Proteases induce secretion of collagenase and plasminogen activator by fibroblasts

(plasmin/morphology/cell surface glycoprotein/adhesion/serum inhibition)

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ABSTRACT   We have observed that treatment of rabbit synovial fibroblasts with proteolytic enzymes can induce secretion of collagenase (EC 3.4.24.7) and plasminogen activator (EC 3.4.21.-). Cells treated for 2–24 hr with plasmin, trypsin, chymotrypsin, pancreatic elastase, papain, bromelain, thermolysin, or α-protease but not with thrombin or neuraminidase secreted detectable amounts of collagenase within 16–48 hr. Treatment of fibroblasts with trypsin also induced secretion of plasminogen activator. Proteases initiated secretion of collagenase (up to 20 units per 10^6 cells per 24 hr) only when treatment produced decreased cell adhesion. Collagenase production did not depend on continued presence of proteolytic activity or on subsequent cell adhesion, spreading, or proliferation. Routine subculturing with crude trypsin also induced collagenase secretion by cells. Secretion of collagenase was prevented and normal spreading was obtained if the trypsinized cells were placed into medium containing fetal calf serum. Soybean trypsin inhibitor, $α_1$-antitrypsin, bovine serum albumin, collagen, and fibronectin did not inhibit collagenase production. Although proteases that induced collagenase secretion also removed surface glycoprotein, the kinetics of induction of cell protease secretion were different from those for removal of fibronectin. Physiological inducers of secretion of collagenase and plasminogen activator by cells have not been identified. These results suggest that extracellular proteases in conjunction with plasma proteins may govern protease secretion by cells.

Extracellular proteases are believed to be involved in growth, remodeling processes, and cell migration. Cells can be released from density-dependent growth inhibition by treatment with certain proteolytic enzymes (1–6). Protease treatment induces DNA synthesis and cell proliferation, changes cellular morphology, and removes the cell surface glycoprotein fibronectin (LETS protein) (3, 7). Not every protease is capable of eliciting the same array of cellular responses (2, 3), and it is now clear that mitogenesis and morphological changes can be separated (3, 8). Of the cell surface proteins sensitive to proteolysis none has yet been proved to cause a specific cellular response.

Mammalian collagenases catalyze the first major step of collagen breakdown that occurs during remodeling and growth. The activities of extracellular proteases such as collagenase are the net result of secretion from cells, activation of latent forms of the enzymes, and interaction with protease inhibitors. Many examples of protease secretion by transformed and normal fibroblasts have been reported (1, 9–13), but the mechanism(s) that regulates such secretion is poorly understood. Lymphokines (14, 15), colchicine (16), and cytochalasin B (16, 17) may induce protease secretion in cells. Spontaneous secretion of collagenase and plasminogen activator by normal cells has also been reported (9, 13, 18), but physiological factors that regulate collagenase secretion by cells have not been identified.

We reported that plasmin is an activator of the latent form of collagenase (12). In the present study we observed that nonsecreting rabbit fibroblasts were induced by protease treatment to secrete both collagenase (EC 3.4.24.7) and plasminogen activator (EC 3.4.21.-), and that serum prevented induction of the cellular enzymes. These data suggest that extracellular proteases, in conjunction with plasma proteins, may modulate protease secretion by cells under physiological conditions.

MATERIALS AND METHODS

Cell Culture. Rabbit synovial fibroblasts were cultured in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS) (Gibco) (9), subcultured (1:5) weekly with 0.25% trypsin (Gibco), and used between passages 2 and 7. Cells were plated in DME + FCS in 16-mm culture dishes (Costar, 24-well), grown to confluence (approximately $0.5–1 \times 10^5$ cells per well), and washed five times with Hanks' balanced salt solution (Gibco). Then 1 ml of DME containing 0.2% lactalbumin hydrolysate (DME-LH) (Gibco) and the protease of interest was added to each well. After protease treatment, the cells were washed twice and incubated with DME-LH to collect enzyme. A second type of experiment was identical to the first except that cells were plated on ^{3}H-labeled collagen gels (12). In the third protocol, cells in 100-mm dishes were detached with 0.25% crude trypsin, washed with 10 volumes of DME-LH, plated into 16-mm wells with various proteins, and incubated to collect enzyme.

