentrations were employed which produced between 25 and 75% inhibition of collagenase activity. The buffer used for all collagenase assays was 0.05 M Tris, pH 7.5, containing 0.01 M CaCl$_2$.

Preparation of Antiserum to Human Fibroblast Collagenase Inhibitor—Human skin fibroblast collagenase inhibitor purified to homogeneity was used as immunogen in young male white rabbits to obtain a functionally specific antiserum. The antiserum gave a single immunoprecipitin band when reacted in Ouchterlony analysis with either crude skin fibroblast culture medium or pure inhibitor. In addition, a γ-globulin fraction of this antiserum eliminated >90% of the functional inhibitory capacity of fibroblast collagenase inhibitor as compared to <5% reduction in activity produced by a nonimmune γ-globulin preparation (27).

Immunodiffusion—Gel diffusion was performed in 1.0% Ionagar as described by Ouchterlony (31). Staining of the precipitin bands with 0.005% Coomassie blue in 10% methanol, 2.5% acetic acid was performed after extensive washing of the agar gels with cold 0.05 M Tris-HCl, pH 7.5, containing 0.15 M NaCl followed by a short (4-h) wash with cold distilled water.

ELISA Assay for Human Skin Fibroblast Collagenase Inhibitor—200 μl of a 0.1 μg/ml solution of human fibroblast collagenase inhibitor in borate buffer (pH 8.3) was added to each well of a microtitration plate, incubated overnight at 4 °C, and then washed three times in PBS-Tween. The known test solution, which was used for obtaining a standard curve, and the unknown samples, were serially diluted in PBS-Tween-BSA. 200 μl of each dilution was then mixed with 200 μl of rabbit anti-inhibitor serum diluted 1:750 in PBS-Tween-BSA. This yielded 400 μl of a final dilution of 1:1500 antibody and the serially diluted human collagenase inhibitor. The antibody-inhibitor mixtures were incubated at 37 °C for 1 h, then at 4 °C overnight to achieve maximal binding. Following incubation, they were centrifuged at 10,000 × *g* for 5 min. 200 μl of each mixture was then added to the pretreated wells and allowed to incubate at 37 °C for 45 min. The plate was washed three times with PBS-Tween after which 200 μl of goat anti-rabbit IgG-alkaline phosphatase conjugate (diluted 1:1000) in PBS-Tween was added to each well and incubated at 37 °C for 1 h. The plates were then washed three times with PBS-Tween, 200 μl of *p*-nitrophenyl phosphate solution (1 mg/ml) in 10% diethanolamine buffer was added to each well to initiate the colorimetric reaction, which was measured after 20–30 min at room temperature using the Dynatech spectrophotometer.

For ELISA assays performed on serum-containing medium (10% fetal calf serum), a blank of 0.01 μg/ml of cross-reactive material was produced. This value was therefore subtracted from the observed ELISA determinations on such medium.

RESULTS

An enzyme-linked immunoadsorbent assay for human skin fibroblast collagenase inhibitor was developed using antigen purified to homogeneity and a functionally specific rabbit anti-inhibitor antibody. In order to test inhibitor binding to the plates and antigen-antibody interaction, polystyrene microtitration plates were coated with serial dilutions of inhibitor antigen and then incubated with serial dilutions of inhibitor-specific antibody. Goat anti-rabbit IgG conjugated to alkaline phosphatase was added, followed by incubation with substrate. A family of curves was thereby established, as shown in Fig. 1. In order to utilize the assay for measuring antigen, indirect inhibition ELISA methodology (32) was employed. Test solutions of either known amounts of pure inhibitor or various unknowns were pre-incubated with the specific antiserum prior to its addition to the wells which contained antigen coated to their surface. Using this tech-

