Changes in Cell Shape Correlate with Collagenase Gene Expression in Rabbit Synovial Fibroblasts

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ABSTRACT Induction of the neutral proteinase, collagenase, is a marker for a specific switch in gene expression observed in rabbit synovial fibroblasts. A variety of agents, including 12-O-tetradecanoylphorbol-13-acetate, cytochalasins B and D, trypsin, chymotrypsin, poly(2-hydroxyethylmethacrylate), and trifluoperazine induced this change in gene expression. Induction of collagenase by these agents was always correlated with a marked alteration in cell morphology, although the cells remained adherent to the culture dishes. The amount of collagenase induced was positively correlated with the degree of shape change produced by a given concentration and, to some extent, with the duration of treatment. Altered cell morphology was required only during the first few hours of treatment with inducing agents; after this time collagenase synthesis continued for up to 6 d even when agents were removed and normal flattened cell morphology was regained. All agents that altered cell morphology also produced a characteristic switch in protein secretion phenotype, characterized by the induction of procollagenase (M_r 53,000 and 57,000) and a neutral metalloproteinase (M_r 51,000), which accounted for approximately 25% and 15% of the protein secreted, respectively. Secretion of another neutral proteinase, plasminogen activator, did not correlate with increased collagenase secretion. In contrast, synthesis and secretion of a number of other polypeptides, including the extracellular matrix proteins, collagen and fibronectin, were concomitantly decreased. That changes in cell shape correlated with a program of gene expression manifested by both degradation and synthesis of extracellular macromolecules may have broad implications in development, repair, and pathologic conditions.

The neutral proteinase, collagenase, which specifically degrades interstitial collagens, is induced by a variety of agents, including the phorbol diester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)[1] (2, 10), cytochalasin B (CB) (23), and proteolytic enzymes (52), as well as by phagocytosis (54). We have demonstrated that TPA triggers an alteration in gene expression in rabbit synovial fibroblasts that is marked by induction of several secreted proteins, including collagenase and a wide-spectrum neutral metalloproteinase, and by reduction of the synthesis of other secreted proteins, including collagen and fibronectin (2). When we analyzed this switch in gene expression by following the induction of collagenase by TPA, several characteristics of this phenomenon were evident (2). Collagenase induction required only a brief exposure to TPA, and no detectable collagenase was produced until 6–12 h after addition of TPA. Once begun, collagenase synthesis continued for >48 h after TPA was removed. Translatable mRNA for collagenase was present only in induced cells. Finally, concentrations of TPA that induced collagenase always caused alterations in cell morphology. In the present study we have used other agents reported to alter cell morphology to test the hypothesis that changes in cell shape play an important role in initiating the switch in gene expression observed with TPA treatment (2). We now report that TPA, CB, and proteinases, as well as two additional agents, poly(2-hydroxyethylmethacrylate) (polyHEMA) and trifluoperazine (TFP), induce changes in cell shape in rabbit synovial fibro-

[1] *Abbrevations used in this paper:* CB, cytochalasin B; DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; kd, kilodalton; PA, plasminogen activator; polyHEMA, poly(2-hydroxyethylmethacrylate); preproCL, preprocollagenase; proCL, procollagenase; TFP, trifluoperazine; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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blasts that are correlated with induction of this specific secretion phenotype.

MATERIALS AND METHODS

Except for the following, all methods used were as described in the accompanying paper (2).

Cell Culture: Rabbit synovial fibroblasts were cultured in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum as described previously (2). For most experiments, cells (~10^5 cells per well) plated in 16-mm culture dishes (Costar, Boston, MA 24-well) were incubated at 37°C for 16–48 h, and then placed in serum-free DME supplemented with 0.2% lactalbumin hydrolysate for treatment with various agents. For some experiments, cells were incubated at 37°C for various periods of time in the continuous presence of inducing agents; in other experiments, inducing agents were included for the first 6–12 h of incubation only, after which the cells were returned to serum-free medium for collection of secreted proteins. Sodium ascorbate (50 μg/ml) was added to some cultures to facilitate collagen synthesis. TPA (Sigma Chemical Co., St. Louis, MO) was dissolved at 1–5 mg/ml in ethanol; CB (Sigma Chemical Co.) at 1 mg/ml in dimethyl sulfoxide; TFP (Sigma Chemical Co.) at 3 mM in phosphate-buffered saline; and monensin (Eli Lilly and Co., Indianapolis, IN) at 2.5 mM in DME containing 1% ethanol. All stock solutions were stored at −20°C until used.

To study the effects of culturing cells on polyHEMA (Hydron Corp., New Brunswick, NJ) (19), plates were prepared by dissolving polyHEMA (12% wt/vol) in 95% ethanol overnight at 37°C and then diluting this stock solution to the appropriate concentration with 95% ethanol and adding 200 μl/well to 16-mm culture wells. The plates were dried on a leveling platform at 25°C for 2–3 d. Final polyHEMA coating ranged from 50–1,200 μg/cm². Fibroblasts were trypsinized, plated into coated wells in DME-10% fetal bovine serum, and incubated for 18–24 h at 37°C before being washed with saline and placed in serum-free medium.

Cell morphology was monitored by phase microscopy, and the degree of morphologic change after various treatments was scored from 0–4 on an arbitrary scale. For scanning electron microscopy, cells plated onto 12-mm-diam glass coverslips coated with polyHEMA were fixed and processed as described previously (3).

Plasminogen Activator Assay: Plasminogen activator (PA) activity was measured with an ^{125}I-labeled fibrin substrate in the presence or absence of 6 nM purified bovine plasminogen (5, 50). Each well contained 2–5 × 10^4 cpm and 20 μg/ml fibrin substrate. 1 U of activity releases 5% (1 μg) of the available substrate per hour. Release of labeled peptides in the absence of plasminogen was always <5%, indicating that the activity reported was PA.

