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Journal

Journal of Cell Biology, 103(3)

ISSN

0021-9525

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Publication Date

1986-09-01

DOI

10.1083/jcb.103.3.1021

Peer reviewed

Reorganization of Polymerized Actin: A Possible Trigger for Induction of Procollagenase in Fibroblasts Cultured in and on Collagen Gels

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Abstract. Changes in cell shape are postulated to modulate gene expression during differentiation of a number of cell types, including rabbit synovial fibroblasts, which are inducible for expression of the zymogen form of the metalloendopeptidase, collagenase. In the work presented here, fibroblasts cultured on and within hydrated collagen gels were allowed to contract by release of the gels from the sides of the culture dish. Within 24 h of cell release, synthesis and secretion of procollagenase was initiated in the absence of any chemical manipulation. Fibroblasts grown in and on collagen also responded to 12-O-tetradecanoylphorbol-13-acetate and cytochalasin B with morphologic change and induced procollagenase. However, colchicine, which altered morphology to varying degrees in cells on plastic, on collagen, and within collagen gels, did not induce procollagenase expression. In all cases, the enzyme was induced only after reorganization of polymerized actin, rather than after a change in cellular morphology per se. As a first approach to identifying other aspects of the stimulated phenotype that could affect collagen turnover, the expression of collagen and endogenous metalloproteinase inhibitors in relation to procollagenase secretion was investigated. Collagen secretion by fibroblasts decreased when procollagenase secretion was induced by the pharmacologic agents, but not when cells were stimulated by contraction on or within collagen gels. The expression of two endogenous inhibitors was not coordinately regulated with induction of procollagenase. Therefore, the extracellular matrix and the cellular actin cytoskeleton may transduce signals that modulate the tissue remodeling phenotype of fibroblasts.

During development and growth, the interaction of cells with the extracellular matrix changes continuously, and the matrix is remodeled as tissue structure is altered. In a reciprocal way, the matrix influences cellular behavior (Bissell et al., 1982). The major components of the matrix are collagens; under physiologic conditions these are resistant to proteolytic attack except by a specific metalloendopeptidase, collagenase, which is produced by a number of cell types, including fibroblasts (Werb and Burleigh, 1974; Shinkai and Nagai, 1977; Woessner, 1979; Heath et al., 1984). Positive or negative control of collagenase activity in the appropriate loci is essential for normal tissue development, function, and architecture, and its abnormal expression may contribute to disease processes such as tumor metastasis and rheumatoid arthritis. Yet, regulatory mechanisms involved in the synthesis, activation, and inhibition of collagenase are not well understood.

A differentiated phenotype characterized by expression of the zymogen form of collagenase, procollagenase (proCL),[1] is inducible in rabbit synovial fibroblasts (RSFs) (Werb and Burleigh, 1974; Aggeler et al., 1984*a*). A number of pharmacologic agents, including 12-O-tetradecanoylphorbol- 13-acetate (TPA) and cytochalasin B (CB), cause this induction, which is also marked by synthesis of other new polypeptides, including the metalloproteinase, stromelysin (Chin et al., 1985; Werb et al., 1986). Because induction of procollagenase expression is strongly correlated with a change in cell morphology, this shape change has been hypothesized to play a role in the switch in cell phenotype, as it has been in a number of other systems, including chondrogenesis (Benya and Shaffer, 1982; Zanetti and Solursh, 1984) and adipogenesis (Spiegelman and Farmer, 1982; Spiegelman and Ginty, 1983).

The connective tissue matrix and the cytoskeleton are mutually organizing and important in maintenance of cellular morphology and differentiated function. Allowing cells to grow on or in biological matrices is frequently permissive for, or causes, dramatic changes in the phenotype of a number of cell types (Bissell et al., 1982). In the present study, we took advantage of the fact that RSFs can be grown on and within collagen gels to investigate induction of proCL expression under these conditions. By manipulating RSFs un-

1. *Abbreviations used in this paper*: CB, cytochalasin B; DME, Dulbecco's modified Eagle's medium; proCL, procollagenase; RSF, rabbit synovial fibroblast; TIMP, tissue inhibitor of metalloproteinases; TPA, 12-O-tetradecanoylphorbol-13-acetate.

der these culture conditions, we found an inducing stimulus that may be relevant to events occurring during morphogenesis (Stopak and Harris, 1982). Furthermore, we separated the influences of cell shape, reorganization of actin microfilaments, and extracellular matrix on the secretion of procollagenase, of its substrate, collagen, and of its endogenous inhibitors.

Materials and Methods

Cell Culture

RSF between passages 1 and 6 were cultured in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum. Cells (2×10^5) were plated in 16-mm wells (Costar, Cambridge, MA) for 24 h in this medium before washing and replacement with serum-free DME supplemented with 0.2% lactalbumin hydrolysate for experiments. Cells were seeded directly onto plastic or onto type I collagen gels or embedded within type I collagen (Vitrogen; Collagen Corp., Palo Alto, CA) in a 8:1:1 mix with 10× Hanks' balanced salt solution and 0.2 N NaOH. 24 h after cells were seeded on collagen, half of the collagen cultures were released by rimming the gel away from the sides of the dish with a small spatula. Cells (2×10^5) were embedded within 0.5 ml of collagen by diluting a 10× suspension of cells 1:10 in the Vitrogen mixture and plating it into 16-mm wells prelayered with a thin film of hydrated collagen. This collagen film prevented any cells from settling onto plastic during polymerization of the gel. The polymerized gels were overlaid with DME supplemented with 10% fetal bovine serum.

Drugs

TPA (Sigma Chemical Co., St. Louis, MO) was dissolved at 2 mg/ml in ethanol and administered at 50–100 ng/ml. CB (Sigma Chemical Co.) was dissolved at 1 mg/ml in dimethyl sulfoxide and used at a concentration of 4 μg/ml. Colchicine was used at 1 μM. Stock solutions were stored at −20°C until use. Duration of treatment ranged from 24–72 h. Ascorbate (25 μg/ml) (Sigma Chemical Co.) and β-aminoproprionitrile (80 μg/ml) (Sigma Chemical Co.) were made up immediately before experiments.

Collagenase Assay

Collagenase activity was measured by means of a [^{14}C]collagen fibril assay (Werb and Burleigh, 1974; Aggeler et al., 1984*a*). Conditioned medium was collected at various time points after drug treatments or after release of collagen gels. Release of the gels increased the volume of medium by ~10% and therefore did not change enzyme activity levels dramatically. All medium samples were activated with L-1-tosylamide-2-phenylethylchloromethyl ketone trypsin (10 μg/ml) for 30 min at 25°C. 1 U of collagenolytic activity hydrolyzed 1 μg of collagen per min at 37°C.

Biosynthetic Labeling of Secreted Proteins

Cells were biosynthetically labeled with 25 μCi/ml of [^{35}S]methionine (sp act, 1265 Ci/mmol) at a number of time points before and after collagen gel release or drug treatments. Cells on plastic or on collagen were labeled in methionine-free DME for 2–4 h at 37°C (Aggeler et al., 1984*a*). 48 h after the start of drug treatments, cells embedded within collagen gels were continuously labeled for 18 h. In experiments assaying for collagen secretion, ascorbate and β-aminoproprionitrile were added to the labeling mixture. Proteins were precipitated from the medium with quinine sulfate–SDS (Werb et al., 1986*a*), washed, and resuspended in Laemmli sample buffer containing 5% β-mercaptoethanol (Laemmli, 1970), and then boiled.

SDS PAGE

SDS PAGE was performed according to the method of Laemmli (1970) on discontinuous gradient gels (7–15%) or on 10% gels with a 3% stacking gel under reducing conditions. Molecular mass standards used were lactoglobulin (18 kD), carbonic anhydrase (30 kD), immunoglobulins (25 and 50 kD), bovine serum albumin (68 kD), phosphorylase B (93 kD), and myosin (200 kD). Gels were dried and autoradiographed with Kodak X-Omat AR X-ray film.

