Commitment to Expression of the Metalloendopeptidases, Collagenase and Stromelysin: Relationship of Inducing Events to Changes in Cytoskeletal Architecture

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Abstract. Agents that alter the morphology of rabbit synovial fibroblasts induce synthesis of the metalloendopeptidases, collagenase and stromelysin. We studied the relationship of cytoskeletal changes to the commitment to expression of these metalloendopeptidases. Cells treated with cytochalasin B (CB) or 12-O-tetradecanoylphorbol-13-acetate rounded, and only cells that had lost their stress fibers expressed collagenase and stromelysin, as determined by immunofluorescence. We concentrated on the effects of CB because of its rapid reversibility. When CB was added for 1–24 h, then removed, the cells respread within 30–60 min. The minimum period of CB treatment that committed cells to the subsequent synthesis of collagenase and stromelysin was 3 h. After initial treatment with 2 μg/ml CB for 3–24 h, or with various concentrations of CB (0–2 μg/ml) for 24 h, both enzyme activity and biosynthesis of the proenzymes showed a graded increase when measured at 24 h. Even after treatment with 2 μg/ml CB for only 3 h, >85% of all cells were positive for both collagenase and stromelysin when cells were monitored by immunofluorescence. In contrast, when the dependence of collagenase and stromelysin expression on the inducing concentration of CB was examined, there was a dose-dependent increase in the number of cells positive for collagenase and stromelysin, as determined by immunofluorescence. Thus, at low concentrations of CB (<0.5 μg/ml), a heterogeneous population response was observed. These results suggest that the commitment of fibroblasts to induction of the metalloproteinases is a stochastic process in which a second signal that correlates with the disruption of the actin cytoskeleton may be rate-limiting for collagenase and stromelysin gene expression.

An emerging body of knowledge from a variety of experimental systems indicates that there is a correlation between cytoskeletal architecture and the expression of certain genes (2, 4, 6, 7, 11, 12, 15, 24–28, 31). Many of these cases have involved the terminal differentiation of developing systems with a permanent reorganization of the actin cytoskeleton. For example, in induction of chondrogenesis (6, 25, 27, 31) and adipogenesis (24) and in mammary epithelial differentiation (11, 15), the acquisition of a rounded cell shape and the reorganization of the actin cytoskeleton are prerequisites for initiation of expression of genes that encode differentiated cell products.

In previous work using rabbit synovial fibroblasts (1, 2, 9, 16) we studied a reversible alteration in collagenase gene expression that correlated with changes in cell shape. Recently, we described a second metalloendopeptidase, stromelysin (9, 13), that is secreted by fibroblasts along with collagenase. Our data suggested a model in which an agent must be present initially for several hours for induction of the proteinases to take place and must cause alterations in cell morphology during this period. Synthesis and secretion of collagenase and stromelysin, as their zymogen precursors procollagenase (proCL)[1] and prostromelysin (proSL), were not detected until 6–20 h after inducers were added to cells. We inferred that a second inducing signal must be generated within the cells, because proCL and proSL synthesis and secretion were initiated even when agents were removed and cells flattened down. The amount of enzyme secreted depended on the time of treatment and the concentration of agent used.

Our system of induction of metalloendopeptidase expression by agents that alter cell shape has advantages over some other models. We can study the nature of morphological changes committing the fibroblasts to their response in the absence of continued shape change. Because the alterations

[1] *Abbreviations used in this paper:* CB, cytochalasin B; DME-LH, Dulbecco's modified Eagle's medium with lactalbumin hydrolysate; proCL, procollagenase; proSL, prostromelysin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

induced are not part of a terminal differentiation response, we can also determine the factors that are required for maintenance of the response. The present study was an immunocytochemical investigation of the commitment of fibroblasts to the coordinate regulation of the two metalloendopeptidases, proCL and proSL, in relation to alterations in the actin cytoskeleton induced by cytochalasin B (CB) or 12-O-tetradecanoylphorbol-13-acetate (TPA).