Collagen Fibril Assay. Before assay, samples of medium were activated with 10 μg of trypsin per ml for 30 min at 25°; the reaction was stopped with 50 μg of soybean trypsin inhibitor (STI) per ml (12). Reconstituted fibrils from [^{14}C]collagen (200 μg) were incubated at 37° with 100 mM Tris·HCl buffer (pH 7.8), 10 mM $CaCl_2$, and 150 μl of activated sample for 2–18 hr, and solubilized ^{14}C-labeled peptides were assayed for radioactivity; 1 unit hydrolyzes 1 μg of collagen per min (9, 12, 19). The collagenase activity reported is the sum of active and latent enzyme as measured in activated samples. The proportion of latent collagenase was determined by sham activation with trypsin mixed with STI (12).

Collagenase Cleavage Products. Medium from cells cultured for 48 hr after trypsinization was activated and incubated 18 hr at 25° with 100 μg of collagen in 100 mM Tris·HCl buffer, pH 7.8. Samples were prepared and run on 7% sodium dodecyl sulfate/polyacrylamide slab gels to examine cleavage peptides of collagen (20, 21).

Collagen Plate Assay. Cells were plated onto [^{3}H]acetyl-

Abbreviations: FCS, fetal calf serum; DME, Dulbecco's modification of Eagle's medium; DME-LH, DME supplemented with 0.2% lactalbumin hydrolysate; STI, soybean trypsin inhibitor; M_r, molecular weight.
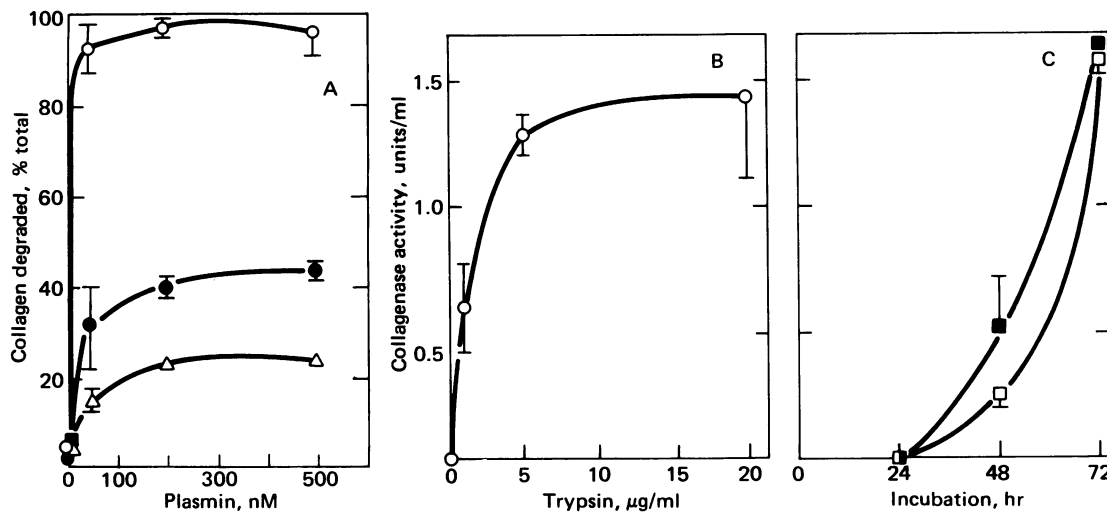
FIG. 1.   Secretion of collagenase after incubation with trypsin or plasmin. (*A*) Cells were plated on [^{3}H]collagen layers and treated with plasmin for the duration of the experiment. Media were sampled at 16 (△), 24 (●), and 40 (○) hr. Cell-free controls were determined with plasmin; 500 nM released 17% of the collagen after 40 hr, and lower concentrations released less. (*B*) Cells plated in 16-mm dishes were washed free of serum, incubated with trypsin for 24 hr, washed free of protease, and incubated for 48 hr; then media were assayed with [^{14}C]collagen fibrils. (*C*) Cells plated in 16-mm dishes were washed and incubated with 20 μg of trypsin per ml (□) or 30 nM plasmin (■). Medium was removed and assayed for collagenase activity on [^{14}C]collagen fibrils at the times indicated. Points and bars represent the mean ± SD or range of 2–8 replicate cultures. Error bars less than the size of the symbols were omitted.

collagen gels (12) in DME + FCS, grown overnight, then washed with Hanks' balanced salt solution and incubated with plasmin. Aliquots were removed to monitor release of ^{3}H-labeled peptides. Control wells for release of radioactivity from the collagen by plasmin were included in each experiment (12). Results are expressed as percentage of total radioactivity determined at the end of the experiment with bacterial collagenase.