ProCL was immunoprecipitated from [^{35}S]methionine-labeled secreted proteins with 10 μg of sheep monospecific anti-collagenase IgG (Aggeler et al., 1984*a*; Chin et al., 1985; Werb et al., 1986*a*) using formalin-fixed *Staphylococcus aureus* (Zysorbin; Zymed Laboratories, San Francisco, CA) by the method of Jones (1980). Normal sheep IgG (20 μg) replaced the immune IgG as a negative control in parallel experiments. Antibody was incubated with *S. aureus*–preabsorbed secreted proteins for 30 min at 37°C. The precipitated immune complexes were washed, resuspended in Laemmli sample buffer, and centrifuged, and the supernatants were analyzed by SDS PAGE. Gels were dried and autoradiographed.

To assay for secretion of collagenous proteins, we incubated one half of the conditioned medium from [^{35}S]methionine-labeled cells with 100 U/ml of purified bacterial collagenase (Advanced Biofactures Corp., Lynbrook, NY), the other half with buffer only, for 2–3 h at 37°C. Proteins from the conditioned medium were then precipitated with quinine sulfate–SDS, resuspended in Laemmli sample buffer, and electrophoresed on a 7% acrylamide gel under reducing conditions. Total cpm in samples from treated cells were comparable to or lower than cpm in medium from control cells. To quantify the percent collagen (collagenase-sensitive bands) and the percent proCL, the autoradiographs were scanned densitometrically.

Detection of Proteinase Inhibitors

Electrophoresis in a 10% acrylamide gel impregnated with 1 mg/ml type I gelatin was used to demonstrate the presence of a tissue inhibitor of metalloproteinases (TIMP) in a modification (Herron et al., 1986*a*) of the substrate gel method described previously (Heussen and Dowdle, 1980; McKerrow et al., 1985). Unconcentrated conditioned culture medium was mixed with Laemmli sample buffer (lacking β-mercaptoethanol and modified to contain a final concentration of 2.5% SDS) and applied, without boiling, to an SDS-polyacrylamide Mini-Slab gel (Idea Scientific, Corvallis, OR) containing 1% gelatin, then electrophoresed under nonreducing conditions. SDS was eluted from the gel in 2% Triton X-100 for 30 min at 37°C before immersion into 4-aminophenylmercuric acetate–activated culture medium from TPA-treated fibroblasts for 1 h at 37°C with shaking. The gels were then rinsed and incubated overnight at 37°C in substrate buffer (50 mM Tris-HCl buffer, pH 8, 5 mM $CaCl_2$). After staining with Coomassie Blue R250, gelatin-degrading enzymes present in the conditioned medium were identified by their ability to clear the substrate at their respective molecular weights. Inhibitors of gelatin-degrading proteinases were visualized as dark blue bands against a paler blue background. Conditioned culture medium from rabbit brain capillary endothelial cells, which secrete large amounts of these inhibitors, and partially purified TIMP (Herron et al., 1986*a*, *b*) were used as standards.

Cytochemistry

Cells were cultured for 24–48 h in 16-mm plastic wells or on collagen gels. Cells embedded within collagen were examined by preparing thin layers of gels containing cells on Vitrogen-coated coverslips. Cells were fixed in 2.5% paraformaldehyde for 20 min at 25°C. Actin filaments were stained with 1-nitrobenz-2-oxa-1,3-diazolyl-phallicidin (Molecular Probes, Inc., Junction City, OR) at a concentration of 20 U/ml.

For microtubule staining, cells were fixed with methanol at −20°C and stained for indirect immunofluorescence with mouse monoclonal anti-alpha tubulin (Amersham Corp., Arlington Heights, IL) followed by rhodamine-labeled anti-mouse Ig (Cappel Laboratories, Cochranville, PA).

For procollagenase localization, cells were fixed in 2% paraformaldehyde for 10 min, made permeable with 0.1% Triton X-100 for 10 min, and incubated with the IgG fraction of a monospecific sheep anti-collagenase antiserum for 40 min (Hembry et al., 1986; Murphy et al., 1985; Werb et al., 1986*b*). This was followed by staining with fluorescein-labeled Fab fragment of pig anti-sheep Fab second reagent (gift of R. Hembry, Strangeways Research Laboratory, Cambridge, England) for 40 min. Cells were examined and photographed on a Zeiss Photomicroscope III fitted for epifluorescence.

Results

TPA, CB, and Inherent Tonic Contraction Induce Procollagenase Expression in RSFs

RSFs grown on collagen gels were generally elongated and slightly more refractile than their counterparts grown on plastic (Fig. 1, *a* and *b*). Within 6 h of TPA or CB administra-

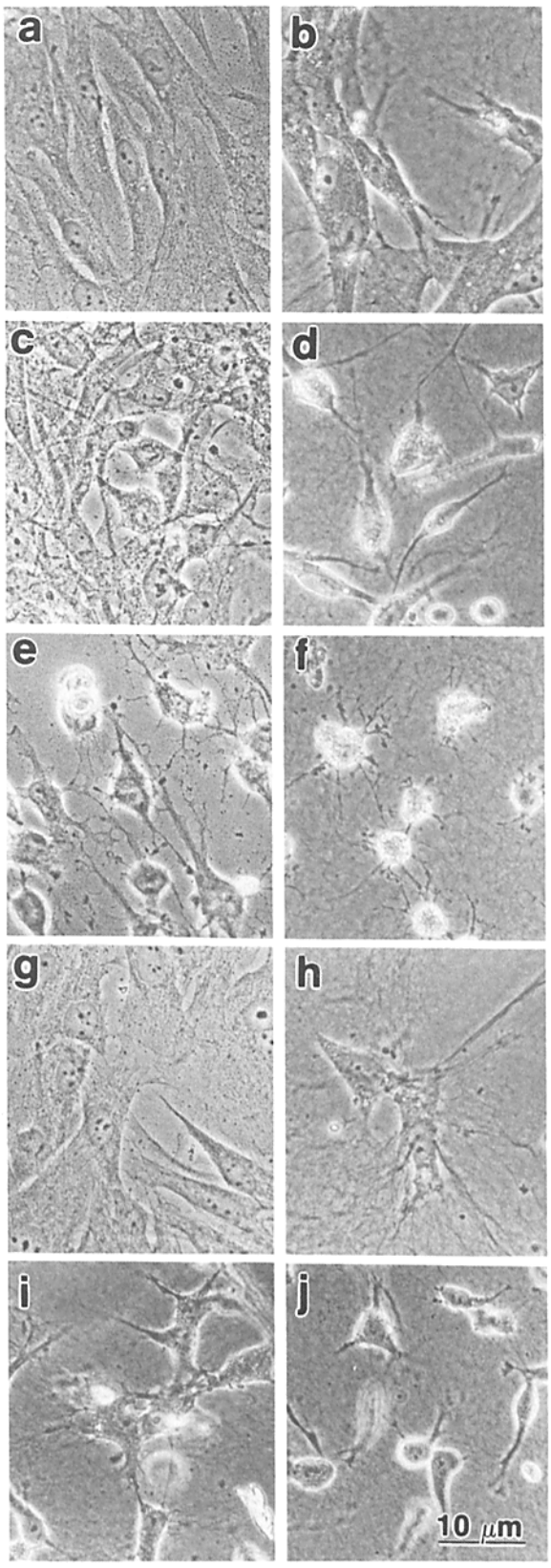


Figure 1. Morphologic effects of TPA, CB, and colchicine on RSFs grown on plastic and collagen, and morphologic effects of gel release on RSFs grown on collagen. Phase-contrast micrographs taken 24 h after no treatment (*a* and *b*) or treatment with TPA (*c* and *d*), CB (*e* and *f*), or colchicine (*g* and *h*) of RSFs cultured on plastic (*a*, *c*, *e*, and *g*) or on collagen (*b*, *d*, *f*, and *h*) show shape changes caused by TPA or CB but not by colchicine. These morphologic changes were evident within 6 h of drug administration and persisted throughout the study period. Release of the collagen gel substrate caused cell rounding at 1 h (*i*) and 4 h (*j*) compared to RSFs on unreleased gels (*b*).