Materials and Methods

Methods for isolation of rabbit synovial fibroblasts, their culture, treatment with inducing agents, and biosynthetic labeling with [^{35}S]methionine were described previously (1, 2). Cell shape change index was determined as described by Aggeler et al. (2). ProCL and proSL secreted by fibroblasts were quantified by scanning fluorographs, prepared from SDS PAGE of [^{35}S]methionine-labeled secreted protein, with a Hoefer gel scanner and Hewlett-Packard integrator (Hewlett-Packard Co., Palo Alto, CA).

Antibodies

The monospecific sheep antibodies to the rabbit metalloproteinases collagenase and stromelysin (9, 17a, 19) and the method for preparation of IgG from these antisera (17) were described previously. The antisera were shown to be specific by double immunodiffusion, by immunoprecipitation of ^{125}I-labeled or [^{35}S]-methionine biosynthetically labeled crude and purified enzyme preparations, by immunoblotting, and by enzyme inhibition. Their specificity in immunoblotting is shown in Fig. 1.

Intracellular Localization of Collagenase and Stromelysin by Indirect Immunofluorescence

Fibroblasts actively engaged in secretion of collagenase and stromelysin were identified by intracellular localization with specific IgG. Cultured fibroblasts on 12-mm-diam glass coverslips were incubated with the inducing agents for various lengths of time and then incubated in fresh medium for 3 h with or without 1 μM monensin to accumulate secretion products intracellularly (19). Coverslip cultures were fixed for 10 min in 2% paraformaldehyde in phosphate-buffered saline, then washed in buffer that contained 20 mM NH_4Cl. The cells were then made permeable by a 10-min incubation with 0.1% Triton X-100 and incubated for 45 min with 0.05–5 μg/ml of nonimmune or immune IgG. Two alternative procedures were used to provide a fluorescent second antibody: the cells were incubated for 45 min with either fluorescein-conjugated Fab fragments from a pig antiserum to sheep Fab′ fragments, or with biotinylated rabbit anti-sheep IgG (Vector Laboratories, Inc., Burlingame, CA) followed by either Texas Red– or fluorescein-labeled streptavidin (Amersham Corp., Arlington Heights, IL). For double immunofluorescence, cells were first stained with anti-collagenase and the fluorescein-labeled second antibody, then with anti-stromelysin with the biotinylated second antibody and Texas Red–labeled streptavidin. Coverslips were observed by epiillumination and photographed on Ilford HP-5 film rated at 800 ASA with a Zeiss Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY). At least 200 cells were scored for intracellular immunofluorescence directly at the microscope. Controls included (*a*) substitution of nonimmune IgG for immune IgG, (*b*) nonpermeable cells, and (*c*) immune IgG adsorbed with an excess of enzyme.

Fluorescent Staining of Filamentous Actin

Cultured fibroblasts on 12-mm-diam glass coverslips were incubated with inducing agents for 1–3 h and then with fresh serum-free medium for 15 min to 24 h. Cells were then washed and fixed with 3.7% paraformaldehyde. Actin filaments were visualized in rabbit fibroblasts with rhodamine-phalloidin (5) as described by the manufacturer (Molecular Probes, Junction City, OR). For dual localization of enzymes and actin, the cells were first stained for enzyme immunofluorescence using biotinylated anti-sheep IgG and fluorescein-labeled streptavidin, then stained for actin with rhodamine-phalloidin.

Results

ProCL and ProSL Secretion by Induced Rabbit Fibroblasts Is Detectable by Immunofluorescence

Agents that cause an alteration in rabbit fibroblast morphology induce proCL and proSL synthesis. Fibroblasts treated with CB or TPA secreted proCL of M_r 57,000 and 53,000 and proSL of M_r 51,000, which could be selectively visualized by immunoblotting with monospecific antibodies raised against collagenase and stromelysin, respectively (Fig. 1). To obtain detailed information about the response of individual cells, we visualized intracellular proCL or proSL by indirect immunofluorescence. Fibroblasts treated with CB or TPA, but not control fibroblasts, had demonstrable proCL or proSL concentrated in the perinuclear region of the cells (Fig. 2). In some cells bright punctate staining, corresponding to secretion vesicles, and more diffuse staining were visible (Fig. 2, *d* and *e*). When the fibroblasts were treated with 1 μM monensin for 3 h before fixation and immunofluorescent labeling, staining in the Golgi region was increased markedly, making it possible to visualize cells that were synthesizing these enzymes