Plasminogen Activator Assay. Direct conversion of ^{125}I-labeled plasminogen to the heavy and light chains of plasmin was determined (10, 13). Cells were incubated with 10 μg of trypsin per ml at 37° for 17 hr, and then STI (50 μg/ml), 200 units of Trasylol, and 2 μl of ^{125}I-labeled plasminogen (4 × 10^5 cpm) were added to each well. Medium was sampled after 4 hr and run on 11% sodium dodecyl sulfate/polyacrylamide gels; plasmin peptides were identified by radioautography (22).

Proteolytic Activity. Relative proteolytic activity of the proteases was determined with ^{125}I-fibrin (10, 13) or azocasein (23) as substrate. One unit of fibrinolysis represents hydrolysis of 1 μg of fibrin per hr, and 1 unit of azocaseinolytic activity represents a ΔA_{366} of 0.1 per hr.

Column Chromatography. For gel filtration of FCS, Bio-Gel A-1.5m (2.6 cm × 31.4 cm, 167 ml) (Bio-Rad) was equilibrated with 25 mM sodium phosphate buffer containing 0.08 M NaCl, pH 7.4. Protein concentration was determined assuming A_{280} = 1.0 for 1 mg/ml.

Materials. Materials were obtained from the following sources. Worthington: TPCK-trypsin (224 units/mg, EC 3.4.21.4), neuraminidase (28 units/mg, EC 3.2.1.18), α₁-antitrypsin. Calbiochem: urokinase (2330 Plough units/ampoule, EC 3.4.99.26), thermolysin (8080 Plough units/mg, EC 3.4.24.4), Trasylol. Sigma Chemical Co.: bromelain (840 units/mg, EC 3.4.22.4, formerly 3.4.4.24), chymotrypsin (41 units/mg, EC 3.4.21.1), pancreatic elastase (17 units/mg, EC 3.4.21.11), papain (31 units/mg, EC 3.4.22.2), bovine serum albumin, STI, fetuin (Type IV, lot 27C-8701). Accurate Chemical & Scientific Corp: α-protease (from *Crotalus atrox*, EC 3.4.24.1). Amersham/Searle: Na^{125}I. Purified thrombin (EC 3.4.21.5) was a gift of Bruce R. Zetter, University of California, San Francisco. Plasmin (EC 3.4.21.7) was prepared by incubating bovine plasminogen (12, 24) with 10 Plough units of urokinase per ml for 30 min at 25° (12). Bovine fibronectin (25) was a gift of E. Engvall, City of Hope Medical Center, Duarte, CA.

RESULTS

Induction of Collagenase Secretion by Proteases. After treatment of rabbit synovial fibroblasts with plasmin or trypsin, collagenase activity could be detected by monitoring degradation of collagen fibrils by living cells (Fig. 1A) or by assaying the enzyme activity secreted by the cells into medium (Fig. 1B). The amount of secreted collagenase increased with enzyme concentration and with time of exposure of the cells to the inducing protease (Fig. 1; Table 1). Secretion of up to 20 units of collagenase per 24 hr per 10^6 cells was induced by protease treatment. Prolonged exposure to protease (trypsin, bromelain, or chymotrypsin) sometimes decreased the secretion of collagenase. Collagenase was detected in the medium of protease-treated cells by 16–48 hr. This lag period, characteristic of the protease-mediated induction of collagenase, is not observed when cytochalasin B is used as inducer (17). Of other enzymes tested, only thrombin and neuraminidase did not induce collagenase secretion (Table 1). Collagenase secretion was also induced when cells were detached by a 10-min treatment with crude trypsin and then washed and replated (as occurs during routine cell passage) (Table 2). The inducing ability of the proteases did not correlate with their general proteolytic activity (Table 1). Thermolysin was very active against azocasein at 10 μg/ml, but was able to induce secretion only at 100 μg/ml. However, some proteolytic activity was necessary because plasminogen, or trypsin mixed with STI, failed to induce secretion.

