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# Human fibroblast collagenase: Glycosylation and tissue-specific levels of enzyme synthesis

(phorbol diester/gene expression/metallo-endoprotease inhibitor/collagen degradation)

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**ABSTRACT** **Human skin fibroblasts secrete collagenase as two proenzyme forms (57 and 52 kDa). The minor (57-kDa) proenzyme form is the result of a partial posttranslational modification of the major (52-kDa) proenzyme through the addition of N-linked complex oligosaccharides. Human endothelial cells as well as fibroblasts from human colon, cornea, gingiva, and lung also secrete collagenase in two forms indistinguishable from those of the skin fibroblast enzyme. *In vitro* tissue culture studies have shown that the level of constitutive synthesis of this fibroblast-type interstitial collagenase is tissue specific, varies widely, and correlates with the steady-state level of a single collagenase-specific mRNA of 2.5 kilobases. The tumor promoter, phorbol 12-myristate 13-acetate, apparently blocks the control of collagenase synthesis resulting in a similarly high level of collagenase expression (≈3–7 μg of collagenase per $10^6$ cells per 24 hr) in all examined cells. The constitutive level of synthesis of a 28-kDa collagenase inhibitor does not correlate with that of the enzyme. Phorbol 12-myristate 13-acetate stimulates the production of this inhibitor that in turn modulates the activity of collagenase in the conditioned media. As a result, the apparent activity of the enzyme present in the medium does not accurately reflect the rate of its synthesis and secretion.**

Specific enzymes, vertebrate collagenases (EC 3.4.24.7), are required for the initiation of collagen degradation (1). These enzymes play an essential role in the maintenance of the extracellular matrix during tissue development and remodeling. Two types of interstitial collagenase can be distinguished; one from human skin fibroblasts (2, 3) and the other from human granulocytes (4, 5). These enzymes differ immunologically (6), in substrate preference (7, 8), and in molecular weight (2, 5). The interstitial collagenase from human skin fibroblasts has been purified and characterized enzymatically (2, 7). This enzyme is secreted by cultured skin fibroblasts as two closely related proenzyme forms, 57 and 52 kDa, both of which can be activated by limited digestion with trypsin to produce lower molecular weight active enzyme species (3) that have identical amino-terminal sequences (9).

Regulation of fibroblast collagenase activity may occur at several points, which include biosynthesis of the proenzyme (10, 11), zymogen activation (12, 13), and interaction of the active enzyme with a 28-kDa metallo-endoprotease inhibitor (14, 15). We have determined the complete primary structure of the enzyme as predicted from the sequence of the cDNA clone pCol 185.2 (9). Human skin fibroblast collagenase is synthesized as a preproenzyme, $M_r$ 54,092, with a 19-amino acid signal peptide. The amino acid sequence of the proenzyme contains two potential N-glycosylation sites positioned near the amino terminus of the proteolytically activated enzyme. Here we report that the minor, 57-kDa, proenzyme is the result of a partial posttranslational modification of the major, 52-kDa, proenzyme through the addition of complex N-linked oligosaccharides. In addition, we present data showing that a number of human cell lines derived from different connective tissues secrete collagenase indistinguishable from that of human skin fibroblasts. The constitutive level of enzyme secretion is tissue specific, although treatment with the tumor promotor phorbol 12-myristate 13-acetate (PMA) stimulates transcription of a single collagenase mRNA and enzyme synthesis and secretion up to a level comparable to that of fibroblasts derived from adult skin.