tion, a change in morphology from flat to rounded was evident in cells grown on both substrates (Fig. 1, *c–f*). Colchicine, in contrast, did not cause a morphologic change in cells grown on plastic (Fig. 1 *g*) but did cause some aggregation and reduction in diameter in cells grown on collagen (Fig. 1 *h*). Release of the collagen gel substrate also resulted in fibroblast rounding. Rimming the collagen gel 24 h after plating 2×10^5 cells resulted in rapid contraction of the hydrated lattice so that it had shrunk to 20% of its original volume 8 h later. The decrease in gel area was reflected in a change in RSF shape from flat to rounded (Fig. 1, *i* and *j*). RSFs did not enter the gel with significant frequency either before or after treatment.

48 h after the start of the treatments, the profiles of proteins secreted by cells cultured on plastic or collagen were analyzed after biosynthetic labeling with [^{35}S]methionine. Two polypeptides at 53 kD and 57 kD were induced by TPA or CB treatments and immunoprecipitation with specific anti-collagenase antibody showed them to be proCL (Fig. 2).

ProCL was also expressed by RSFs after gel release in the absence of any chemical manipulation. Immunoprecipitable proCL secreted by RSF was detected by 24 h and continued up to 72 h after gel contraction, as demonstrated by biosynthetic pulse-labeling of the cells (Fig. 3, *A* and *B*). Little or no protein was immunoprecipitated from medium from unreleased cultures. In agreement with this time course, proCL measured as trypsin-activatable enzyme in the fibril assay was first detected in culture medium 24 h after gel release (Fig. 3 *C*). As enzyme continued to be secreted, activity accumulating in the medium increased to 4.2 U/10^6 cells by 48 h. Little or no proCL was detected in unreleased cultures. By indirect immunofluorescence, specific anti-collagenase immunoglobulin localized proCL in the Golgi region of cells on released but not unreleased cultures (Fig. 4).

RSFs that were induced for proCL secretion by TPA or CB had markedly altered patterns of filamentous actin visualized by staining with 1-nitrobenz-2-oxa-1,3-diazolyl–phallicidin, whereas untreated RSF or those treated with colchicine for 24 h had prominent arrays of actin fibers (Fig. 5, *a–d*). In contrast, the fibrillar nature of microtubules was not altered by the inducing stimuli, but staining was predictably diffuse in colchicine-treated RSFs (Fig. 5, *e–h*).

RSFs induced for proCL expression by gel contraction also showed alterations in polymerized actin. Within 1 h of gel release, stress fibers became attenuated in length and girth, concomitant with the formation of actin aggregates, which were often seen in register with remaining fibers (Fig. 6). Residual filaments were often visualized in arborized configuration in lamellipodia of the contracting cells. At 4 h, actin was predominantly organized in a cortical sheath punctuated by aggregates. Cell contraction did not alter the filamentous nature of microtubules (data not shown).

Actin Reorganization, Not Cell Shape, Is Correlated with ProCl Induction

RSFs were cultured within a hydrated collagen lattice at den-

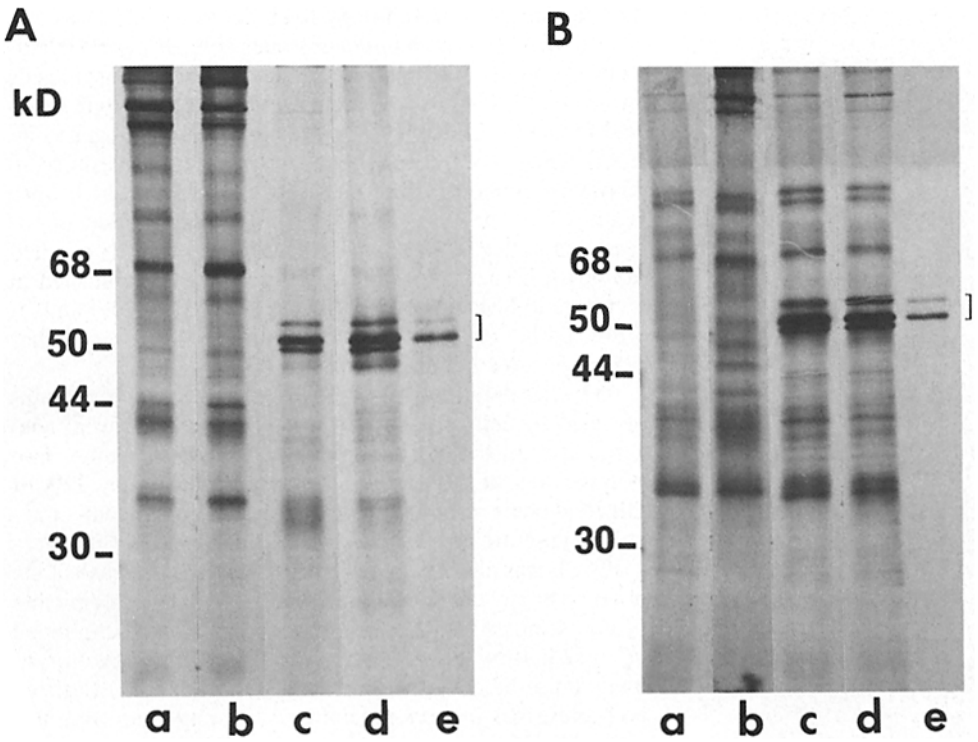


Figure 2. Induction of proCL secretion by CB or TPA in RSFs cultured on plastic (*A*) or on collagen (*B*). RSFs cultured on either substrate were treated for 48 h with no agents (lanes *a*), colchicine (lanes *b*), CB (lanes *c*), or TPA (lanes *d*). RSFs were biosynthetically labeled with [^{35}S]methionine for 2 h. Labeled secreted proteins were precipitated with quinine sulfate–SDS, resuspended in Laemmli sample buffer, and boiled. Equal amounts of radioactivity were loaded in each lane. The 53/57-kD proCL doublet (bracket) is evident in TPA- and CB-treated cells. Molecular mass markers ($\times 10^{-3}$) are indicated at the left.

sities low enough not to cause spontaneous release and contraction of the gel. RSFs allowed to spread within the collagen gel for 24 h displayed a three-dimensional configuration ranging from bipolar to polygonal. Treatment with colchicine, TPA, or CB radically distorted the shape of cells grown within a collagen gel (Fig. 7, *a–d*), in contrast to cells grown on plastic or on collagen. Exposure to colchicine usually resulted in a more spherical configuration than exposure to either TPA or CB, which often left cells dendritic.

Untreated RSFs embedded within collagen had few stress fibers. Instead, filamentous actin was found predominantly in their cortical regions (Fig. 8 *a*). In cells rounded by TPA or CB, the actin sheath collapsed into peripheral aggregates (Fig. 8, *b* and *c*). Similar changes in actin were caused by contraction of cells within the gel (data not shown). In contrast, colchicine, which also rounded the cells, did not alter the smooth, cortical distribution of actin, which was modified only to accommodate the change in cell shape (Fig. 8 *d*). Linear arrays of microtubules were found in untreated cells (Fig. 8 *e*) and in cells induced by TPA or CB (Fig. 8, *f* and *g*). Staining of colchicine-treated cells was diffuse (Fig. 8 *h*).