RESULTS

Alteration of Cell Shape in Rabbit Fibroblasts

To test the hypothesis that shape change is required for induction of collagenase, we used a variety of agents that have been reported to alter morphology of various cell types (Table I) and developed a semi-quantitative scale for scoring alterations in cell shape. Agents that were effective in altering the flattened morphology of rabbit synovial fibroblasts typically caused concentration- and time-dependent changes. The scale is illustrated for TPA treatment in Fig. 1. With increasing concentrations of TPA, the normally well-spread cells showed progressive alteration in morphology, such as retraction of lamellipodia, increased membrane ruffling and blebbing, and rounding of cell bodies. Although the area of cell contact with the substratum was reduced by all agents, even cells that appeared by phase microscopy to be completely rounded remained adherent to the culture dish under our experimental conditions (43).

Cells treated with different agents were not morphologically identical; nevertheless, similar grading of changes in shape was possible for all agents. TPA- and trypsin-treated cells were fusiform or rounded (Figs. 1 and 2) (52), whereas cells treated with CB had the stellate appearance typically caused by this agent (Fig. 2). It should be noted that the concentration of agent used and the duration of treatment were interrelated such that treatment with a high concentration for a short period of time was roughly equivalent to treatment with a low concentration for a longer time. PolyHEMA has been used previously to produce gradations of shape change in anchorage-dependent cells (19). Rabbit fibroblasts plated on intermediate thicknesses of polyHEMA were typically fusiform and displayed increased surface microvilli and ruffles with focal attachments to the coverslip where there were small holes in the polyHEMA layer (Fig. 2). TFP, a psychotropic phenazine derivative that inhibits Ca^{2+} binding to calmodulin (33), has been reported to alter the shape of human erythrocytes (38) and platelets (28). Low concentrations of TFP (7–8 μM) produced TPA-like morphologic changes in rabbit fibroblasts (Fig. 3*b*). TFP displayed a very limited range of effective concentrations; at <6 μM, cells remained flat and well-spread, and at >9 μM the cells rounded up completely and detached from the dish. This range corresponds well to the concentrations of calmodulin normally found in mammalian cells (12).

TABLE I

Induction of Morphologic Alteration and Collagenase Secretion in Rabbit Fibroblasts

Agent	Morphologic change	Activatable collagenase
		*U**
None	0	0.05
TPA, 10 ng/ml	1	3.04
20 ng/ml	2	4.80
100 ng/ml‡	4	11.70
4-α-Phorbol didecanoate, 20 ng/ml	0	0.00
Cytochalasin B, 1 μg/ml	3	3.63
Cytochalasin D, 5 μg/ml§	2	1.30
Trypsin, 5 μg/ml‖	2	1.29
PolyHEMA, 200 μg/cm²	4	4.45
Trifluoperazine, 4 μM‡	0	0.08
6 μM‡	2	0.91
9 μM‡	4	2.39
Monensin, 10 μM‡	0	0.01
Colchicine, 1 μM	0	0.03
Epidermal growth factor, 100 ng/ml	0	0.11

Rabbit synovial fibroblasts were incubated in serum-free medium in 24-well plates for 72 h in the presence or absence of agents as indicated. Medium was then collected, activated, and assayed for collagenase activity. Degree of morphologic change was scored at 24 h by phase-contrast microscopy as described in the legend to Fig. 1.

* 1 U of collagenase activity is equivalent to ~100 ng of procollagenase protein.

‡ Assayed after 48 rather than 72 h.

§ Cytochalasin D was more toxic to rabbit fibroblasts and less effective at inducing collagenase than cytochalasin B at concentrations from 0.5–5 μg/ml.

‖ Trypsin was present for the first hour of incubation only.

Agents That Alter Cell Shape Induce Secretion of Collagenase Activity

We next compared the ability of agents to alter rabbit fibroblast morphology with their ability to induce collagenase (Table I). TPA was an effective inducer of collagenase activity, producing secretion of 1–5 U per 10^5 cells per 24 h over a concentration range of 10–100 ng/ml. Significantly, the degree of change in cell shape caused by increasing either time of exposure or concentration of TPA was positively correlated

with the amount of collagenase activity secreted at each time or concentration. 4-α-Phorbol didecanoate, a nontumor-promoting analogue of TPA, did not affect cell morphology or protein secretion. Low concentrations (1–5 μg/ml) of both CB and cytochalasin D caused rounding of rabbit fibroblasts and induced collagenase production.

When cells were cultured on polyHEMA, the degree of morphologic change observed was directly correlated with the amount of collagenase induced by increasing concentrations (Table I, Fig. 4). Similarly, the concentration-dependent increase in collagenase secretion caused by TFP (Table I) corresponded precisely to the limited range of concentrations over which this agent produced morphologic alterations in these cells (Fig. 3). At high concentrations of polyHEMA (>800 μg/cm^2) or TFP (>10 μM), cells became detached from the surface of the dish and floated into the medium. Collagenase secretion was greatly reduced or absent from these suspended cells (Fig. 4), reflecting the inhibition of overall protein synthesis observed in detached anchorage-dependent cells (18, 40). In view of the observation that TFP decreases exocytosis by cultured cells (29), it is notable that collagenase accumulated within TFP-treated rabbit fibroblasts, as detected by a small but reproducible amount of collagenase associated with cell layers (0.3 U per 10^6 cells after 40-h exposure to 7 μM TFP). The large number of small vesicles observed in TFP-treated fibroblasts provided further evidence for partially inhibited secretion in these cells (Fig. 3). When cells were treated with monensin, another inhibitor of protein secretion (48), they remained flattened and did not produce collagenase (Table I). Monensin added to TPA-treated cells reduced, but did not eliminate, the accumulation of collagenase in the medium (data not shown).

Agents that did not alter cell shape did not induce collagenase. Although disruption of microtubules by colchicine causes rounding of some cell types (21, 22, 24), it did not alter the morphology of rabbit synovial fibroblasts under our culture conditions, and neither colchicine (1 μM) (Table I) nor vinblastine (1 μM) (not shown) induced collagenase production in these cells. (Occasionally, for reasons that are not well understood, colchicine will cause fibroblasts to round, and in these cases induction of collagenase secretion is observed [22, 24].) Epidermal growth factor and platelet-derived growth factor, which cause transient changes in the morphology of cultured epidermal cells (13), neither changed the morphology of rabbit fibroblasts nor induced collagenase production by these cells.