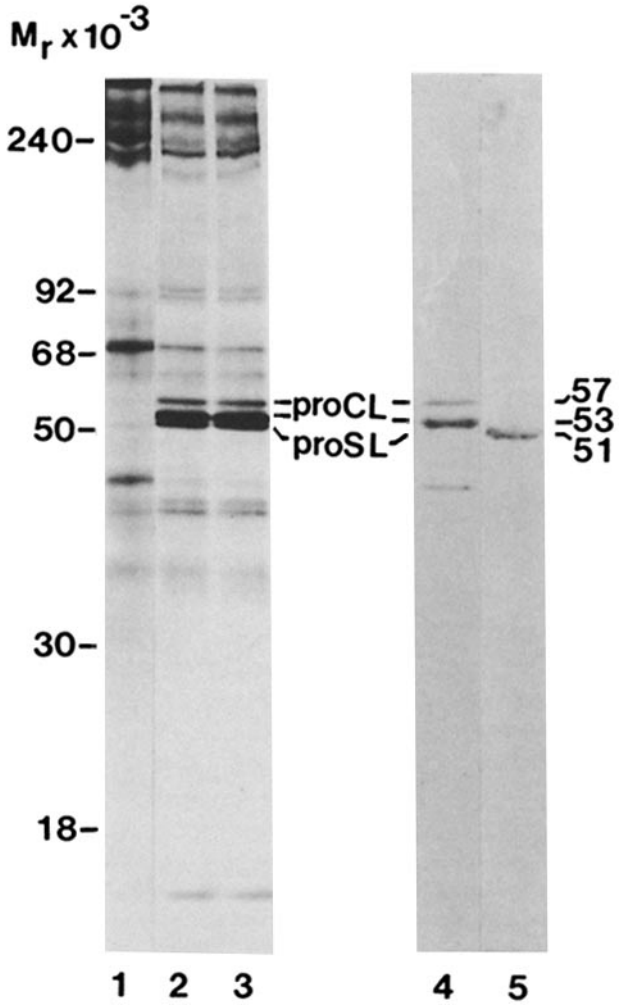


Figure 1. Demonstration of proCL and proSL in conditioned culture medium of rabbit fibroblasts. Lanes *1–3*, total [^{35}S]methionine-labeled secreted proteins from control (lane *1*), CB-treated (lane *2*), or TPA-treated (lane *3*) cells. Lanes *4* and *5*, Western immunoblots of concentrated secreted proteins from TPA-treated fibroblasts visualized with anti-collagenase (lane *4*) or anti-stromelysin (lane *5*). Migration of proCL and proSL is shown. Some active collagenase and stromelysin are also seen in the immunoblots of concentrated conditioned medium. Molecular weight markers ($M_r \times 10^{-3}$) are indicated on the left.