By 48 h after administration of TPA or CB, but not colchicine, RSFs secreted immunoprecipitable proCL into their medium, as demonstrated by biosynthetic labeling with [^{35}S]methionine (Fig. 9). Induction of proCL in these cells was confirmed by detection of activatable collagenase in culture media using the fibril assay. Activity in conditioned medium 72 h after treatment measured 1.6 U/10^6 cells after TPA, 1.9 U/10^6 cells after CB treatment, and <0.1 U/10^6 cells after colchicine addition or in untreated cultures.

Table I. Summary of ProCL Induction and Structural Changes in RSF under Three Culture Conditions

Culture condition	Treatment	Cell shape change	Actin reorganization	ProCL induction
On plastic	None	–	–	–
	TPA	+	+	+
	CB	+	+	+
	Colchicine	–	–	–
On collagen	None	–	–	–
	Release	+	+	+
	TPA	+	+	+
	CB	+	+	+
	Colchicine	–	–	–
In collagen	None	–	–	–
	Release	+	+	+
	TPA	+	+	+
	CB	+	+	+
	Colchicine	+	–	–

Table II. Secretion of ProCL and Collagen by RSF

Culture condition	Treatment	ProCL	Collagen
		%	%
On plastic	None	0	23.5
	TPA	17.3	8.5
On collagen	None	0	27.3
	Release	7.3	28.0
	TPA	13.8	5.1
In collagen	None	0	16.3
	Release	10.5	13.5
	TPA	17.2	0
	CB	13.9	0
	Colchicine	0	14.0

Samples were prepared and analyzed as described in Materials and Methods. Percentages were obtained by scanning densitometry of the gels. The percentage of total secreted protein represented by proCL and collagen are tabulated.

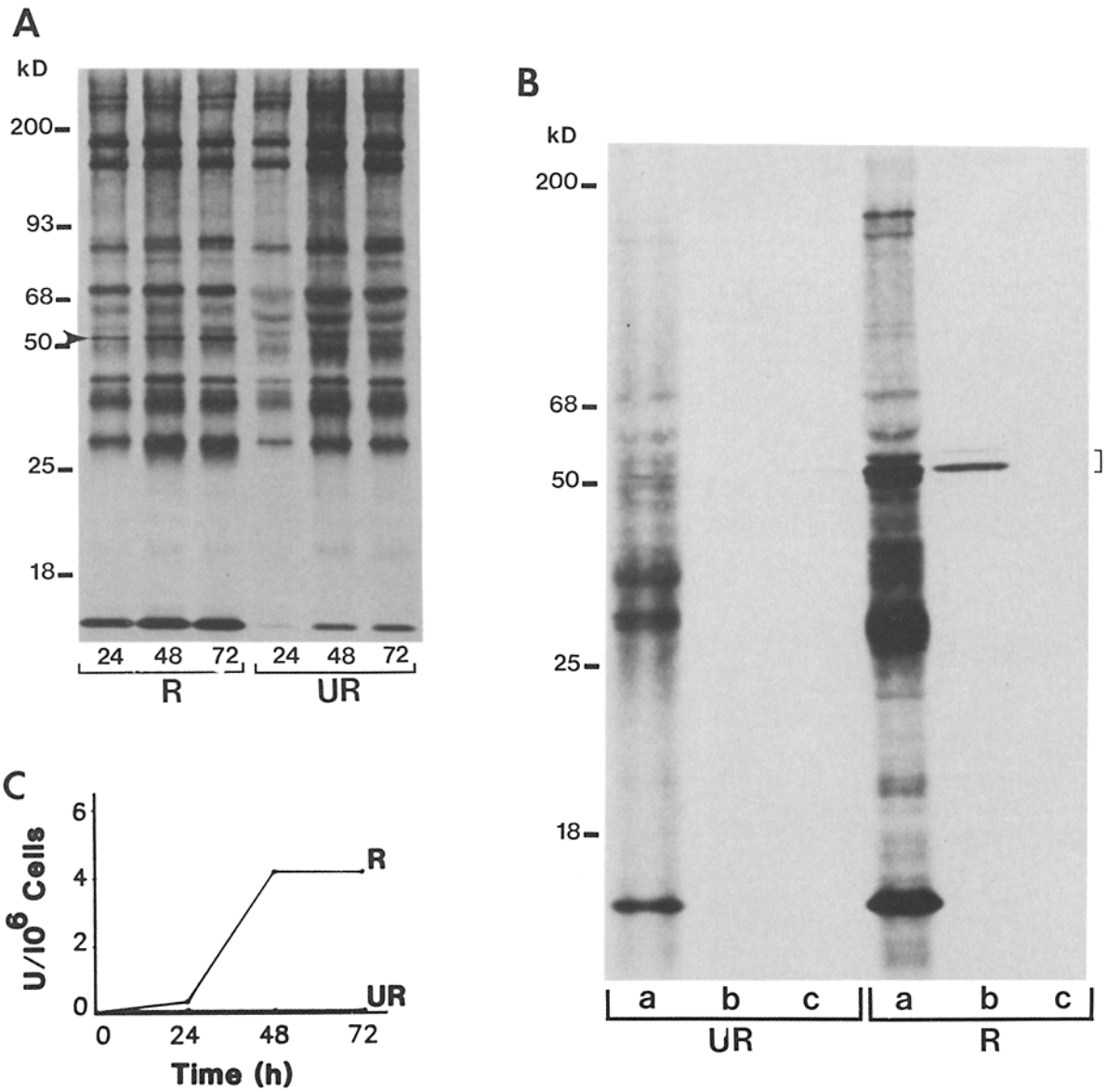


Figure 3. Detection of proCL in medium from released collagen cultures. (*A*) Rate of proCL secretion was determined from pulse labeling studies with [^{35}S]methionine. The 53-kD proCL band (*arrow*) was detected at 24 h, 48 h, and 72 h in released (*R*) but not unreleased (*UR*) cultures. (*B*) After immunoprecipitation of proCL bands by specific anti-collagenase IgG, total quinine sulfate–SDS-precipitated secreted proteins from released (*R*) cultures included the 53/57-kD proCL doublet (bracket) (lanes *a*), which was immunoprecipitable with a monospecific anti-collagenase antibody (lanes *b*). Equal volumes of culture medium from unreleased (*UR*) cultures failed to show significant amounts of the doublet either before (lanes *a*) or after (*R*, lane *c*) specific immunoprecipitation. Nonimmune sheep IgG failed to precipitate any proteins from the medium of either released (*R*, lane *c*) or unreleased (*UR*, lane *b*) cultures. Molecular mass markers ($\times 10^{-3}$) are indicated at the left. (*C*) Accumulation of proCL in the medium of released but not unreleased cultures was detected in the [^{14}C]collagen fibril assay. Activity was detectable at 24 h and increased to 4.2 U/10^6 cells by 48 h.

Treated and control cells were observed by immunofluorescence with anti-collagenase IgG (Fig. 7, *e–h*). More than 80% of TPA- and CB-treated cells embedded within the gel contained intracellular proCL concentrated in their perinuclear Golgi areas (Fig. 7, *f* and *g*). Control RSFs and those treated with colchicine had no detectable intracellular proCL, indicating an absence of synthesis rather than failure to secrete the enzyme (Fig. 7, *e* and *h*).

These results and those of the previous section are summarized in Table I. Cell shape change and actin microfilament organization are dissociable when RSFs are cultured within collagen gels and treated with colchicine. ProCL is induced only when microfilament organization is altered.