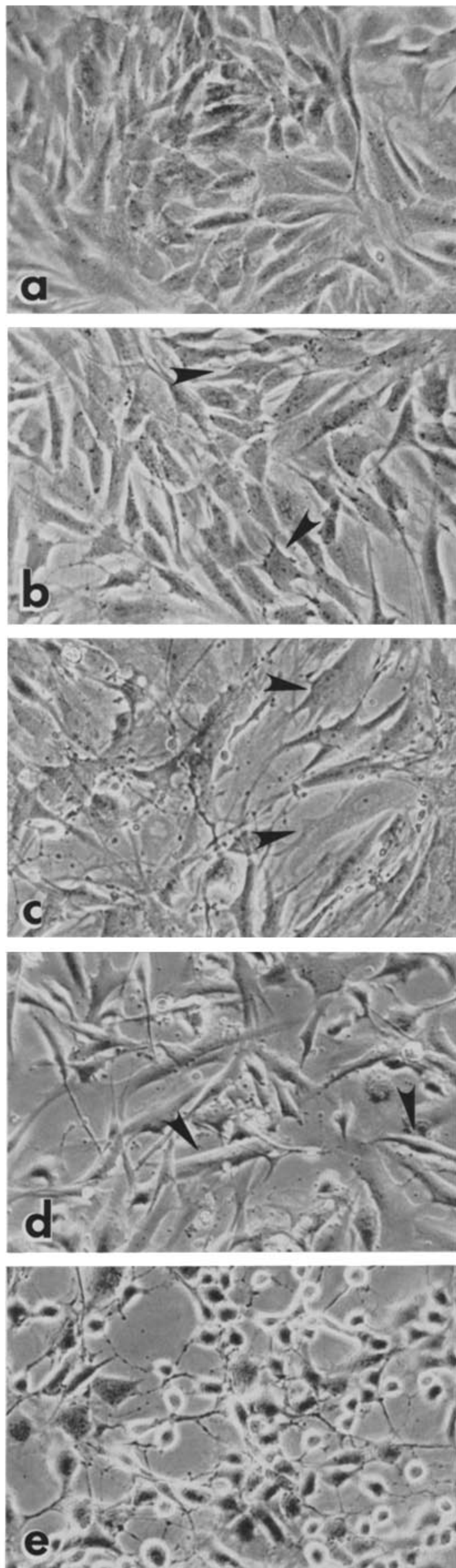

FIGURE 1 Cell rounding produced by TPA, illustrating the grading of morphologic changes. Rabbit synovial fibroblasts were incubated in the presence of various concentrations of TPA for 72 h. (*a*) Untreated control cells are well spread and in broad contact with each other at their margins (score = 0). (*b*) Cells treated at a low concentration (2 ng/ml) are beginning to retract at their margins (arrowheads) and lose contact with one another (score = 1). (*c*) After treatment at an intermediate concentration (20 ng/ml), many cells have retracted from their neighbors, although some remain quite well spread (arrowheads) (score = 2). (*d*) Most cells treated at a high concentration (100 ng/ml) have lost contact with each other and become fusiform (arrowheads) (score = 3). (e) After prolonged treatment at a high concentration, cells are usually rounded but still adherent, and often form clusters and clumps (score = 4). Phase contrast. × 350.

FIGURE 2 Morphologic changes in rabbit synovial fibroblasts treated with TPA, CB, and polyHEMA. Compared with the extremely flat, smooth-surfaced control cells (*a*), cells plated in 20 ng/ml TPA (*b*), in 2 μg/ml CB (*c*), or onto 200 $\mu g/cm^2$ polyHEMA (*d*) appear fusiform or stellate and display many microvilli and ruffles on their surfaces. Small holes are observed in the polyHEMA layer (*d*) (asterisks), and cells appear to attach selectively to them (arrow). In the well-spread control cells (*a*) nuclei (*Nu*) and stress fibers (arrowheads) are plainly visible. × 1,400.

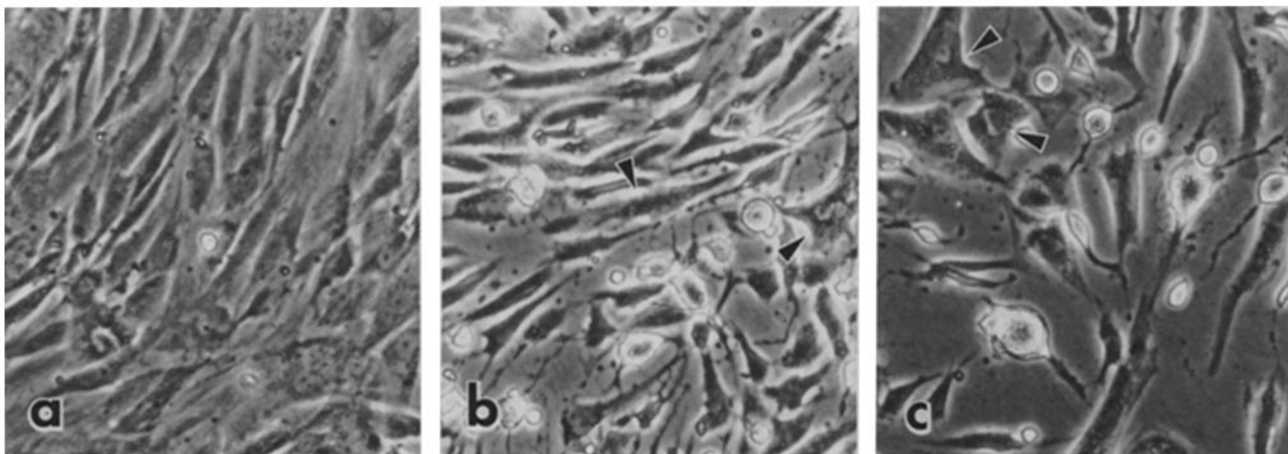

FIGURE 3 Morphologic changes produced by TFP. Rabbit synovial fibroblasts were incubated for 6 h in the presence or absence of TFP; the TFP was then removed and the cells were returned to serum-free medium for 20 h before being photographed by phase-contrast microscopy. Control cells (*a*) are well-spread; cells treated with 7 (*b*) or 8 μM TFP (*c*) are increasingly rounded. Arrowheads (*b* and *c*) indicate the marked accumulation of small vesicles in TFP-treated cells. × 350.