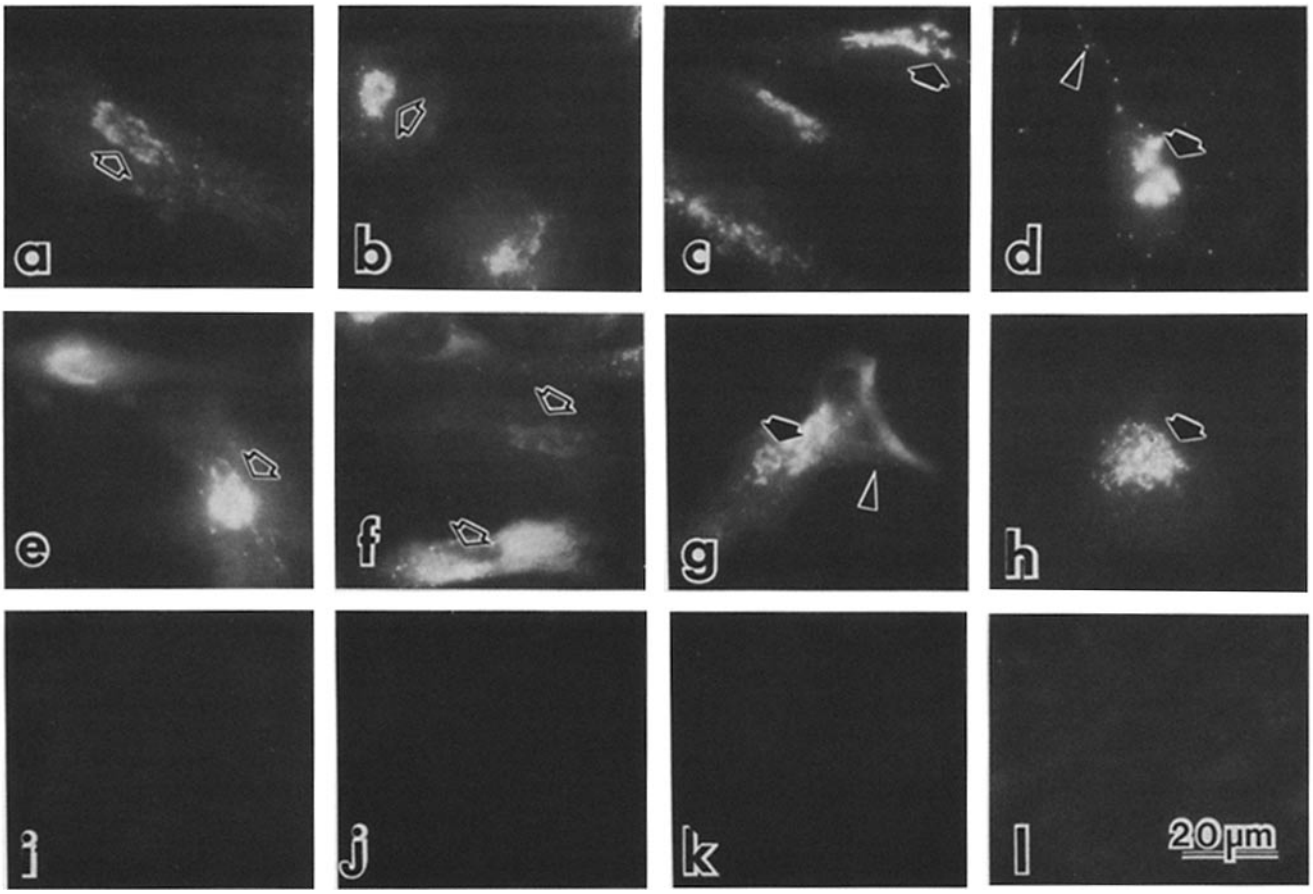


Figure 2. Detection of intracellular proCL and proSL in rabbit fibroblasts by indirect immunofluorescence. Cells were incubated for 24 h in DME-LH (Dulbecco's modified Eagle's medium with lactalbumin hydrolysate) with either no additions, 2 μg/ml of CB, or 50 ng/ml of TPA. The medium was then removed and cells were incubated for an additional 3 h in DME-LH in the absence or presence of 1 μM monensin. At 27 h cells were fixed, made permeable, and then incubated with 5 μg of nonimmune sheep IgG or IgG specific for collagenase or stromelysin followed by a fluorescent-tagged second antibody. CB-treated cells incubated without monensin (*a* and *b*) or with monensin (*c* and *d*) were stained with anti-collagenase (*a* and *c*) or anti-stromelysin (*b* and *d*). TPA-treated cells incubated without monensin (*e* and *f*) or with monensin (*g* and *h*) were also stained with anti-collagenase (*e* and *g*) or anti-stromelysin (*f* and *h*). Fluorescence was seen in the perinuclear region (open arrows), which appeared in swollen and fragmented vesicles (filled arrows) after treatment with monensin; accumulated small secretion vesicles (arrowheads) were also visible. Under these conditions virtually every cell was positive for either anti-stromelysin or anti-collagenase; however, the fluorescence varied somewhat in intensity. Control fibroblasts were also stained with anti-collagenase (*i*) or anti-stromelysin (*j*); some cells were treated with CB (*k*) or TPA (*l*) and then incubated with monensin and stained with nonimmune IgG. Exposures for the fluorescence micrographs were 30 s for cells without monensin and 10 s for cells with monensin.