Secretion of Procollagenase, Collagens, and Metalloproteinase Inhibitors Is Not Coordinately Regulated

The relationship of proCL secretion to soluble collagen secretion in induced and control RSFs was next examined. Procollagens, identified by SDS PAGE as [^{35}S]methionine-labeled bands susceptible to bacterial collagenase digestion, were secreted by control cells on plastic, on collagen, and

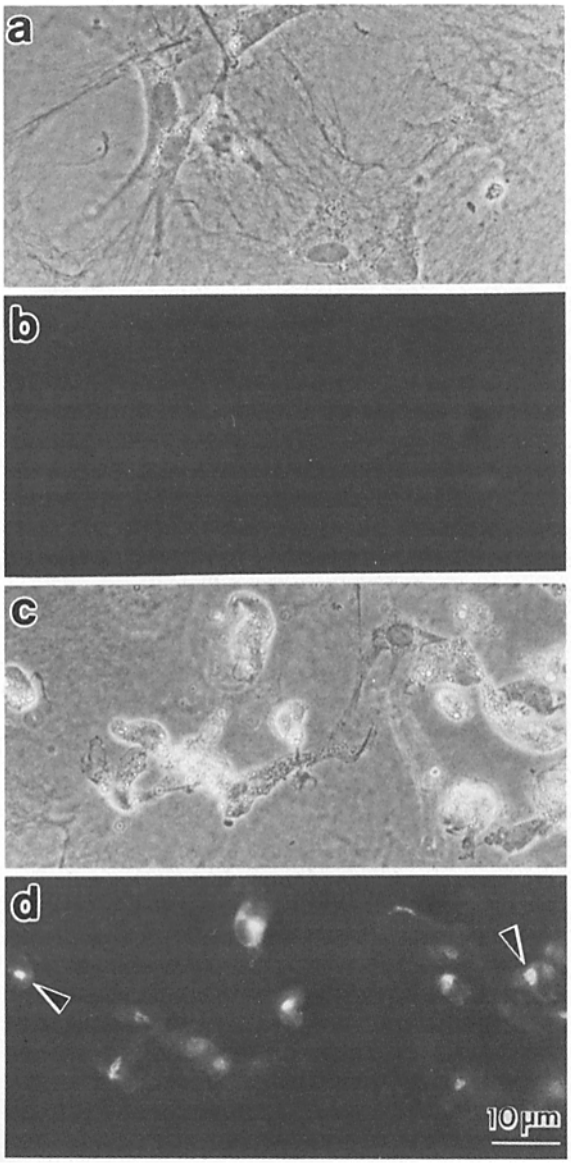


Figure 4. Immunofluorescent localization of proCL in RSFs on released collagen cultures. RSFs on unreleased (*a* and *b*) and released (*c* and *d*) collagen gels at 48 h were stained for indirect immunofluorescence with a monospecific antibody to collagenase. Cells on released (*d*) but not unreleased (*b*) cultures stained positively for proCL in the perinuclear Golgi areas (*arrowheads*). The same fields as seen by phase-contrast microscopy are shown in *a* and *c*.

within collagen (Table II). The secretion of collagen as a percentage of total secreted protein was slightly less in RSFs grown in collagen than in RSFs grown on plastic or on collagen. Stimulation of RSFs by TPA produced a decrease in collagen secretion when the cells were grown on plastic, on collagen, or within collagen gels. However, a decrease in collagen secretion was not a universal correlate of proCL induction. Release of collagen cultures resulted in proCL secretion but did not significantly alter the secretion of collagen. Total protein secreted by treated RSF was comparable

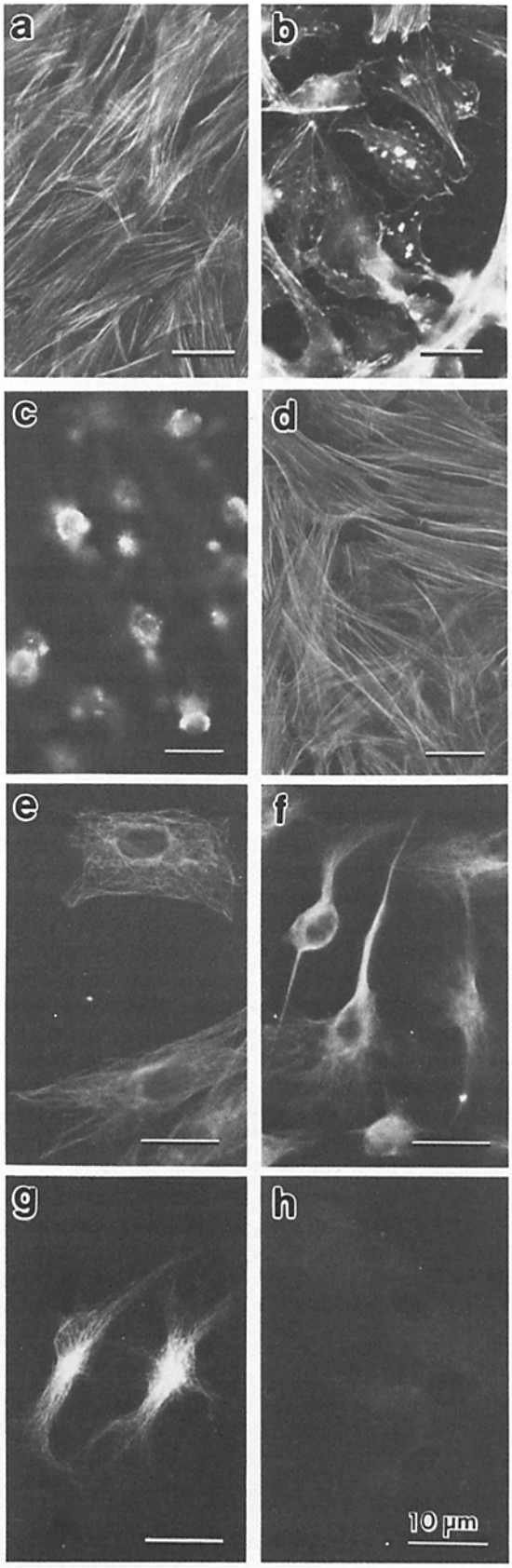
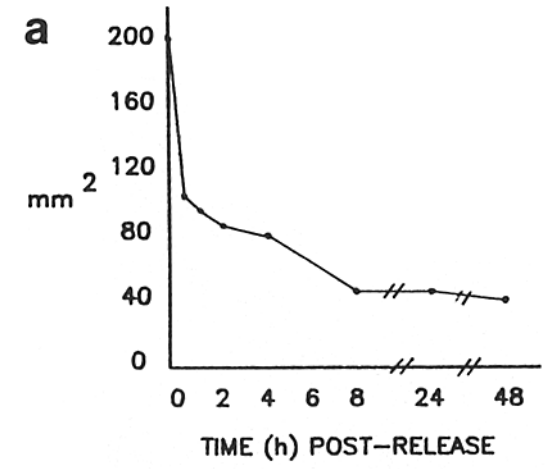


Figure 5. Actin microfilament and tubulin organization in RSFs cultured on plastic. RSFs plated on plastic for 24 h were treated for 24 h with no agents (*a* and *e*), TPA (*b* and *f*), CB (*c* and *g*), or colchicine (*d* and *h*) and then stained for microfilaments (*a–d*) or tubulin (*e–h*). Actin microfilaments were replaced by aggregates in TPA- and CB-induced RSFs. Microtubules retained their fibrillar form in induced cells, whereas staining was diffuse in colchicine-treated RSFs.