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[1] The abbreviations used are: PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
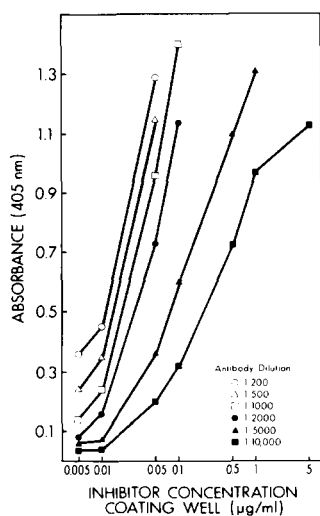
FIG. 1. **Effect of solid phase antigen concentration on the ELISA.** Increasing concentrations of human skin fibroblast inhibitor in borate buffer were bound to a microtitration plate overnight at 4 °C. The inhibitor-coated wells were then incubated with serial dilutions of rabbit anti-inhibitor antibody in PBS-Tween-BSA for 1 h at 37 °C. Goat anti-rabbit IgG conjugated to alkaline phosphatase was then added, followed by substrate. Absorbance readings were measured 25 min after the addition of substrate.

nique, the combination of coating antigen and rabbit inhibitor-specific antibody which resulted in the highest degree of accuracy with sufficient sensitivity for most purposes was 0.1 μg/ml of inhibitor and a 1:1500 dilution of antibody. Under these conditions, a typical standard curve for the measurement of human skin fibroblast collagenase inhibitor was produced as shown in Fig. 2. The minimum sensitivity for the ELISA was 10 ng/ml, or 2 ng of inhibitor protein.

Several experiments were performed to assess the correlation of immunoreactive material as measured by ELISA with functional inhibitor activity and also to evaluate the accuracy and precision of the assay. Serum-free medium from four replicate culture flasks of a single human skin fibroblast line was examined for both immunoreactive inhibitor protein and functional collagenase inhibitory capacity. Table IA shows the correlation between the two, as measured by inhibitor activity per immunoreactive protein. The variation around the mean of approximately 20% was expected because of the inherent errors of the ELISA and functional assays combined, and the error involved in concentrating the medium protein for the latter assay. To test the accuracy of the ELISA, known chemical quantities of human skin fibroblast collagenase inhibitor were measured by this method. Despite widely varying inhibitor concentrations, the ELISA determinations were accurate to within 10% (Table IB). Precision or reproducibility was examined by using the same aliquoted sample of inhibitor in three ELISA assays performed on three different days. All measurements were within 10% of the mean (data not shown).

In order to perform comparative studies, immunoreactive material was measured in cell and explant culture media from a variety of human connective tissues. Serum-free media from confluent monolayer cultures of corneal, adult and fetal lung, and gingival fibroblasts all contained immunoreactive inhibitor protein in concentrations similar to the levels found in human skin fibroblast cultures (Table II). When tested for functional inhibitory activity, an approximate 1:1 stoichiometry of collagenase inhibition per immunoreactive protein was observed, just as for the skin fibroblasts (Tables IB and II). Furthermore, all samples displayed an immunologic reaction of identity against pure inhibitor protein in Ouchterlony gel
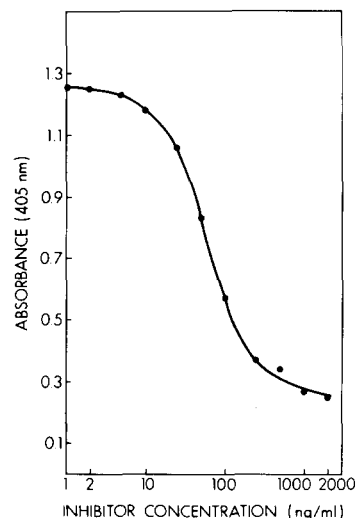

FIG. 2. **Indirect inhibition ELISA for human skin fibroblast collagenase inhibitor.** A typical standard cycle is shown using a coating inhibitor concentration of 0.1 μg/ml and an antibody dilution of 1:1500. Note that the sensitivity of the assay is approximately 10 ng/ml, or 2 ng of inhibitor protein. Details of the ELISA methodology are given under "Experimental Procedures."

TABLE IA

Correlation of inhibitor activity and immunoreactive protein as measured in the ELISA

Four replicate cultures of a single human skin fibroblast cell line were placed in serum-free medium for 24 h. Immunoreactive protein was determined by ELISA. Functional inhibitory activity was measured as detailed under "Experimental Procedures."