at low rates (Fig. 2, *c*, *d*, *g*, and *h*). Fibroblasts treated with CB, a reversible actin filament–disrupting agent, for as long as 24 h flattened down within 3 h of transfer to fresh medium while continuing to synthesize proCL and proSL at high rates. It is interesting to note that many of the cells incubated with monensin in the absence of CB for this same 3-h period failed to respread (compare Fig. 2*b* with Fig. 2*d*), possibly because of reduced secretion of an adhesion protein such as fibronectin (20). Cytochalasin D (1 μg/ml) gave essentially the same data as CB.

Time of Treatment with CB Influences the Induced Rate of Synthesis of ProCL and ProSL

When fibroblasts were treated with CB for 0–24 h, a graded increase in synthesis and secretion of proCL was observed at 24 h by biosynthetic labeling and SDS PAGE (Fig. 3). The minimum time required to commit the cells to subsequent proCL secretion was 3 h.

The nature of this graded response was investigated by immunocytochemical localization of proCL and proSL. More than 85% of all the cells were positive for both proCL and proSL when observed at 24–27 h of incubation, whether they had been treated with CB for 24 h or for only 3 h (Fig. 3*a*). Onset of proCL secretion at about 12 h of CB treatment was also coordinated in the majority of the cells (data not shown). Cells treated with CB for only 1 h were uniformly negative for proCL and proSL. When the intensity of the immunofluorescence was estimated, all the cells in the population showed increased intracellular and secreted proCL and proSL with increasing time of exposure to CB (Fig. 3). Taken together, the biosynthetic labeling and immunofluorescence data indicate that the rate of proCL and proSL synthesis expressed by individual cells was related to the length of their exposure to CB. They also confirm our previous observation (2) that cells once exposed to CB for a critical induction period were committed to the subsequent expression of proCL and proSL even when they flattened down.

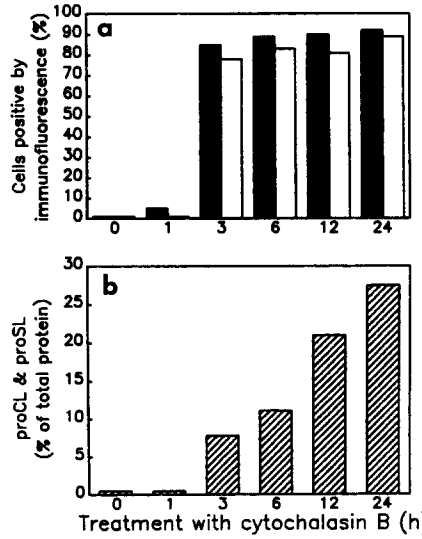


Figure 3. Time dependence of proCL and proSL expression by CB-treated fibroblasts. Rabbit synovial fibroblasts were incubated for 0–24 h with 2 μg/ml CB in DME-LH. CB was removed at the times indicated and replaced with DME-LH. (*a*) After 24 h the medium of cells cultured on coverslips was removed, and 1 μM monensin in DME-LH was added to all samples for an additional 3 h to prevent protein secretion and to increase the sensitivity of immunodetection. At 27 h, cells were fixed, made permeable, and then prepared for indirect immunofluorescence. The percentage of cells staining for anti-collagenase IgG (■) or anti-stromelysin IgG (□) is indicated. The relative intensity of intracellular fluorescence on an arbitrary scale of 0–4+ was 0 for cells treated with CB for 0 or 1 h, 1+ for cells treated for 3 h, 2+ for 6 h, 3+ for 12 h, and 4+ for 24 h. (*b*) After 24 h, the cells were biosynthetically labeled for 2 h with 25 μCi/ml of [^{35}S]-methionine, ^{35}S-labeled secreted proteins were separated by SDS PAGE, and gels were scanned with a densitometer. The percentage of the total secreted proteins in the M_r of 57,000, 53,000, and 51,000 bands after subtraction of control values (<4%) is indicated.