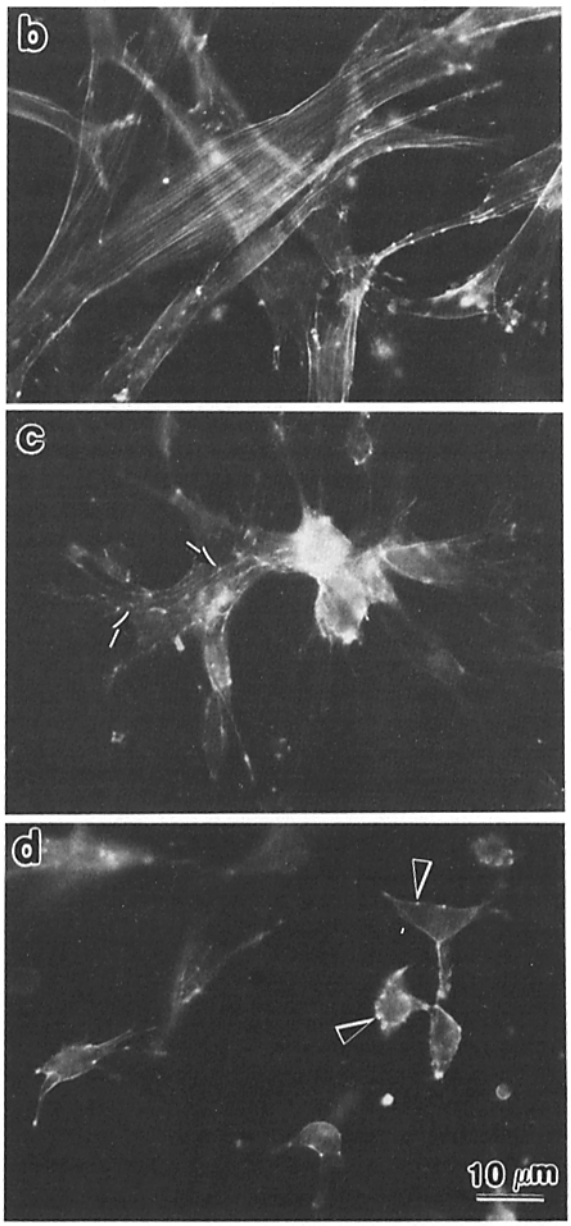


Figure 6. Actin microfilament organization during cell contraction in RSFs cultured on collagen. RSFs contracted the gel to 20% of its original surface area within 8 h of release (*a*). RSFs allowed to contract for 0 (*b*), 1 (*c*), or 4 (*d*) h were fixed and stained for actin microfilaments. At 1 h, aggregates of actin were present (*arrows*), often in register with the remaining stress fibers. By 4 h, microfilaments were predominantly cortical, punctuated by cortical foci (*arrowheads*).

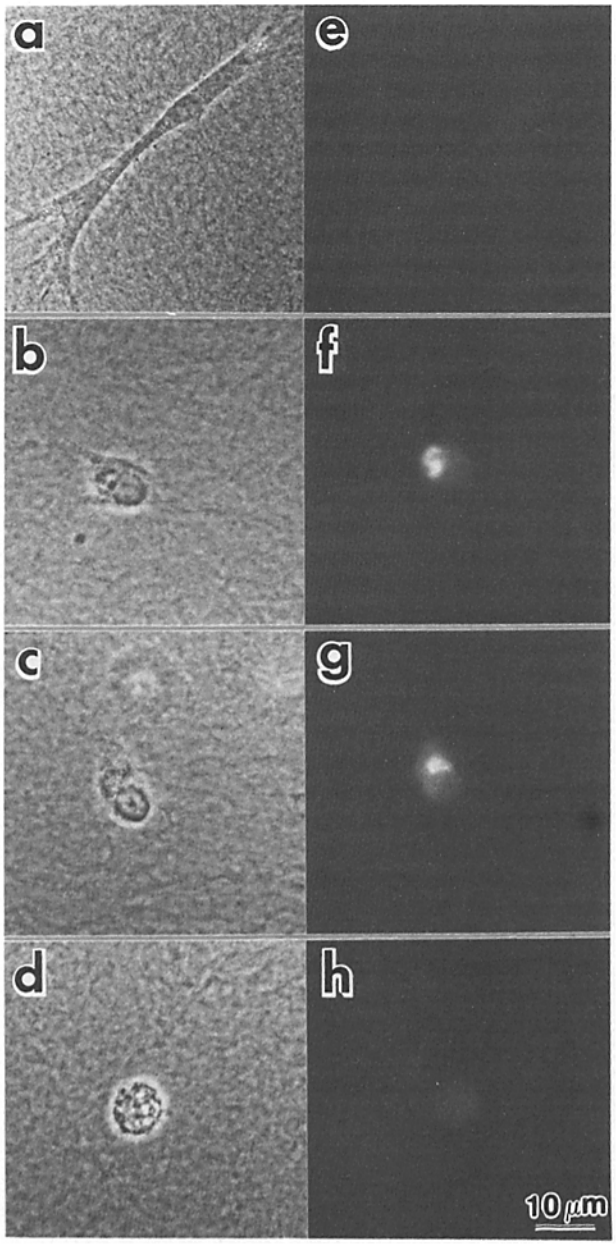


Figure 7. Immunofluorescent localization of proCL in TPA- and CB-treated RSFs embedded within collagen. RSFs grown within collagen gels for 24 h were treated for 48 h with no agents (*a* and *e*), TPA (*b* and *f*), CB (*c* and *g*), or colchicine (*d* and *h*) before fixation and indirect immunofluorescence staining with a monospecific antibody for collagenase (*e–h*). The cells as seen by phase-contrast microscopy are shown in *a–d*. Positive staining is seen in cells treated with TPA or CB but not colchicine.

to or lower than that secreted by control RSF, so that any decreases in the percentage of collagen secreted reflected decreases in the absolute amounts of collagen secreted.

In view of the dissociation in the regulation of proCL and collagen expression in RSF, the relationship between production of proCL and metalloproteinase inhibitor was also