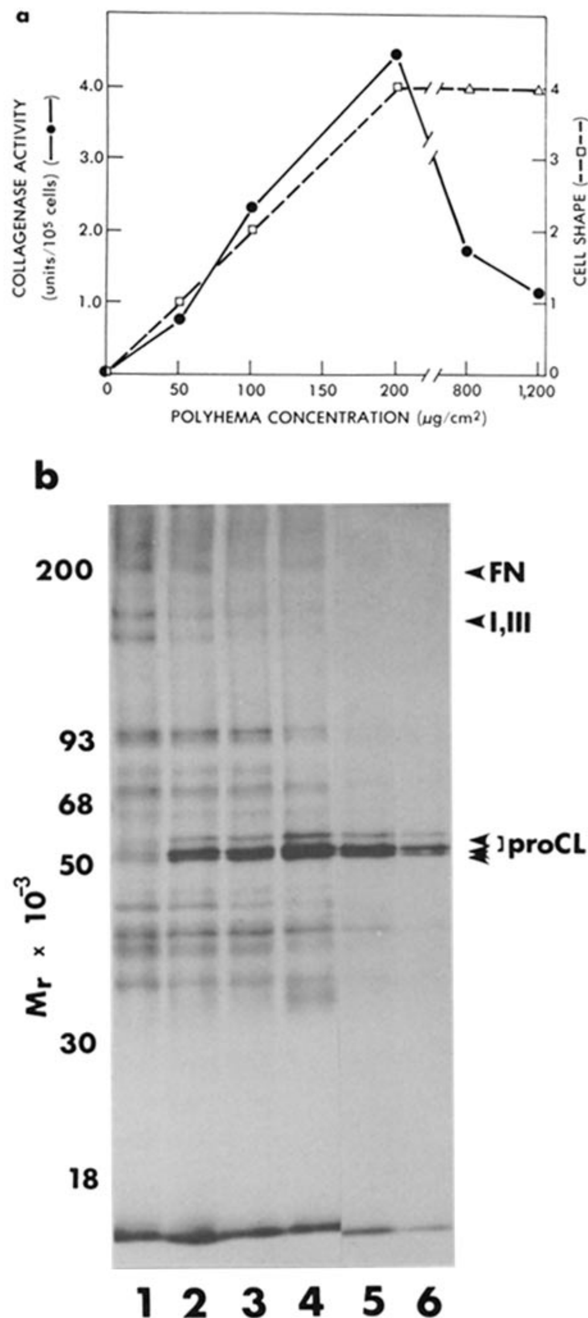

FIGURE 4 Induction of collagenase activity by polyHEMA. Rabbit synovial fibroblasts were incubated for 72 h in serum-free medium in the absence or presence of increasing concentrations of polyHEMA, and medium was collected, activated, and assayed for collagenase activity. Washed cells were then incubated with [^{35}S]-methionine for 4 h, and labeled secreted proteins were acid precipitated and prepared for SDS PAGE (2). (*a*) Collagenase activity in medium (•); cell shape score (□); Δ indicates the presence of rounded cells that had become detached from the culture dish and were found floating in the medium. 1 U of collagenase activity is equivalent to ~100 ng of proCL protein (2). (*b*) [^{35}S]Methionine-labeled secreted proteins of cells incubated in the absence (lane *1*) or presence of polyHEMA at 50 μg/cm^2 (lane *2*); 100 μg/cm^2 (lane *3*); 200 μg/cm^2 (lane *4*); 800 μg/cm^2 (lane *5*); or 1,200 μg/cm^2 (lane *6*). Brackets indicate migration of the 53- and 57-kd proCL bands. The positions of fibronectin (*FN*) and procollagens type I and III (*I, III*) are indicated by arrowheads, and molecular weight markers are indicated at left.

Changes in Cell Shape Trigger a Major Switch in Synthesis of Secreted Proteins by Rabbit Fibroblasts

The induction of collagenase by TPA can be easily monitored by the appearance of newly synthesized procollagenase protein (proCL), which migrates as a doublet of 53 and 57 kilodaltons (kd) on SDS polyacrylamide gels and represents 20–25% of the secreted protein of TPA-treated cells (2). In addition to the proCL doublet, a 47-kd polypeptide and a 51-kd neutral metalloproteinase (2, 20) that represents ~15% of the secreted protein are induced. Along with a marked decrease in the synthesis of procollagen and fibronectin, these changes represent a major switch in the secretion phenotype of rabbit synovial fibroblasts (2). When the SDS PAGE patterns of cells treated with other agents that alter cell shape were examined, a similar switch in polypeptide secretion phenotype was observed (Fig. 5). Cells treated with CB, chymotrypsin, polyHEMA, or TFP synthesized proCL and the 51-kd metalloproteinase as their major secretion products. Immunoprecipitation of proCL from both the medium and cell lysates of TFP-treated cells with antibodies specific for collagenase confirmed that the material migrating at 53 and 57 kd was proCL (Fig. 5*b*). The 47-kd polypeptide appeared to be TPA-specific, because it was not observed after treatment with any other agent at the concentrations tested. Similar to TPA, reduced secretion of procollagen and fibronectin, identified as ^{35}S-labeled bands, was found after treatment with CB, polyHEMA, chymotrypsin, and TFP (Fig. 5). Collagen synthesis was reduced by 90% (from 3.96×10^4 dpm to 0.39×10^4 dpm) in cells treated with CB (2 μg/ml), as determined by measuring incorporation of hydroxyproline into secreted proteins (27). Addition of 50 μg/ml sodium ascorbate to facilitate procollagen processing and secretion partially reversed the decrease in procollagen secretion (Fig. 5*a*). In addition, unidentified polypeptides of 95, 92, 75, 70, 45, 40, and 35 kd were secreted in reduced amounts. When mRNA from cells treated with CB for 24 h was translated in a rabbit reticulocyte lysate, the polypeptide pattern was indistinguishable from that for TPA-treated cells, and preprocollagenase (preproCL) appeared as a major band of 55 kd.