Alteration of the Actin Cytoskeletal Architecture of Rabbit Fibroblasts during Treatment with TPA or CB Correlates with Induction of Proteinase Expression

When rabbit fibroblasts were doubly stained with rhodamine-phalloidin, a fluorescent derivative of a fungal alkaloid that binds specifically to assembled actin microfilaments (30), and with anti-collagenase, untreated cells showed prominent bundles of microfilaments or stress fibers but no collagenase immunofluorescence (Fig. 4, *a* and *b*). Upon addition of TPA or CB, cells became rounded, stress fibers disappeared from most cells, and after 24 h the little remaining actin staining was localized in the cell cortex and in dense clumps at the ventral cell surface (Fig. 4, *c* and *e*), as has been observed previously (22, 23), and most of the cells were positive for collagenase immunofluorescence (Fig. 4, *d* and *f*). The occasional nonsecreting cells within the population of cells treated with TPA had retained their prominent stress fibers (Fig. 4, *c* and *d*). After 3–24 h in the presence of CB, cells rapidly respread and reformed stress fibers when placed in fresh medium, and cells with both collagenase immunofluorescence and stress fibers were observed (data not shown).

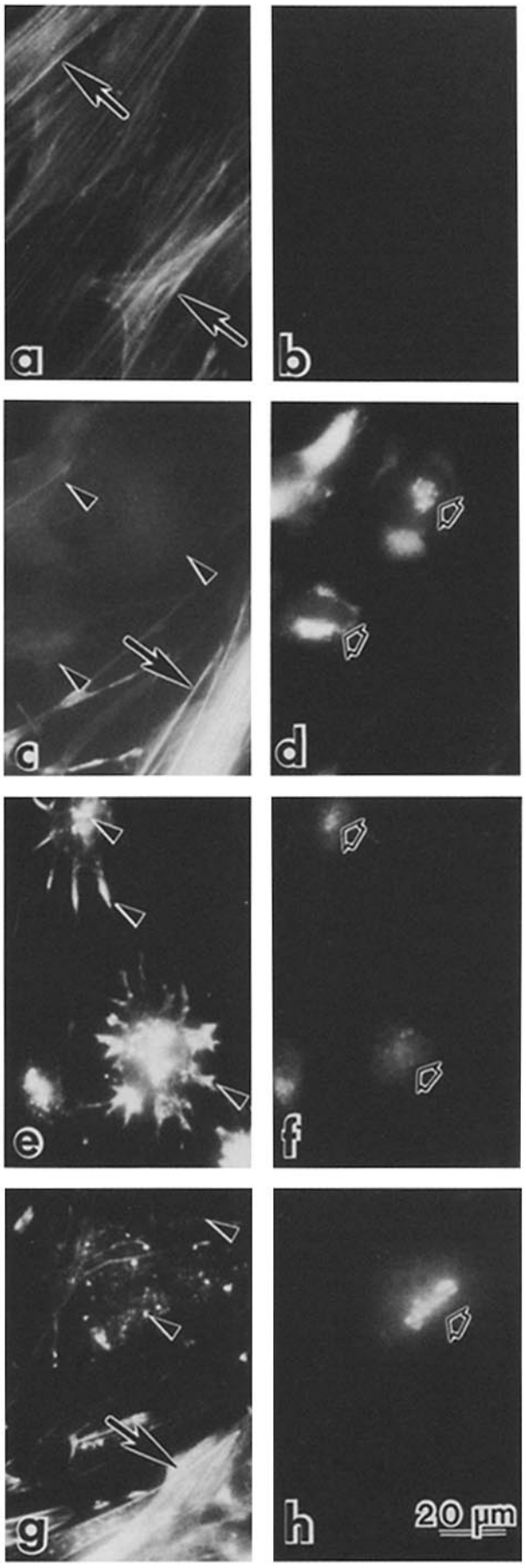
ProCL and ProSL Secretion in Response to Increasing Concentrations of CB Is Heterogeneous within the Fibroblast Population