## MATERIALS AND METHODS

**Cell Culture and Enzyme Assays.** Human fetal skin fibroblast cell strains AG4431, AG4392, AG4525, and GM1381 were obtained from the Human Genetic Mutant Cell Repository. Adult human colon fibroblasts (CRL 1459) and gingival fibroblasts (CRL 1292) were obtained from the American Type Culture Collection. Adult human skin (WU 80547), corneal (WU 77245), and lung fibroblasts (WU 827605, gift of J. Clark) were cultured from biopsies obtained from healthy volunteers after informed consent. Human umbilical vein endothelial cells were a gift from J. Olander (Monsanto). Cells were cultured and treated with PMA as described (16). Tunicamycin (1 mg/ml in 25 mM NaOH) was added to the medium to a final concentration of 0.25 μg/ml where indicated. Collagenase activity was measured using [$^{14}$C]glycine-labeled collagen (5 × $10^4$ cpm/mg) as substrate (17). Proteolytic activation of collagenase by limited digestion with trypsin was carried out as described (18). One unit of enzyme activity represents the amount of enzyme capable of degrading 1 μg of collagen per min at 37°C in 50 mM Tris·HCl (pH 7.5) and 10 mM $CaCl_2$. Collagenase and the 28-kDa metallo-endoprotease inhibitor were assayed by indirect enzyme-linked immunosorbent assay (ELISA) (19, 20). These measurements were performed in parallel on confluent cultures of similar cell density and passage under identical culture conditions (16). IgG from specific anti-collagenase and nonimmune serum was purified by DEAE Affi-Gel Blue chromatography (21).

To measure *de novo* collagenase synthesis, cells were incubated for 16 hr in serum-free media minus leucine that was supplemented with [$^3$H]leucine at 20 μCi/ml (154 Ci/mmol; 1 Ci = 37 GBq). Incorporation of [$^3$H]leucine into the trichloroacetic acid-insoluble fraction of the cell layer and conditioned medium was determined along with total protein (22). Aliquots from [$^3$H]leucine-labeled conditioned media containing the same number of counts were immunoprecipitated and subjected to $NaDodSO_4$/PAGE as described (16).

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Abbreviation: PMA, phorbol 12-myristate 13-acetate.

**Endoglycosidase Digestions.** A preparation enriched in the 57-kDa form of human skin fibroblast procollagenase was prepared by chromatography on blue-dextran-Sepharose and proteolytically activated using the trypsin-Sepharose method (3). Samples were dialyzed against 0.1 M acetic acid, lyophilized, redissolved in 10 mM Tris·HCl (pH 6.8) containing 1% $NaDodSO_4$ and 5% (vol/vol) 2-mercaptoethanol, and heated for 2 min at 100°C. Samples containing 10 μg of protein were diluted 1:10 with endoglycosidase assay buffer (23, 24) and digested with endoglycosidase F (New England Nuclear, 0.5 unit) and endoglycosidase H (Miles, 25 milliunits) for 16 hr at 37°C. After digestion samples were subjected to $NaDodSO_4$/PAGE and stained for immunoreactive enzyme as described below.

**Immunoblotting.** Immunoblotting experiments were carried out according to the method of Burnette (25). Samples (3 ml) of conditioned serum-free medium from control and PMA-treated cultures were dialyzed against 5 mM Tris·HCl (pH 7.5), 0.1 mM $CaCl_2$, and 0.01% Brij-35, lyophilized, redissolved in 300 μl of the $NaDodSO_4$ sample buffer (1× sample buffer = 60 mM Tris·HCl (pH 6.8), 2.3% (wt/vol) $NaDodSO_4$, 10% (vol/vol) glycerol, and 2% (vol/vol) 2-mercaptoethanol), or 150 μl of buffer containing 50 mM Tris·HCl (pH 7.5), 10 mM $CaCl_2$. The latter were subjected to a limited digestion with trypsin (25 μg/ml for 10 min at room temperature) followed by the addition of at least a 5-fold molar excess of soybean trypsin inhibitor and an equal volume of 2× $NaDodSO_4$ sample buffer. Samples were subjected to $NaDodSO_4$/PAGE and transferred onto nitrocellulose filters (25). For immunostaining, filters were incubated in 20 mM Tris·HCl (pH 7.5), 150 mM NaCl (TBS) and 3% (wt/vol) bovine serum albumin for 1 hr at room temperature and rinsed with TBS containing 0.05% Tween 20. Reaction with anticollagenase IgG (1:250 dilution in TBS/1% bovine serum albumin) was for 2 hr at room temperature. Filters were washed twice with TBS, containing 0.05% Tween 20, and once with TBS. Reaction with secondary antibody (1:3000 dilution of goat anti-rabbit IgG in TBS with 1% bovine serum albumin) conjugated to peroxidase (Bio-Rad) was for 1 hr at room temperature. Filters were rewashed as above and incubated for 10–15 min at room temperature in TBS containing 0.15% $H_2O_2$, 15% (vol/vol) methanol, and 4-chloronaphthol at 0.5 mg/ml until the color developed.