Induction of Collagenase Is Not Correlated with Secretion of PA

Coordinate secretion of collagenase and the serine proteinase, PA, has been reported in endothelial cells (37) and in rheumatoid synovial cells (53). Quigley (41) has hypothesized that PA secreted by TPA-treated fibroblasts is responsible for shape changes in these cells. However, when we examined secretion of PA by rabbit fibroblasts treated with TPA, CB, EGF, polyHEMA, or TFP, no correlation with induction of collagenase was observed (Tables I and II). Although TPA, polyHEMA, and TFP stimulated secretion of both proteinases to various degrees, CB stimulated secretion of collagenase only, and EGF caused an increase in PA activity alone (32). Examination of the timing of PA stimulation in TPA-treated cells showed that secretion of this proteinase increased to a maximum within 6 h after addition of TPA, in parallel with an increase in overall protein synthesis and secretion (Fig. 6). This pattern was distinct from the consistent pattern of delay observed in the induction of collagenase secretion (Fig. 6*b*)

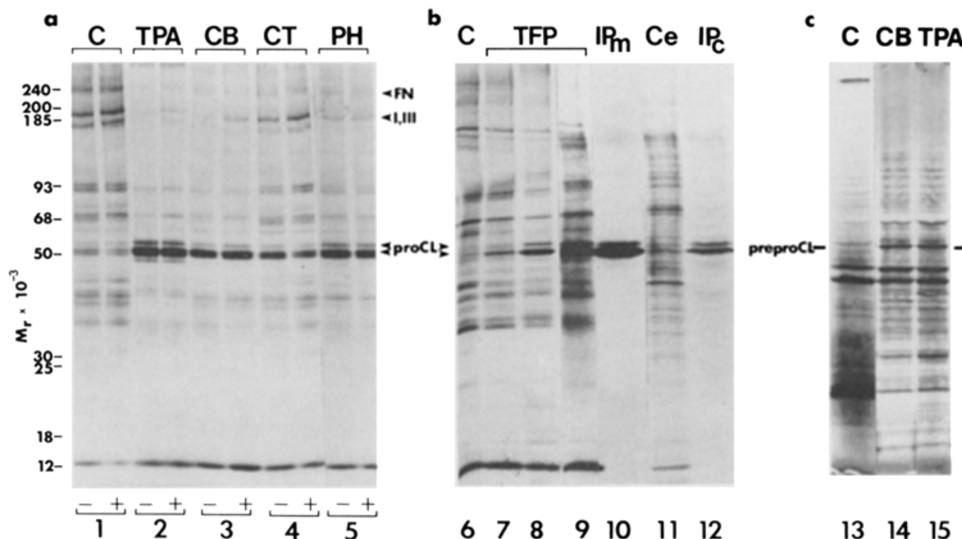

FIGURE 5 Comparison of the proteins of rabbit synovial fibroblasts treated with various agents. Rabbit synovial fibroblasts were induced to synthesize collagenase, and [^{35}S]-methionine-labeled, secreted proteins were analyzed on SDS PAGE gels (2). (*a*) Cells were incubated for 72 h in the presence of serum-free medium (*C*) (lane *1*); 20 ng/ml TPA (lane *2*); 2 μg/ml CB (lane *3*); 5 μg/ml chymotrypsin (*CT*) (lane *4*); or 100 μg/cm^2 polyHEMA (*PH*) (lane *5*), and then labeled with [^{35}S]methionine for 4 h. The − and + at bottom indicates the absence or presence of 50 μg/ml sodium ascorbate during the incubation. The positions of fibronectin (*FN*) and procollagens type I and III (*I*, *III*) are indicated by arrowheads. (*b*) Cells were incubated for 44 (lanes *6–8*), or 6 h (lanes *9–12*) in the presence of TFP, and secreted and cellular proteins were analyzed by SDS PAGE. (In lanes *9–12*, samples were labeled with [^{35}S]methionine after 28 h of treatment [6 h in TFP followed by 22 h in serum-free medium].) Lane 6, untreated controls (*C*); lane *7*, 6 μM TFP; lane *8*, 7 μM TFP; lanes *9–12*, 8 μM TFP. Lane *10*, immunoprecipitation of proCL from medium (*IP$_m$*); lane *11*, cellular protein (*Ce*); lane *12*, immunoprecipitation from cells (*IP$_c$*). In these gels the 51-kd polypeptide and the 53-kd proCL band were not resolved, so the relative contribution of each to the overall secretion pattern cannot be ascertained unambiguously. The two proCL bands of 53 and 57 kd are indicated with brackets. (*c*) Cells were incubated for 24 h in serum-free medium in the presence or absence of 5 μg/ml CB or 100 ng/ml TPA, and RNA was extracted. SDS PAGE demonstrated the [^{35}S]methionine-labeled proteins from the cell-free translation of 5 μg total RNA isolated from control (*C*) (lane *13*), CB-treated (*CB*) (lane *14*), or TPA-treated (*TPA*) (lane *15*) cells. Migration of preproCL (55 kd) is indicated by an arrowhead. Molecular weight markers are indicated at the left.