Characteristics of Secreted Collagenase and Plasminogen Activator. The collagenase induced in protease-treated rabbit fibroblasts specifically cleaved collagen as indicated by the appearance of three-quarter- and one-quarter-length collagen degradation products on polyacrylamide gels (Fig. 2A). In some instances some of the induced collagenase was found in the medium in active form (Tables 1 and 2). It is not yet clear why active collagenase appeared in these cases, but it is possible that

Table 1. Collagenase secreted by rabbit fibroblasts after protease treatment

	Proteolytic treatment			Collagenase secreted	
Protease	Conc., μg/ml	Time, hr	Proteolytic activity, units	Units/ml	Latent form, % total
Control	—	24	0	0.01 ± 0.01 (5)	—
Plasminogen	2.7	24	0	0.04 ± 0.03 (3)	—
Plasmin	2.3	24	3.5 ± 0.1 (2)	1.12 ± 0.34 (7)	0
Trypsin	20	24	49.6 ± 2.4 (2)	1.25 ± 0.41 (3)	0
	5	2	—	0.06 ± 0.03 (3)	—
	5	24	12.4 ± 0.6 (2)	1.29 ± 0.09 (2)	0
	1	2	—	0 (2)	—
	1	24	2.5 ± 0.1 (2)	0.75 ± 0.15 (2)	0
Chymotrypsin	10	24	16.8 ± 0.5 (2)	1.03 ± 0.02 (2)	—
	5	24	8.4 ± 0.3 (2)	1.53 ± 0.34 (2)	58
Elastase	10	24	5.3 ± 0.8 (3)	1.32 ± 0.50 (2)	85
Papain	6	24	2.5 ± 1.1 (2)	1.58 ± 0.58 (3)	97
Bromelain	1	24	0.4 ± 0.1 (2)	0.59 ± 0.15 (3)	93
Thermolysin	100	24	153 ± 16 (2)	1.23 ± 0.46 (2)	100
	10	24	15.3 ± 1.6 (2)	0.02 ± 0.02 (2)	—
α-Protease	5	24	4.1 ± 0.2 (3)	0.74 ± 0.02 (2)	100
Thrombin	0.5	24	—	0 (5)	—
Neuraminidase	50	24	0 (2)	0.04 ± 0.02 (2)	—

Cell monolayers in 16-mm dishes were incubated with protease for 2 or 24 hr, washed free of protease, and incubated for 48 hr to collect collagenase before assay on [^{14}C]collagen fibrils. Azocasein was used as substrate to determine relative activities of the proteases. Activities are expressed as the mean ± SD or range, with the number of replicate cultures in parentheses. Cell-free wells treated with protease and then incubated with DME-LH gave <0.05 unit per well in the fibril assays. Little residual proteolytic activity remained in the cultures after washing (0–0.1 unit per well with ^{125}I-fibrin, and none with azocasein as substrate). The percentage of collagenase found in latent form was determined as described in *Materials and Methods.*

some of the inducing protease was retained on the cell surface or in the cell, consistent with observations on thrombin (26).

Cells treated with trypsin secreted a plasminogen activator that cleaved ^{125}I-labeled plasminogen to plasmin heavy and light chains (Fig. 2B). Trypsin plus STI did not induce secretion of plasminogen activator (Fig. 2B).

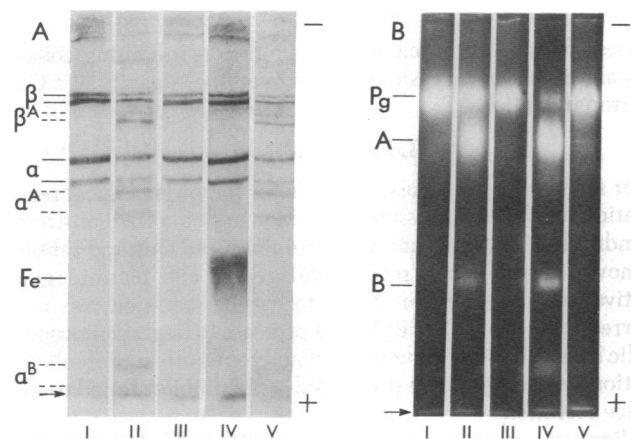**Morphology of Protease-Treated Fibroblasts.** The changes