Culture	Immunoreactive protein	Inhibitory activity/immunoreactive protein[a]
	μg/ml	
1	1.15	0.85
2	1.07	1.18
3	0.89	1.07
4	0.97	0.74
Mean ± S.E.	1.02 ± 0.11	0.96 ± 0.20

[a] Molecules of collagenase inhibited/molecule of immunoreactive inhibitor protein.

TABLE IB

Recovery of known quantities of inhibitor in the ELISA

The concentration of immunoreactive inhibitor protein was measured by ELISA in three different samples containing known amounts of human fibroblast inhibitor.

Experiment	Inhibitor added	Inhibitor measured	Difference
	μg/ml	*μg/ml*	*%*
1	10.00	10.51	+5
2	1.00	1.07	+7
3	0.10	0.092	−8
			Mean = 7

diffusion (Fig. 3). Similar results were obtained for tumor cells grown in monolayer culture from a human fibrosarcoma (Table II and Fig. 3). Serum-free medium from confluent monolayer cultures of human fetal osteoblasts likewise contained easily measurable quantities of immunoreactive inhibitor protein by ELISA. Functional collagenase inhibitory activity per immunoreactive material was the same as for the skin fibroblast inhibitor, and a line of immunologic identity was also observed in Ouchterlony analysis (Table II and Fig. 3). Confluent monolayer cultures of human uterus smooth muscle cells produced significant amounts of immunoreactive material only in the presence of serum-containing medium

TABLE II

Immunoreactive inhibitor protein in cell and explant cultures of human tissues

Immunoreactive protein in confluent cell culture or explant medium was measured by ELISA assay following incubation for 24–48 h under serum-free conditions.

Inhibitor source	Immunoreactive protein in culture medium	Inhibitory activity/immunoreactive protein[a]
	$\mu g/ml$	
Cells		
Skin fibroblasts	1.3	1.0
Corneal fibroblasts	1.2	1.1
Lung fibroblasts		
Adult	3.1	1.2
Fetal	3.0	0.9
Gingival fibroblasts	0.8	0.6
Fibrosarcoma	1.0	0.9
Uterus smooth muscle	0.6[b], 0.05[c]	—[d]
Fetal osteoblasts	0.25	0.8
Keratinocytes	0.03[b], 0.03[c]	—
Explants		
Tendon	1.5	0.7
Articular cartilage	1.1	1.0
Skin	0.11	—

[a] Molecules of collagenase inhibited/molecule of immunoreactive inhibitor protein.

[b] Immunoreactive protein in confluent cell cultures containing 10% fetal calf serum.

[c] Immunoreactive protein in confluent cell cultures cycled for 24–48 h in serum-free medium.

[d] —, not performed due to insufficient quantities of inhibitor present in serum-free medium.
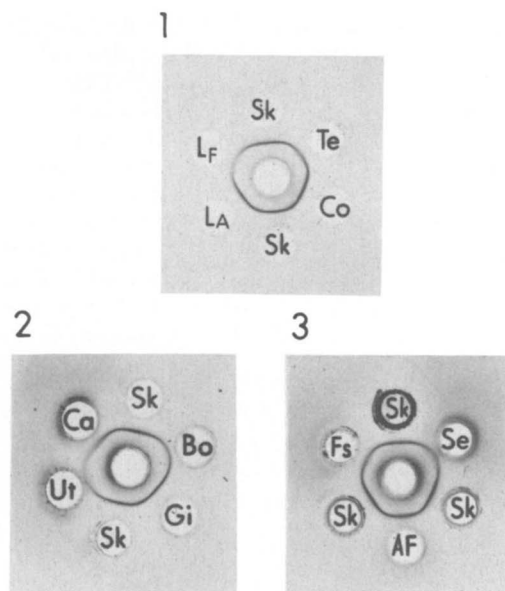