TABLE II
Stimulation of Plasminogen Activator Secretion by Agents Inducing Morphologic Change

Agent	Morphologic change	Plasminogen activator
		U
Experiment 1		
None	0	24.5
TPA, 10 ng/ml	2	247.9
Cytochalasin B, 1 μg/ml	2	24.3
Epidermal growth factor, 100 ng/ml	0	74.8
Experiment 2		
None	0	52.5
PolyHEMA, 100 μg/cm^2	2	97.0
Trifluoperazine, 6 μM	2	96.8

Rabbit synovial fibroblasts (10^5/well) were incubated in 24-well plates for 72 h in the presence or absence of agents as indicated. For determination of PA activity, 50 μl of conditioned medium was diluted in 500 μl of 0.05 M Tris-HCl (pH 7.8) in 16-mm wells containing 25,000 cpm ^{125}I-labeled fibrin in the presence or absence of 6 nM plasminogen.

(2, 52). In addition, it should be noted that a moderate amount of PA was secreted even by well-spread untreated fibroblasts (Table II), and it thus seems unlikely that production of these two proteinases is under close coordinate control in rabbit synovial fibroblasts.

Alteration in Cell Shape Is Required for Collagenase Induction, But Not for Continued Collagenase Production

The data so far presented indicate that alterations in cell morphology are required to trigger a specific switch in secretion phenotype in rabbit synovial fibroblasts. We wished next to determine whether change in shape acts only at the induction step, or is also necessary to sustain the altered secretion phenotype. In experiments described thus far, treatments were continuous, and cell morphology remained altered throughout. Previous experiments using brief treatments with trypsin (52) or TPA (2) suffered from the limitation that these agents persist within cells, making it difficult to control the duration of exposure precisely. We therefore monitored the synthesis of proCL by fibroblasts that were treated with CB for a limited time and then transferred to medium without CB. Under these experimental conditions, cells respread within 30 min of removal of CB, showing that the duration of CB treatment and of cell rounding corresponded closely. In cells treated with CB for either 6 or 12 h, proCL was first detected at 12 h by SDS PAGE (Fig. 7). It is clear that, although an initial alteration of cell shape (score = 4) was required for collagenase to be induced by CB, continued cell rounding was not required for subsequent synthesis and secretion of collagenase. Cells that had reverted to normal flattened morphology (score = 0) after 6 or 12 h of treatment with CB showed the same pattern of proCL secretion at 48 h (Fig. 7) as did continuously treated cells (Fig. 5 *a*).

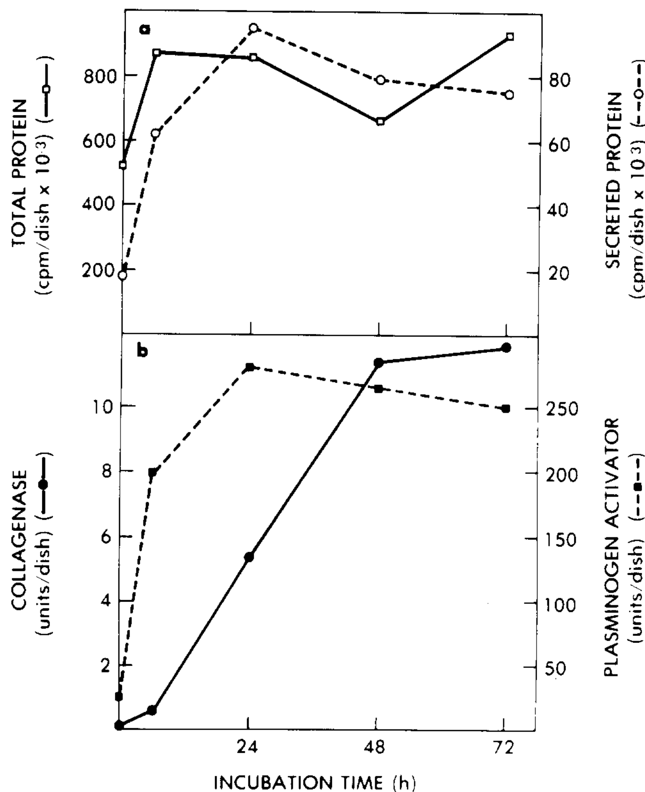

FIGURE 6 Timing of protein secretion by TPA-treated cells. Rabbit synovial fibroblasts were incubated in serum-free medium for 6 h in the presence of 100 ng/ml TPA, washed, and then returned to 37°C in fresh medium without TPA. At the times indicated, the medium was collected for enzyme assay and cells were radiolabeled with [^{35}S]methionine (25 μCi/ml) for 2 h. Incorporation of [^{35}S]-methionine into cellular and secreted proteins was determined as previously described (2). (*a*) Incorporation of [^{35}S]methionine into cellular (□) or secreted (○) proteins. (*b*) Activity of secreted collagenase (●) or plasminogen activator (■).

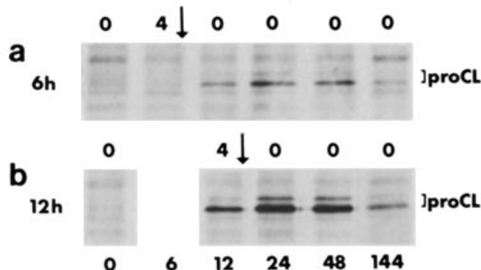

FIGURE 7 Collagenase secretion by respread fibroblasts. Rabbit synovial fibroblasts were treated with 2 μg/ml CB for 6 (*a*) or 12 h (*b*), washed, and incubated in serum-free medium for up to 144 h. At 24-h intervals, cells were pulse-labeled for 2 h with [^{35}S]methionine and labeled proteins were analyzed by SDS PAGE. Only the central portion of the gels containing the proCL bands (53 and 57 kd) and the neutral proteinase (51 kd) is shown. Elapsed time after initial CB treatment is indicated at the bottom, and the time of removal of CB is marked by an arrow at the top of each gel. The degree of morphologic change is indicated above each lane (see legend to Fig. 1 for explanation of scoring).