FIG. 2. Characteristics of collagenase and plasminogen activator induced by trypsin treatment. The secreted fibroblast enzymes were characterized by examination of specific cleavage of substrate proteins as described in *Materials and Methods.* (A) Collagen in solution was incubated in DME-LH (I) alone; (II) from trypsin-treated cells; (III) from control cells; (IV) from trypsin-treated cells + 3.5 mg of fetuin per ml; (V) from trypsin-treated cells + 0.5 mg of fetuin per ml. β, α, uncleaved subunits of collagen; β^A, α^A, three-quarter-length cleavage fragments; α^B, one-quarter-length cleavage fragments; Fe, fetuin. (B) ^{125}I-labeled plasminogen was incubated with (I) DME-LH alone; (II) DME-LH + 10 units of urokinase per ml; (III) cells incubated in DME-LH; (IV) cells preincubated with 10 μg of trypsin per ml; (V) cells preincubated with (10 μg of trypsin + 50 μg of STI) per ml. Pg, intact plasminogen; A, A chain (heavy chain) of plasmin; B, B chain (light chain) of plasmin.

in morphology produced by proteolytic treatment of the cells always preceded collagenase secretion (Fig. 3 a–f); those proteases that did not cause the typical trypsinized morphology (retraction of pseudopods, rounding, and decreased adhesion of cells) did not initiate secretion. Treatment that rounded fibroblasts also removed iodinatable membrane glycoprotein; however, glycoprotein was removed by concentrations of trypsin (0.1 μg/ml, 3 hr) too low to produce a morphological

Table 2. Inhibition of collagenase secretion by serum components

Inhibitory treatment			Collagenase secreted	
Serum component	Conc., mg/ml	Trypsin inhibited, μg/ml	Units/ml	Latent form, % total
DME-LH	2.0	0	1.10 ± 0.87 (14)	33
Bovine albumin	3.5	0	1.68 ± 0.94 (16)	61
α$_1$-Antitrypsin	0.1	27.2	0.64 ± 0.31 (3)	0
Fibronectin	0.07	0	0.98 ± 0.14 (2)	68
Fetuin (partially purified)	1.0	0	1.44 ± 1.19 (5)	26
	3.5	0	0.03 ± 0.02 (3)	—
FCS fraction, M_r 40,000	0.19	13.6	2.00 ± 0.38 (3)	36
FCS	0.1%	1.1	0.13 ± 0.14 (6)	—
	1.0%	11.2	0.15 ± 0.19 (15)	94
	10.0%	—	0.02 ± 0.03 (6)	—