FIG. 3. **Immunologic comparison of inhibitors from human connective tissues and body fluids by Ouchterlony gel diffusion.** Cell culture medium from human connective tissues, serum, and amniotic fluid was prepared as described under "Experimental Procedures." *1: Sk*, skin fibroblast inhibitor (pure); *Te*, tendon explants; *Co*, corneal fibroblasts; *L_A*, adult lung fibroblasts; *L_F*, fetal lung fibroblasts; *2: Sk*, skin fibroblast inhibitor (pure); *Bo*, fetal bone osteoblasts; *Gi*, gingival fibroblasts; *Ut*, uterine smooth muscle; *Ca*, articular cartilage explants; *3: Sk*, skin fibroblast inhibitor (pure); *Se*, serum; *AF*, amniotic fluid; *Fs*, fibrosarcoma.

(Table II). Nevertheless, when such medium was concentrated, an immunologic band of identity was seen against fibroblast collagenase inhibitor in Ouchterlony gel diffusion (Fig. 3). On the other hand, monolayer cultures of an epithelial

cell strain, human epidermal keratinocytes, produced <5% of the ELISA-reactive material generally observed in the mesodermally derived cell lines (Table II). Culture of these cells in the presence of 10% fetal calf serum did not alter the amount of immunoreactive protein detected by ELISA assay.

Explant cultures of human skin, tendon, and articular cartilage were also examined for the production of immunoreactive inhibitor protein by ELISA. The tendon and cartilage explants released significant quantities of immunoreactive protein into the culture medium that was functionally equivalent to skin fibroblast inhibitor (Table II) and that showed an immunologic reaction of identity with the latter in Ouchterlony gel diffusion (Fig. 3). The skin explants, on the other hand, produced only about 10% of the immunoreactive protein found in monolayer skin fibroblast cultures (Table II). This observation is consistent with our previous difficulty in detecting functionally active inhibitor in such explant cultures (10).

The presence of immunoreactive inhibitor protein was also examined in several human body fluids (Table III). As shown in the preceding paper (27), human serum contains a β-globulin collagenase inhibitor that behaves identically to skin fibroblast inhibitor when subjected to several of the chromatographic procedures used to purify the latter (27). When a sample of serum partially purified in this manner was studied in Ouchterlony gel diffusion, an immunologic line of identity with human skin fibroblast collagenase inhibitor could readily be discerned (Fig. 3). Additionally, when functional collagenase inhibitory activity was examined in this sample, which was free of any α_2-macroglobulin, the stoichiometry of collagenase inhibition per immunoreactive material was 1:1 (Table III). The amount of immunoreactive inhibitor protein present in the sera of seven healthy individuals was measured by ELISA and is shown in Table III. Normal human serum contains approximately 1 $\mu g/ml$ of this material with a standard deviation of 25% around the mean. Apparently, very little of this protein is excreted by the kidney, since its concentration in urine was <0.02 $\mu g/ml$ in all cases.

TABLE III

Immunoreactive inhibitor protein in human body fluids

The concentration of immunoreactive inhibitor protein was measured in serum, amniotic fluid, and urine, as detailed under "Experimental Procedures." Pooled samples of serum or amniotic fluid were used to test for functional collagenase inhibitory capacity. The serum sample was partially purified to remove any α_2-macroglobulin.

Body fluid and sample number	Immunoreactive protein	Inhibitory activity/immunoreactive protein[a]
	$\mu g/ml$	
Serum		
1	0.86	
2	1.08	
3	1.05	
4	1.57	
5	1.10	
6	0.81	
7	0.74	
Mean ± S.D.	1.03 ± 0.27	0.9
Amniotic fluid		
1	4.91	
2	2.29	
3	5.10	
4	3.21	
Mean ± S.D.	3.88 ± 1.36	1.1
Urine		
1–3	<0.02	

[a] Molecules of collagenase inhibited/molecule of immunoreactive inhibitor protein.