Several other aspects of collagenase induction by agents that alter cell shape are also illustrated by these data. First, the timing of the onset of proCL synthesis was independent of the duration of CB treatment. Cells first synthesized proCL at 12 h (Fig. 7) and subsequently reached a maximum rate of proCL synthesis and secretion between 24 and 48 hr after initial treatment with CB (Table III), whether they were treated for 6 or 12 h, or continuously (Fig. 5 *a*). Once induced, the secretion phenotype was stable for several days, and proCL was still being synthesized at approximately one-third the maximum rate 144 h after the initial CB treatment (Fig. 7). In contrast, the maximum accumulation of newly synthesized collagenase depended to some extent on the duration of treatment; cells treated for 12 h with CB synthesized more proCL at 24 and 48 h than did cells treated for only 6 h (Fig. 7). The timing of both the onset and the maximum rate of proCL secretion varied with the inducing agent used. As illustrated in Table IV, CB and TPA produced a rapid induction, whereas the effects of polyHEMA and trypsin were somewhat delayed.

TABLE III
Persistence of Collagenase Secretion after Reversal of Morphologic Change

Time after removal of CB	[^{35}S]Methionine incorporation	
	51 + 53 kd*	57 kd
h	*cpm × 10^3*	*cpm × 10^3*
0	0.3 (0.8)‡	0.2 (0.6)
12	2.4 (14.4)	0.3 (1.9)
24	4.2 (19.9)	1.1 (5.2)
48	4.5 (20.7)	1.0 (4.5)
72	1.8 (13.4)	0.3 (2.2)
144	1.3 (8.8)	0.3 (1.7)

Rabbit fibroblasts plated in 16-mm wells were exposed to 2 μg/ml CB for 12 h and then washed and reincubated in serum-free medium. At the times indicated, cells were incubated with [^{35}S]methionine for 2 h and labeled; secreted proteins were prepared for SDS PAGE as previously described (2).
* These samples were prepared for SDS PAGE using a conventional Laemmli sample buffer containing 5% β-mercaptoethanol. As noted in the accompanying paper (2), this concentration of β-mercaptoethanol did not permit resolution of the 51 kd neutral proteinase and the 53 kd proCL bands. Therefore, they have been grouped together for this analysis.
‡ Numbers in parentheses indicate the percentage of the total labeled protein appearing in each polypeptide band.

TABLE IV
Time of Onset and Maximum Rate of Collagenase Expression after Treatment with Inducing Agents

Agent	Onset	Maximum
	h	*h*
TPA, 100 ng/ml	6	24–48*
CB, 2 μg/ml	12	24–48
PolyHEMA, 100 μg/cm	20	92
Trypsin, 20 μg/ml	12–20	72‡

Rabbit fibroblasts were treated with agents under standard culture conditions, and collagenase synthesis was monitored by measuring activity secreted into the medium or by [^{35}S]methionine incorporation into the 53 and 57 kd polypeptides.
* Data from accompanying paper (2).
‡ Data from reference 52.

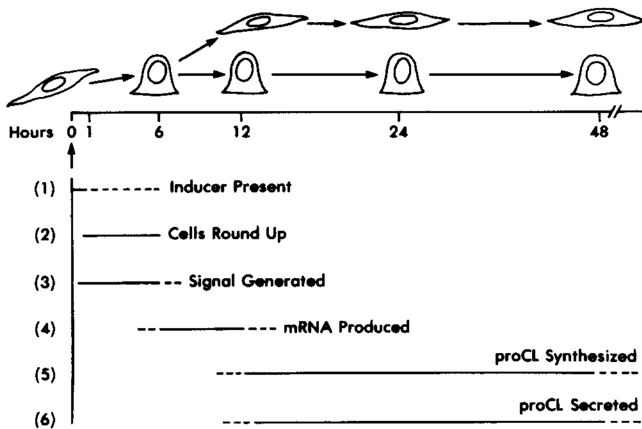

FIGURE 8 Model of collagenase induction by agents that alter cell morphology. (*1*) The inducer must be present for 1-6 h for induction to take place. (*2*) The inducer must cause morphologic changes during this initial 1-6 h. (*3*) An inducing signal, related in some way to alteration of cell shape, is generated. (*4*) Between 6 and 12 h after the inducing signal is generated, translatable collagenase mRNA is produced. (*5* and *6*) Starting at 12-24 h after addition of inducing agent, collagenase is synthesized and secreted. Synthesis and secretion can continue for many days after an initially short period of treatment, and this sustained secretion does not depend on continued morphologic alteration. Arrow at 0 h indicates the time of addition of inducing agents. Dashed lines indicate variability of timing of individual events in cells treated with different agents.

DISCUSSION

The data presented in this and the accompanying paper (2) can be used to define a timetable for the induction of collagenase synthesis and secretion by agents that alter cell morphology. As diagrammed in Figure 8, an agent must be present for several hours for induction to take place, and it must cause alterations in cell morphology during this initial induction period. Because some agents persist in cells, the duration of the induction period is not precisely known. Nevertheless, we can infer that a stable inducing signal, related in some way to morphologic alteration, must be generated within the cells during this period, because subsequent collagenase synthesis and secretion occur even after agents are removed. Likewise, persistence of the inducing signal is not dependent upon continued alteration of cell shape. The biochemical nature of this signal is not known. Because synthesis and secretion of collagenase is not detected until 6-12 h after initial treatment in our cell culture system, we postulate that translatable mRNA for collagenase becomes available during this period. This is consistent with the finding that α-amanitin inhibits the induction of collagenase by urate crystals when added during the first 24 h of treatment (9). For all inducing agents the amount of collagenase secreted depends on the time of treatment and the concentration of agent used. The exact timing of the onset of collagenase secretion, of the maximum rate of collagenase synthesis, and of the duration of synthesis depends to some extent on which agent is used as inducer, but it should be emphasized that all agents that alter cell shape induce collagenase according to the sequence of events outlined here. In view of these observations, it is likely that specific mRNA for collagenase is rapidly induced after the initial change in shape, and that once induced it is relatively stable, disappearing from cells with a half-life of 2-3 d. Experiments to test this directly are under way.