Cells were detached from 100-mm dishes with crude trypsin, washed, and replated at 1 × 10^5 cells per 16-mm well with serum components. The inhibitors were washed off after 24 hr and the cells were placed in DME-LH for 48 hr to collect collagenase. None of the materials added to the cultures inhibited collagenase activity after activation. Capacity of the serum components to inhibit trypsin was determined with azocasein as substrate and is expressed as trypsin inhibited (μg/ml) at the concentration used in the experiment. Results are expressed as the mean ± SD, with the number of replicate cultures in parentheses.
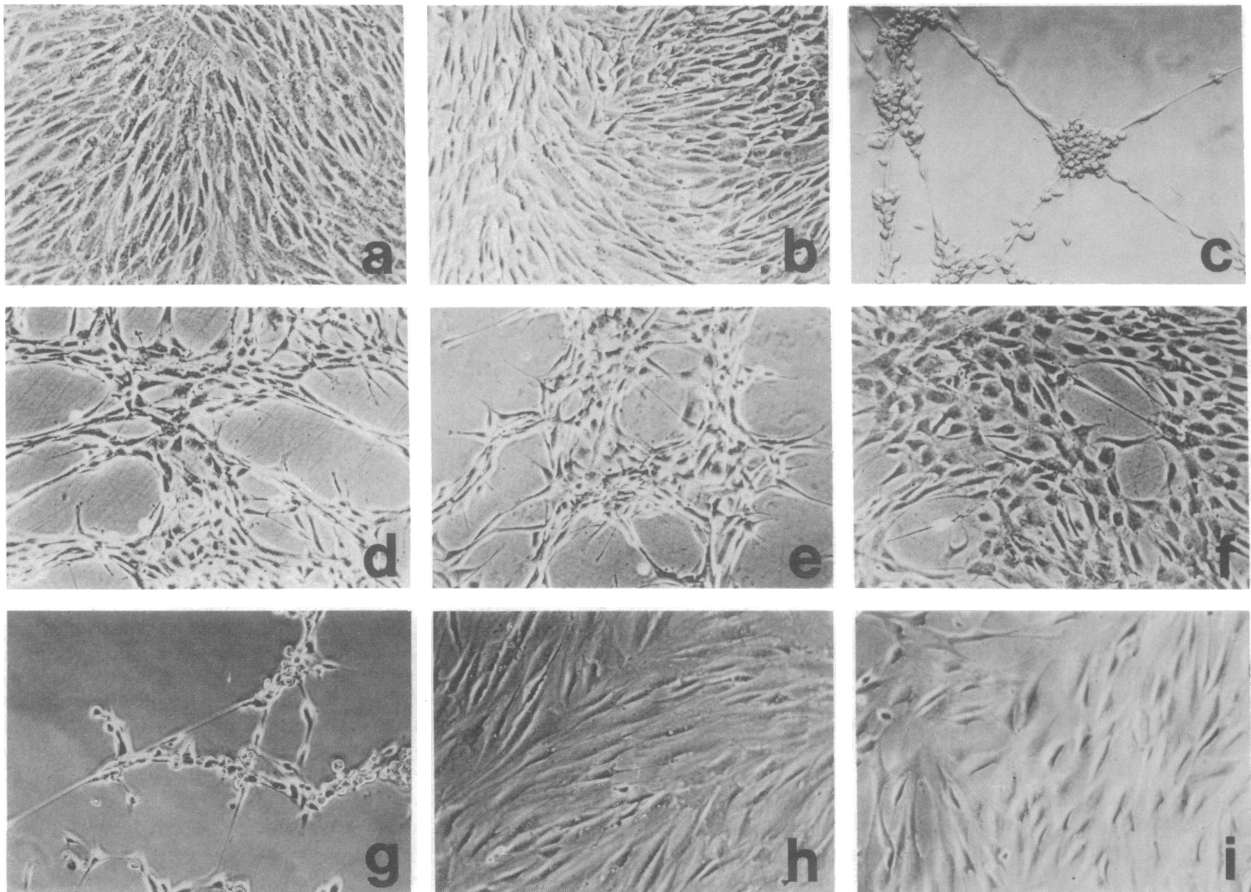
FIG. 3.   Morphology of fibroblasts treated with proteases and inhibitors. Phase contrast. (×80.) Cells were grown to confluence in 16-mm dishes, washed 5 times with Hanks' balanced salt solution, and incubated at 37° for (a) 24 hr in DME-LH, (b) 3 hr with 1 μg of trypsin per ml, (c) 24 hr with 1 μg of trypsin per ml, (d) 3 hr with 10 μg of trypsin per ml, (e) 24 hr with 6 μg of papain per ml, or (f) 24 hr with 1 μg of bromelain per ml. Trypsinized cells were plated into 16-mm wells containing (g) 3.5 mg of bovine serum albumin per ml, (h) 1.0% FCS, or (i) 0.2 mg of FCS fraction (M_r 40,000) per ml, and incubated 24 hr at 37°.

effect or to induce secretion of collagenase (data not shown). Once morphology was altered, the protease could be washed off or inhibited without altering subsequent collagenase secretion.

Regulation of Protease-Induced Collagenase Secretion by Serum Proteins. Although trypsin induced secretion of collagenase, it was apparent that, under normal conditions after culture in DME + FCS, fibroblasts maintained in DME-LH did not contain or secrete collagenase in a detectable form. FCS strongly inhibited secretion (Table 2) and promoted cell adhesion and spreading (Fig. 3h). In contrast, trypsinized cells cultured with bovine serum albumin attached poorly to the dish, but secreted collagenase (Table 2; Fig. 3g). Cells plated into DME + partially purified fetuin attached and spread and, at ≤ 1 μg/ml, secreted collagenase; at higher fetuin concentrations secretion was inhibited (Table 2; Fig. 2A). Trypsin inhibition alone neither allowed the cells to spread, nor stopped collagenase secretion. Although protease treatment of cells removes cell surface glycoproteins, purified bovine fibronectin did not inhibit induction of collagenase secretion (Table 2). Soluble collagen (100 μg/ml) had no effect on spreading or collagenase production. Preliminary characterization of the substances inhibiting secretion was performed by gel filtration of FCS. Only a small proportion of the inhibitory activity of the starting FCS was recovered, but fractions eluting with apparent molecular weight (M_r) 40,000 allowed trypsinized cells to spread and secrete collagenase (Fig. 3i; Table 2). Many lots of FCS spontaneously permitted secretion, perhaps because pro-