Treatment of rabbit fibroblasts with increasing concentrations of CB resulted in a dose-dependent increase in secretion of proCL and proSL. As shown in Fig. 5, collagenase activity increased with increasing drug concentration in parallel with increases in morphological change. An increase in the rate of proCL synthesis was also observed by biosynthetic labeling followed by SDS PAGE (Fig. 5*c*). In contrast to the response with increasing exposure time shown in Fig. 3, however, fluorescent localization showed that, at low drug concentrations (≤0.6 μg/ml), the fibroblast population was heterogeneous in its response (Figs. 4, *g* and *h*, and 5*a*), and only cells that had lost stress fibers were positive for proCL. With increasing concentrations of CB a greater proportion of the cells were positive for proCL and proSL. It is interesting to note that with all treatments a slightly higher percentage of cells were observed to be positive for proCL than for proSL. Similar data were obtained using cytochalasin D, with a maximum response at 0.6 μg/ml. When cells treated with 2 μg/ml CB for 24 h were stained with antibodies against both enzymes, most cells were positive for both. Only a small number (<5% of the total) stained for proCL alone, and <0.5% stained for proSL alone (data not shown).

Discussion

The alterations in cell shape seen during morphogenesis in vivo may trigger secretion of proCL, proSL, and related proteolytic enzymes that then could remodel the extracellular matrix. In this study we investigated the role of cell shape changes in the commitment of rabbit synovial fibroblasts to altered gene expression. We observed the cytoskeletal architecture of these fibroblasts in relation to the induction of expression of the two metalloproteinases, collagenase and stromelysin. The immunofluorescent localization of proCL and proSL revealed that these two enzymes were expressed coordinately by most cells in the population in response to TPA or CB. We also demonstrated that disassembly of actin stress fibers (and/or other complex ordered structures) during a critical induction period was required for commitment of cells to expression of proCL and proSL. Fibroblasts that had reestablished normal actin structure actively secreted these enzymes, and, in the case of a 3-h treatment with CB, the cellular architecture was indistinguishable from that of control cells for nearly 6 h before initiation of enzyme secretion, which occurs about 12 h after initial addition of CB (2), suggesting that the putative second messenger may persist for some time after the beginning of cell flattening. That a change in the actin cytoskeleton itself, rather than a change in overall cell morphology, correlates with induction of gene expression has been shown by related experiments using fibroblasts grown in collagen gels; these cells undergo a shape change with agents that depolymerize microtubules, but proCL secretion is not induced (28). However, changes in cytoskeletal architecture may not be the only inducing stimuli for expression of metalloproteinases (29). Chondrocytes, which are already round, can be stimulated to secrete these enzymes by interleukin-1 (17a), whereas other cells, such as human lung cells, may change shape, but only a small proportion of cells are stimulated to express collagenase (19).

Two lines of evidence suggest that commitment occurs as the result of a stochastic process in which a second signal that correlates with the disruption of the cytoskeleton must reach a critical threshold before proCL and proSL expression is