Amniotic fluid was obtained from four patients undergoing amniocentesis at ≥36 weeks of gestation to assess fetal lung maturity prior to cesarean section. Considerably higher levels of immunoreactive inhibitor protein as compared to serum values were measured by ELISA (3.88 ± 1.36 *versus* 1.03 ± 0.27 $\mu g/ml$). When amniotic fluid was concentrated and studied by Ouchterlony gel diffusion, an immunologic reaction of identity could be observed against the pure fibroblast inhibitor (Fig. 3). Since α_2-macroglobulin, the major collagenase inhibitor in serum, is too large to gain access into the amniotic fluid in signficant amounts (33, 34), the latter was examined for functional collagenase inhibitory activity without prior chromatographic separation. The stoichiometry of collagenase inhibition produced per immunoreactive inhibitor protein was 1.1 to 1.0.

DISCUSSION

Human skin fibroblasts in monolayer culture secrete a collagenase inhibitor of $M_r = 28,500$ that stoichiometrically inhibits collagenase activity on a 1:1 molar basis (10, 27). The production of this or other immunologically similar inhibitor proteins in human connective tissues and their presence in human body fluids were studied using pure antigen, a functionally specific antibody, and an enzyme-linked immunoadsorbent assay. The ELISA can measure human skin fibroblast collagenase inhibitor in an accurate and precise manner, and can detect as little as 2 ng of inhibitor protein (Fig. 2).

Monolayer fibroblast cultures from human cornea, lung, and gingiva all secreted immunoreactive inhibitor protein into serum-free medium at levels between 0.8 and 3.1 $\mu g/ml$. Following dialysis in acetic acid or EDTA to destroy any intrinsic collagenase, each fibroblast-derived medium inhibited fibrillar collagenolysis in a stoichiometric manner with respect to subsequently added enzyme, based upon the amount of immunoreactive material measured by ELISA. Thus, functional activity and immunoreactive inhibitor protein showed the same correlation in these fibroblast cultures that was observed for the skin fibroblasts. Furthermore, concentrated medium from all fibroblast lines reacted with immunologic identity against pure human skin fibroblast collagenase inhibitor in Ouchterlony gel diffusion. It would therefore seem apparent that fibroblasts from many human connective tissues produce a collagenase inhibitor that is functionally equivalent to and immunologically identical, and most probably structurally identical, with the inhibitor produced by human skin fibroblasts. Cells from a malignant tumor of connective tissue, a fibrosarcoma, likewise secreted such a collagenase inhibitor. Production of the inhibitor was also evidenced in fetal tissues, as shown by its presence in the medium of fetal lung fibroblasts which was indistinguishable from that found in cultures of adult lung fibroblasts.

Mesodermally derived cell types other than fibroblasts also secrete such a collagenase inhibitory protein. Human smooth muscle cells and human fetal osteoblasts in monolayer culture produced slightly less immunoreactive inhibitor protein than fibroblasts as measured by ELISA; both displayed an immunologic line of identity with human skin fibroblast collagenase inhibitor when tested in Ouchterlony gel diffusion. While explant cultures of human articular cartilage probably contain a heterogeneous population of cells, the level of immunoreactive protein elaborated at least suggests that the inhibitor is produced by the predominant cell type present, the chondrocyte. The inhibitor protein secreted by cartilage explants is also identical with human skin fibroblast collagenase inhibitor with respect to functional activity and immunologic reactivity as assessed by Ouchterlony analysis. On the other hand,

monolayer cultures of an epithelium-derived cell type, the human keratinocyte, produced <5% of the immunoreactive protein synthesized by the fibroblast cultures. It cannot be determined at the present time whether this amount of immunoreactive material represents an extremely low level of inhibitor production by these epithelial cells or trace contamination of low passage cultures with dermal fibroblasts.