teases of coagulation inactivated the factors inhibiting collagenase induction; trypsin also destroyed the capacity of FCS to stop induction.

DISCUSSION

Our study suggests a possible mode for the physiological regulation of connective tissue destruction under normal or stress conditions. Proteases such as plasmin induced cultured rabbit synovial fibroblasts to secrete collagenase and plasminogen activator. Although the ability to induce secretion was not correlated with the ability of the protease to degrade nonspecific substrates, the induction seemed to require a proteolytic action because protease pretreated with inhibitor failed to induce secretion.

Because a variety of proteases with differences in specificity were capable of inducing collagenase secretion, the possible target site on the cell is difficult to establish. The proteases that were active inducers of collagenase secretion are also capable of removing cell surface glycoproteins (fibronectin) (2, 3, 7), but the correlation between removal of fibronectin and induction of collagenase secretion is not clear. It is unlikely that simple removal of fibronectin is the trigger for secretion. Concentrations of protease (e.g., 1.0 μg of trypsin per ml) that remove fibronectin in 10 min (2) did not induce collagenase secretion even after 2 hr (Table 1) and did not change cell morphology (Fig. 3b). However, distribution of this glycoprotein on the cell surface and collagen substratum, rather than its presence or absence, may be the significant determinant of cell

behavior (27–29). Even if fibronectin is closely related to the regulatory processes involved in collagenase secretion, it is not surprising that its exogenous addition did not inhibit the induced secretion or restore altered morphology, because it has recently been shown that little fibronectin adheres to the surface of normal cells once it is shed into conditioned medium (8, 30).

Induction of secretion by proteases was strongly correlated with changes in fibroblast morphology. Decreased adhesion and rounding of cells were necessary for initiation of collagenase secretion. Low concentrations of trypsin took hours to produce rounding of the fibroblasts and induction of collagenase secretion, whereas high concentrations (as in crude trypsin) accomplished this in minutes. Once the morphologic effect occurred, the subsequent collagenase production did not require continued presence of proteolytic activity and did not correlate with cell spreading or cell growth. Cell rounding is also correlated with induction of collagenase secretion by colchicine and cytochalasin B (16, 17). Fractions of serum allowed the fibroblasts to spread after detachment with trypsin but did not prevent the induction of collagenase secretion. Thus, fibroblasts may continue to secrete cellular proteases after proteolytic treatment whether they remain rounded or re-adhere and spread. These observations may explain earlier reports of spontaneous collagenase secretion in cells regularly subcultured with trypsin (9, 18, 21). How proteases act at the cell surface or possibly within cells is not known, but further investigation of distribution and turnover of various cell surface proteins and attachments of cytoskeletal elements may help elucidate these mechanisms.

It is intriguing that components of serum were able to regulate the expression of collagenase activity by fibroblasts. The concentration of these components was greatest in FCS, but they were present in smaller amounts in adult sera from man and rabbit (unpublished data). Proteins copurifying with fetuin promote cell spreading (31, 32), and we have shown that they may modulate collagenase secretion. The proteins involved in this regulation and their mechanisms of action require further investigation.

In many systems collagenase is secreted in an inactive or latent form (9, 11, 12, 18), which may be converted to active enzyme by plasmin (12), which is in turn generated from its zymogen by plasminogen activator (12). It is not known whether plasminogen activator is always secreted concomitantly with collagenase, but trypsin-treated fibroblasts secreted both enzymes. Thus, protease-treated cells may activate plasmin, which could then initiate secretion of more plasminogen activator and collagenase. This feedback amplification could produce collagenase activity that would destroy the extracellular matrix.

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