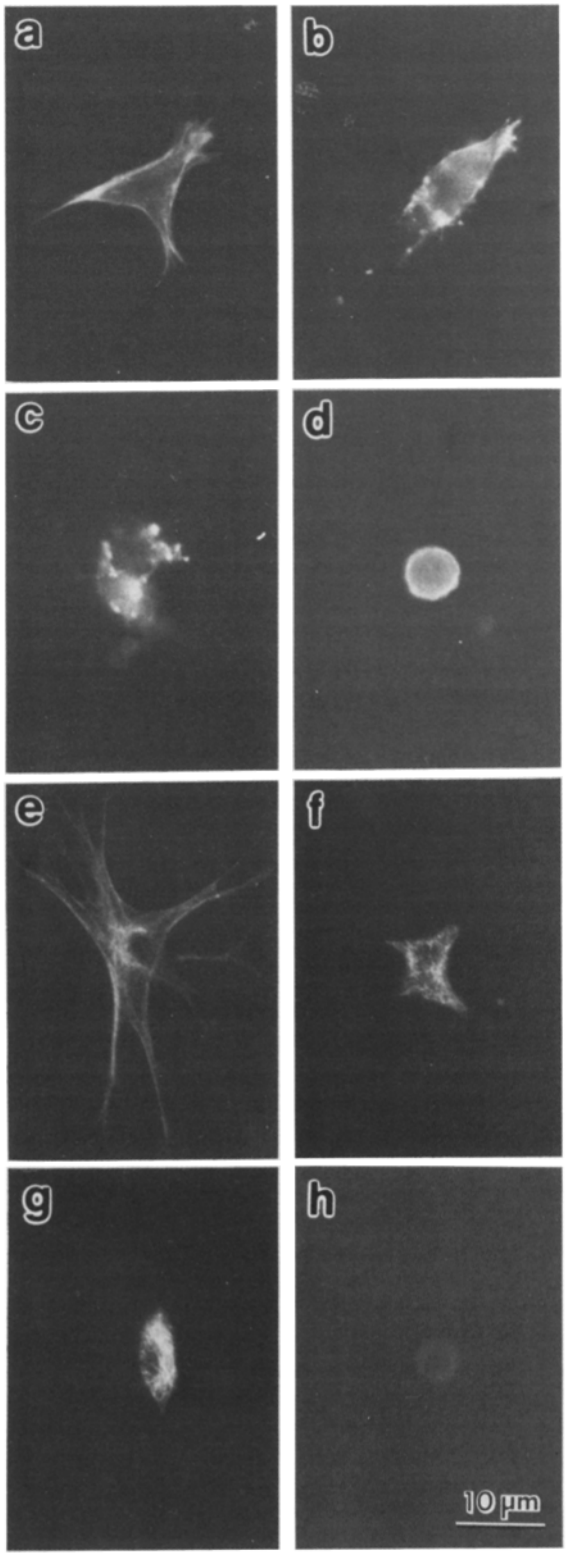


Figure 8. Actin microfilament and tubulin organization in RSFs embedded within collagen. RSFs grown within collagen gels for 24 h were treated for 24 h with no agents (*a* and *e*), TPA (*b* and *f*), CB (*c* and *g*), or colchicine (*d* and *h*). RSFs fixed and stained with 1-nitrobenz-2-oxa-1,3-diazolyl-phallicidin (*a–d*) demonstrated punctate, cortical microfilament staining after TPA or CB but not after colchicine treatment. Fibrillar staining of microtubules, identified by staining with mouse monoclonal anti-α-tubulin (*e–h*), in contrast, was not changed by TPA or CB but was diminished by colchicine.

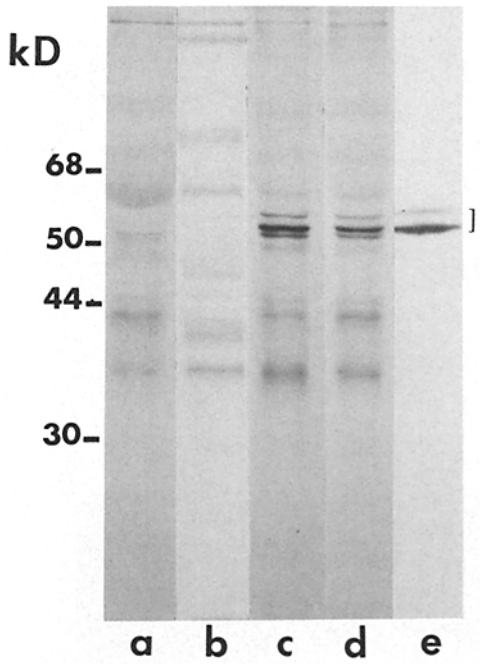


Figure 9. Secretion of proCL by TPA- and CB-treated RSFs embedded within collagen. RSFs grown within collagen gels for 24 h were treated for 48 h with no agents (lane *a*), colchicine (lane *b*), CB (lane *c*), or TPA (lane *d*) before being continuously labeled with [^{35}S]methionine. Labeled proteins were precipitated with quinine sulfate–SDS, resuspended in Laemmli sample buffer, and boiled. Equal amounts of radioactivity were loaded in each lane. TPA- and CB-treated cells secreted the 53/57-kD proCL doublet (*bracket*), which was immunoprecipitable with anti-collagenase antibody (lane *e*). Molecular mass markers ($\times 10^{-3}$) are indicated at the left.

examined. Metalloproteinase inhibitors were identified after separation on an SDS polyacrylamide gel impregnated with gelatin by soaking the gel in medium containing metalloproteinases. The inhibitors were observed as darkly staining bands resolving between 19 and 30 kD. Under all conditions tested, RSFs secreted two of the three inhibitors found in medium conditioned by rabbit brain capillary endothelial cells (Herron et al., 1986 *a* and *b*), which was used as a standard (Fig. 10). The band at 25 kD was identified as TIMP by co-migration with partially purified TIMP. Treatment of RSFs cultured on plastic, on collagen, and in collagen with TPA increased the intensity of the TIMP band but not of the 19-kD polypeptide. CB treatment slightly decreased secretion of TIMP. No other inducing or noninducing regimen altered either inhibitor. It is interesting to note that several bands of gelatinolytic activity were also seen on substrate gels, and some of them were also increased by inducing stimuli. This indicates that stimulated RSFs secrete, in addition to proCL, a family of proteinases, which may include gelatinases that have been described previously (Hibbs et al., 1985).

Discussion

Disruption of Actin Cytoskeleton Precedes ProCL Induction

Our data indicate that secretion of proCL was induced in cultured RSFs only by treatments that modified cellular actin

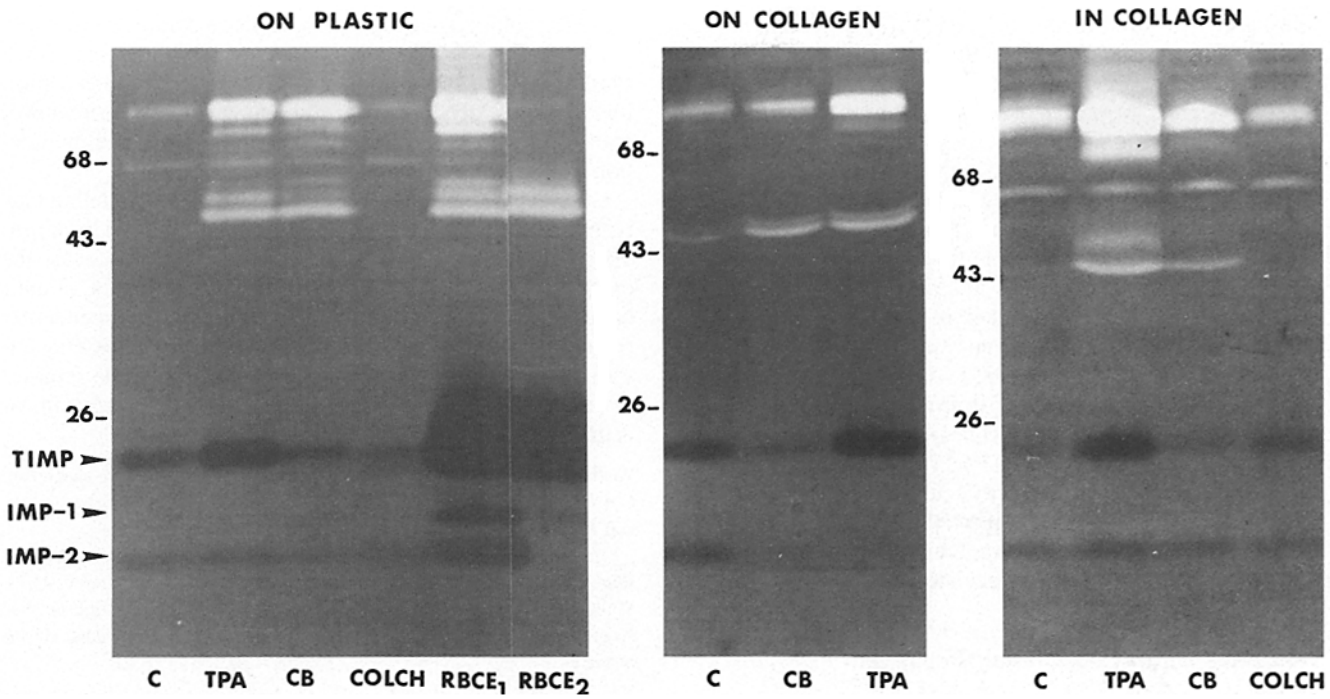


Figure 10. Secretion of two inhibitors of metalloproteinases by RSFs. Conditioned medium from RSFs cultured on plastic, on collagen, and within collagen gels was electrophoresed on 10% polyacrylamide gels impregnated with 1 mg/ml of gelatin and soaked in a solution containing activated metalloproteinases, as described in Materials and Methods. Medium from TPA-treated rabbit brain capillary endothelial cells (*$RBCE_1$*) was used as a standard to indicate three previously described inhibitors, indicated by arrowheads. The band at 25 kD was identified as TIMP by co-migration with partially purified TIMP (*$RBCE_2$*). Cells were treated with no agents (*C*), TPA, CB, or colchicine (*COLCH*). Two inhibitors between 19 and 30 kD that correspond to TIMP and inhibitor of metalloproteinases-2 (IMP-2) were secreted by RSFs under all conditions. TIMP was increased by TPA treatment whether cells were on plastic, on collagen, or within collagen gels. The three gels were electrophoresed separately and show slight differences in migration. Molecular mass markers ($\times 10^{-3}$) are indicated at the left of each gel.