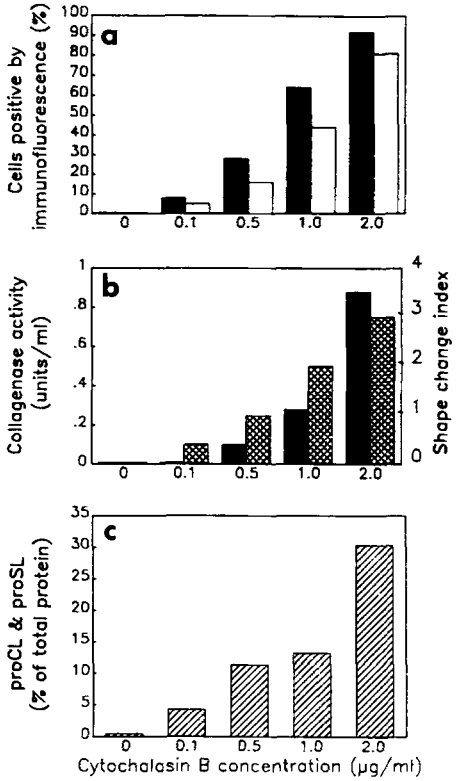


Figure 5. Dose response of proCL and proSL expression. (*a*) Rabbit synovial fibroblasts on glass coverslips were incubated for 24 h in DME-LH that contained 0–2 μg/ml CB, then transferred for 3 h to DME-LH that contained 1 μM monensin. The cells were fixed and stained for indirect immunofluorescence with 5 μg/ml sheep IgG specific for rabbit synovial fibroblast collagenase (■) or stromelysin (□). (*b*) Activatable collagenase activity (■) secreted by fibroblasts treated with CB for 48 h in DME-LH. The shape change index (▩) on a scale of 0–4 was also a function of CB concentration. (*c*) ProCL and proSL bands, as a percentage of total secreted protein, were determined from SDS PAGE of secreted proteins from cells treated for 48 h with CB, then labeled for 2 h with [^{35}S]methionine.

triggered. Varying concentrations of CB produced a mixed population of secreting and nonsecreting cells, suggesting that a threshold was not reached in some cells at lower concentrations. On the other hand, when cells were exposed to a potent inducer (2 μg/ml of CB) for various times, there were time

Figure 4. Dual localization of actin microfilament organization (*a*, *c*, *e*, and *g*) and intracellular proCL (*b*, *d*, *f*, and *h*) in rabbit fibroblasts. The cells were treated with TPA or CB for 24 h with 1 μM monensin added during the last 3 h. The fixed and permeable cells were then treated with anti-collagenase, biotinylated anti-sheep IgG, and fluorescein–streptavidin to demonstrate intracellular proCL localization, followed by rhodamine-phalloidin to reveal the assembled actin cytoskeletons. Control fibroblasts contained abundant stress fibers (arrows) (*a*) but no proCL (*b*). In fibroblasts treated with 100 ng/ml of TPA, most of the stress fibers had disappeared and only weak background actin staining and cortical staining (arrowheads) remained (*c*). Although a proportion of the cells (<20%) retained their stress fibers (arrow) (*c*), only the cells in which the stress fibers had disappeared expressed proCL (open arrows) (*d*). Cells treated with 2 μg/ml of CB had diminished actin staining, with small clumps of staining along the ventral surface of the arborized cells (*e*), and most of these cells were positive for proCL (*f*). Cells treated with 0.6 μg/ml of CB showed heterogeneity in loss of stress fibers (*g*), and the occasional cell stained positively for proCL (*h*).

constraints on the second signal. When the treatment time was too short (<3 h), no secretion was induced, suggesting that sufficient signal had not been generated. Above this threshold, the response was graded to a maximum of 30% of total secreted protein. Similar mechanisms may also operate in other cells in which a change in cell shape triggers differentiation. Transient exposure of limb bud cells to 2 μg/ml of cytochalasin D for 3–24 h produces a reversible loss of actin cables (31). However, the cells are permanently committed to chondrogenic differentiation, which is first observed after 24 h of culture.

The long lag of 6–12 h before induced expression of proCL and proSL (2, 13, 14) contrasts with other systems, such as expression of c-*fos* within 30 min of treatment of cells with growth factors (18). What, then, is the second signal that triggers gene expression? It has been suggested that mRNA translation may be altered by a change in the cytoskeleton (8). We believe that the regulation of proCL and proSL is pretranslational (1, 2, 13, 14). Our data provide no direct evidence about the nature of the signal related to the cytoskeleton, but because of the strong correlation between disruption of the actin cytoskeleton and metalloproteinase induction, we favor a role for actin or an actin-binding protein. Recent studies have implicated G-actin in the regulation of gene transcription (10, 21). Experiments currently in progress with cDNA probes for proCL and proSL, and with microinjection of actin and related proteins into fibroblasts, may allow the elucidation of these mechanisms.

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