All human skin fibroblast lines examined to date secrete detectable amounts of inhibitor when separated from the simultaneously produced procollagenase by chromatographic techniques, and then assayed for functional inhibitory activity. In contrast, explants of human skin in culture have usually failed to yield significant levels of such an inhibitor (10). When human skin explant culture medium was subjected to ELISA assay, the concentration of inhibitor was found to be only 10% of that generally present in the medium of monolayer fibroblast cell cultures. Such quantities would not be readily detectable by functional assay and probably explain the difficulty in previously identifying this protein in skin explant culture medium. The reason for these low levels of inhibitor may be related to the observation that such explants undergo massive collagen resorption in culture (35). It is possible that, under these conditions, regulatory mechanisms would reduce inhibitor production. Since anti-inhibitor antibody reacts equally well with free inhibitor or inhibitor bound to enzyme (data not shown), the presence of active collagenase in such explants would not help to explain the diminished immunoreactive inhibitor levels.

Human serum is a potent inhibitor of mammalian collagenase action (6). Greater than 95% of the collagenase inhibitory capacity of serum has been shown to reside in the general antiprotease, α_2-macroglobulin (7). The remaining few per cent has been attributed to a 40,000-Da β_1-globulin which acts as a specific inhibitor of mammalian collagenase, and has been designated as β_1-anticollagenase (7, 16). As shown in the preceding paper (27), pure human skin fibroblast collagenase inhibitor also migrates in the β-position on immunoelectrophoresis. Furthermore, when human serum was subjected to several of the chromatographic procedures employed for the purification of the fibroblast inhibitor, a serum anticollagenase was identified which behaved in a manner identical with the fibroblast-derived inhibitor (27). Ouchterlony gel diffusion of human serum partially purified by such techniques revealed a line of immunologic identity with the skin fibroblast collagenase inhibitor (Fig. 3); stoichiometry of collagenase inhibition was also identical with the fibroblast protein (Table III). It is therefore possible that human fibroblast inhibitor and β_1-anticollagenase represent the same protein. The concentration of this inhibitor, as measured in the serum of seven healthy volunteers by ELISA, is 1.03 ± 0.27 $\mu g/ml$. Assuming an approximate concentration for α_2-macroglobulin in serum of 2 mg/ml (36), and accounting for differences in molecular weight, human fibroblast inhibitor would be expected to contribute 1–2% of the collagenase inhibitory activity found in serum. It would seem likely that, due to its M_r of ~30,000 and its presence in connective tissues throughout the body, the inhibitor may gain access into the bloodstream from capillaries which bathe the extracellular space of such tissues. However, little of the serum inhibitor is filtered through the renal glomeruli and excreted in the urine (Table III).

A collagenase inhibitor has been reported in human amniotic fluid which has properties similar to human skin fibroblast collagenase inhibitor with respect to molecular weight, carbohydrate content, and thermal stability (17, 18). When four amniotic fluid specimens were tested by ELISA assay, they were found to contain an average of 3–4 $\mu g/ml$ of im-

munoreactive protein. Concentrated amniotic fluid displayed stoichiometric inhibition of collagenase activity, in addition to an immunologic reaction of identity with pure skin fibroblast inhibitor in Ouchterlony gel diffusion. These data, thus, also suggest that the collagenase inhibitor in human amniotic fluid is the same as or a closely related protein to human fibroblast collagenase inhibitor. Since the large majority of proteins found in amniotic fluid originate from maternal serum (33), the latter may be the source of the inhibitor which is contained in such fluid, although a fetal source cannot be excluded at this time.

The results of this study suggest that a similar, if not identical, collagenase inhibitor is produced by human skin fibroblasts, corneal fibroblasts, gingival fibroblasts, adult and fetal lung fibroblasts, uterine smooth muscle cells, fetal osteoblasts, tendon, and cartilage explants. Furthermore, this protein is found in certain body fluids, such as serum and amniotic fluid. Although the inhibitor proteins from all these sources share the same functional activity and an identical immunologic behavior, further studies will be required to assess the exact extent of structural similarity in each case. Nevertheless, the data do suggest that such a collagenase inhibitor is produced by most, if not all, fetal and adult mesodermal tissues in the human organism, and by at least one mesodermally derived malignant tumor. This inhibitor protein may therefore serve to regulate collagenase activity during normal and abnormal collagen turnover in many connective tissues throughout the body.

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