architecture, suggesting that a change in the state of actin assembly may influence proCL expression. ProCL was expressed by contracted RSFs, in which there was marked redistribution of filamentous actin. TPA and CB also caused large changes in cell shape and filamentous actin organization, whether the cells were cultured on a planar substrate or within collagen gels; proCL was induced in all these instances. Significantly, in RSFs embedded within collagen gels, cell shape was dissociable from actin reorganization, and proCL was expressed only when actin was altered.

RSFs were cultured within hydrated collagen matrices because of existing evidence that the cytoskeletal organization of cells grown in this fashion can differ significantly from that of cells grown on plastic (Tomasek and Hay, 1984). In agreement, we found that although colchicine did not markedly distort cells on a planar substrate, it caused collapse of the bipolar or stellate morphology of RSFs grown within the gel into a spherical shape. This suggests that microtubules play an important role in the cytoarchitecture of cells grown within the gel. This hypothesis was borne out by tubulin staining experiments, which showed that collapse of normal morphology caused by colchicine treatment was accompanied by abnormal tubulin staining. In contrast, the change in morphology was independent of alteration of actin microfilament organization, the integrity of which was maintained even in cells distorted by exposure to colchicine. This apparent dependence of morphology of cells in the gel upon microtubule organization allowed us to exploit this culture system to dissociate cell shape from actin microfilament organization.

These results complement those of other investigators who have observed that a change in morphology is often required for a switch in gene expression (Emerman and Pitelka, 1977; Ben-Ze'ev et al., 1980; Benya and Shaffer, 1982; Spiegelman and Farmer, 1982; Spiegelman and Ginty, 1983; Lee et al., 1984; Zanetti and Solursh, 1984). Although this phenomenon is not well understood, perhaps the best-characterized examples of shape-modulated changes in cell behavior are adipogenesis (Spiegelman and Farmer, 1982; Spiegelman and Ginty, 1983) and chondrogenesis (Benya and Shaffer, 1982; Zanetti and Solursh, 1984), in which actin cytoskeletal modification is implicated as an important prerequisite to new protein synthesis.

In all cases of induction of proCL in RSFs, the disposition of actin, whether filamentous or sheath-like, was clearly altered. This change may involve either the state of actin polymerization or filament assembly. TPA or cytochalasin treatment of tissue culture cells causes energy-dependent attenuation of stress fibers, concordant with the formation of actin aggregates (Godman et al., 1980*a*, *b*; Schliwa, 1982; Schliwa et al., 1984). In collagen-embedded RSFs, which develop few stress fibers, TPA and CB probably cause displacement of microfilaments from the cortical meshwork seen by electron microscopy (Tomasek et al., 1982) into foci

similar to those described in cells on plastic. Filamentous actin was also clearly rearranged during cellular contraction. Isotonic contraction coincided with rapid shortening of stress fibers and formation of actin aggregates, often in register with the shrinking fibers.

It is not clear how the reorganization of the collagen matrix and subsequently of the actin cytoskeleton is associated with modulation of proCL expression. Previous work has demonstrated more than a skeletal role for actin in cells; it affects gene transcription (Capco et al., 1982; Robinson et al., 1982; Egly et al., 1984; Scheer et al., 1984), translation (Cervera et al., 1981), and protein secretion (Osawa et al., 1984; Novick and Botstein, 1985). Aggeler et al. (1984*a*, *b*) and Gross et al. (1984) have shown that although resting RSFs grown on plastic have virtually no translatable mRNA for proCL, cells treated with TPA or CB contain high levels of proCL mRNA. Induction, therefore, is likely to be pretranslational. Therefore, it is possible that the extracellular matrix may regulate nuclear function and gene expression via an interplay with the cytoskeleton.

Secretion of ProCL, Collagens, and Metalloproteinase Inhibitors Is Not Coordinately Regulated

Many types of cultured mesenchymal cells are able to secrete collagens (Layman et al., 1971); human skin fibroblasts and endothelial cells are capable of expressing proCL and its inhibitors in concert. We therefore examined the expression of proCL in relation to the production of its substrate, collagen, and its inhibitors. Collagen was actively synthesized and secreted by RSFs when grown on plastic, on collagen, and within collagen gels. Chondrocytes (Gibson et al., 1984) and embryonic corneal epithelial cells (Dodson and Hay, 1974) have been reported to modify their collagen synthetic patterns in response to growth on or within collagen gels. The introduction of a collagen substratum to RSF cultures did not markedly alter their secretion of collagen; however, there was some decrease in collagen secretion in cells grown within the gel.

The production of proCL and its presumptive substrate, collagen, was not coordinately regulated in RSFs. ProCL induction in cells cultured on plastic was accompanied by a marked decrease in collagen production, consistent with a decrease in the secretion of many proteins. When RSFs were induced by gel release, however, secretion of procollagens continued even after stimulation. These results indicate that (*a*) actin reorganization is correlated with proCL induction but has no apparent relation to collagen secretion, and (*b*) reorganization of the matrix, such as that caused by contraction, also appears to promote collagen secretion. It is possible that an alteration in the extracellular matrix triggers a "remodeling response" in which both collagen and collagenase are secreted. Simultaneous secretion of collagen and proCL can be reconciled by postulating selective directional transport of the two proteins. The ability to modulate site-specific deposition of this enzyme and its substrate may have important consequences during tissue remodeling.

Collagen degradation can also be modulated by inhibitors of metalloproteinases found in tissues (Cawston et al., 1983; Welgus and Stricklin, 1983). These inhibitors are glycoproteins of approximately 30 kD that may be coordinately produced by the enzyme-secreting cells (Murphy et al., 1985; Herron et al., 1986*a*, *b*). We found that RSFs are capable of secreting inhibitors under all conditions studied here. Only TPA, which has previously been reported to increase TIMP synthesis (Murphy et al., 1985; Herron et al., 1986*a*), stimulated TIMP production. Although quantities were never enough to negate proCL activity completely, some modulation of enzyme activity may take place locally.

Our data suggest that extracellular matrix remodeling may be mediated by RSFs under conditions that result in the reorganization of actin microfilament architecture. Because the expression of proCL and related metalloproteinases, of their endogenous inhibitors, and of collagens is independently regulated, a variety of matrix turnover phenotypes may ensue. The effects of these phenotypes could include removal of large amounts of connective tissue, focal degradation, or degradation accompanied by matrix resynthesis.

We thank R. Hembry for the gift of fluorescein-labeled pig anti-sheep Fab, Ole Behrendtsen for excellent technical assistance, and Mary McKenney and Patricia Taber for preparation of the manuscript.

This work was supported by the Office of Health and Environmental Research, United States Department of Energy, contract No. DE-AC03-76-SF01012, and by National Institutes of Health National Research Service Award 5-T32-ES07106 from the National Institute of Environmental Health Sciences.

Received for publication 23 September 1986, and in revised form 2 June 1986.

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