In the studies described in this and the accompanying paper, the agents we used to induce collagenase secretion are unlikely to be encountered by cells in other than an experimental situation. In whole tissues, collagenase synthesis, secretion, and activity are known to be controlled by many interacting factors (31, 51), and this complicates the study of collagenase secretion under physiologic conditions. A number of possible physiologic inducers of collagenase secretion have, nevertheless, been described. Phagocytosis can induce collagenase secretion by fibroblasts and macrophages (54), and stimulation of collagenase by urate crystals (9) may be related to their endocytosis by synovial cells. Although changes in cell shape have not been reported for phagocytic stimuli, the membrane activity and cytoskeletal reorganization that accompany endocytosis (42) may be similar to the changes that accompany collagenase induction by agents that alter cell shape. In addition, it should be noted that phagocytic stimuli induce relatively low amounts of collagenase activity (9, 54). This implies that, if morphologic changes occur in these cells, they are likely to be subtle (score <1) and/or transient. There has been a great deal of interest in the interaction of various tissue cell types in stimulating collagenase secretion. Krane et al. (31) have shown that leukocytes of the monocyte-macrophage lineage produce a monocyte cell factor, interleukin 1 (36), that is a potent inducer of collagenase secretion by human synovial fibroblasts. Interestingly, flattened synovial cells became markedly rounded and dendritic in appearance when treated with partially purified monocyte factor (31), and immunofluorescence studies of rheumatoid synovial cells have indicated that it is the dendritic cells in these cultures that actively secrete collagenase (55, 56). In addition, collagenase induction by monocyte factor is potentiated by treatment of rheumatoid cells with TFP (31). Preliminary experiments in our laboratory indicate that human interleukin 1 also alters cell shape and induces collagenase in rabbit synovial fibroblasts. Johnson-Muller and Gross (26) have described the regulation of corneal stromal fibroblast collagenase production by interaction with corneal epithelium. It is interesting to note that the cells must be treated with CB to effect this response.

Because the biochemical and structural mechanisms controlling alterations in cell shape and gene expression are not known, we have defined these changes empirically. Similar regulation of differentiated function by alterations in cell shape has been described in a number of other cell culture systems. The effects of TPA on differentiation are discussed in the accompanying paper (2). Benya and Shaffer (7) have shown that chondrocytes growing in monolayers and secreting only procollagen type I were induced to synthesize cartilage-specific procollagen type II after being rounded in agar. Emerman et al. (16, 17) have demonstrated that mouse mammary epithelial cells can be cultured on floating collagen gels, allowing them to assume a normal, polarized, cuboidal morphology and to synthesize and secrete milk proteins. In a fascinating experiment, Allan and Harrison (6) constructed a series of cell hybrids between erythroleukemia cells and various lymphoid cell lines to determine which parental genotype was dominant in such chimeras. They found that both genotypes could be expressed in many hybrids, but that the erythroid genotype, demonstrated by induction of hemoglobin synthesis, was expressed only when the cells were cultured in

suspension. Spiegelman and co-workers (45, 46) have determined that 3T3 preadipocytes round up and begin synthesizing lipogenic enzymes during differentiation in culture. Both cell rounding and enzyme induction can be blocked by forcing these cells to spread onto a fibronectin-coated substratum. This effect is, in turn, reversed by addition of cytochalasin D, leading these investigators to conclude that the state of assembly of actin can regulate the activation of specific gene expression during differentiation (45). These examples suggest that induction of differentiated function is brought about by cell rounding per se, but induction of differentiation by cell spreading has also been documented. The human tumor cell line HL60 can be induced to spread in culture by treatment with TPA, and under such conditions these multipotential cells differentiate into macrophages (34). Thus, it appears that cells of different tissue origins possess the ability to sense their proper morphology and to modulate their gene expression accordingly.

Although we have documented a strong correlation between changes in cell shape and induction of collagenase, we do not yet know whether altered cell morphology actually causes this induction or is simply a parallel phenomenon. The identity of a hypothetical second messenger is unknown, especially because the agents that we have found to be effective inducers have a wide variety of pharmacologic effects and few, if any, known common actions other than their ability to alter cell morphology. CB (35) and TPA (44) are known to cause disassembly of actin filament bundles, the former by binding directly to microfilaments (35) and the latter by causing the disappearance of vinculin in adhesion plaques (M. Schliwa, personal communication). Recent studies have documented the presence of binding sites for both actin and calmodulin on nonerythrocyte spectrin (49), suggesting to us that TFP, which binds stoichiometrically to calmodulin, may also interfere with cytoskeletal filament-membrane attachments. In addition, a Ca^{2+}-calmodulin–dependent phosphorylation of vimentin has been described (47), indicating that TFP may be able to alter the interaction of cytoskeleton with the plasma membrane by specific phosphorylations. Activation by TPA of a membrane-associated Ca^{2+}- and phospholipid-dependent protein kinase has recently been described (11, 30). This kinase copurifies with the TPA receptor (39). In one of the best-documented examples of transmembrane signaling, the EGF receptor has been shown to be a protein kinase (14), but in this case phosphorylation of cytoplasmic target proteins is not sufficient to induce DNA synthesis, and the nature of the second messenger that communicates with the cell nucleus is unknown. These results suggest that such an intermediate might be produced in cells without prior morphologic change, but no agent that has this effect has yet been identified.

Whatever the nature of the second messenger that ultimately reaches the nucleus, the observations presented in this and the accompanying paper indicate that connective tissue cells possess the ability to sense their surroundings and to respond specifically to external changes. The nature of the cells' contact with the extracellular matrix is still being defined (15, 25), but our data constitute a clear example in which cells, by altering their genetic program in a specific and predictable way, can interact with and modify specific matrix components (8). Rabbit synovial fibroblasts treated with agents that alter cell morphology express a new phenotype, characterized by a decrease in synthesis of a number of secreted proteins, including the structural macromolecules, collagen and fibronectin, and by an increase in synthesis of the degradative enzymes, collagenase and neutral proteinase. Such responses may effect morphogenetic changes during embryonic development, remodeling during tissue repair, and tissue damage in many pathologic processes.

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