**RNA Isolation and Blot Analysis.** Fresh medium was added to cell cultures 24 hr before harvesting for isolation of total cytoplasmic RNA according to a described procedure (26). Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography (27). A total mRNA preparation was fractionated on 1.2% agarose gel containing 2.2 M formaldehyde (28), transferred to nitrocellulose filters, and hybridized to a nick-translated pCol 185.2 cDNA probe as described (9).

## RESULTS

**The Minor, 57-kDa, Form of the Human Skin Fibroblast Collagenase Is the Result of Partial N-Glycosylation of the Single, 52-kDa, Proenzyme Species.** The primary secretion product of human skin fibroblast collagenase is represented by two proenzyme species, a minor (57-kDa) and a major (52-kDa) species. Both the 57- and 52-kDa proenzymes can be activated by limited digestion with trypsin generating their respective 47- and 42-kDa active enzyme forms (2) that have an identical amino-terminal sequence. The two potential N-glycosylation sites, at asparagine-120 and -143 (9), are contained in the active enzyme form. We, therefore, investigated whether the 57-kDa proenzyme form and the corresponding 47-kDa active enzyme, contain N-linked oligosaccharides to provide further evidence that fibroblast procollagenase is coded by a single mRNA species.

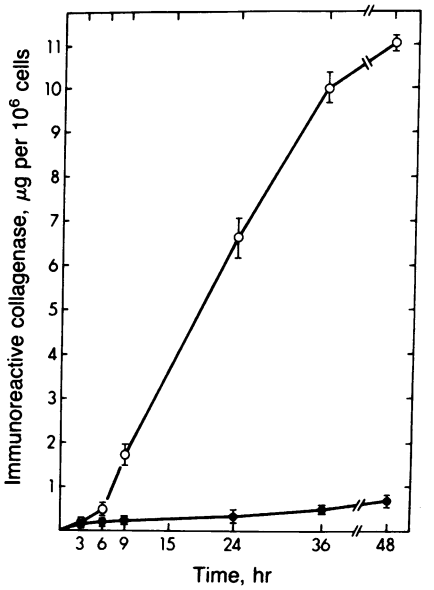


FIG. 1. Time course for the stimulation of collagenase secretion by PMA. Confluent cultures of fetal skin fibroblasts (AG 4431) were incubated in medium containing either 0.01% dimethyl sulfoxide (solid circles) or 0.01% dimethyl sulfoxide and 50 nM PMA (open circles). Aliquots of 0.5 ml were withdrawn at indicated times after PMA addition to determine immunoreactive enzyme protein. The data represent the mean value ± the range obtained from two experiments.

Human fetal skin fibroblasts secrete very low levels of immunoreactive collagenase (29). The rate of enzyme secretion can be stimulated approximately 30-fold by the addition of PMA (Fig. 1). Similar results were obtained using three other cell strains of fetal skin fibroblasts (data not shown). Induction of collagenase secretion by PMA is concomitant with the increase of the steady-state level of a single 2.5-kb collagenase mRNA (Fig. 2) to a level comparable to that found in adult human skin fibroblasts. Although RNA blot analysis reveals induction of a single mRNA species, synthesis and secretion of both 57- and 52-kDa proenzyme forms are stimulated simultaneously (Fig. 3). Fetal human skin fibroblasts were labeled with [$^3$H]leucine, and the secreted proenzyme was precipitated with collagenase-specific antibody and subjected to $NaDodSO_4$/PAGE. As shown in Fig. 3, addition of tunicamycin, an inhibitor of N-linked glycosylation (30), resulted in complete and selective inhibition of the PMA-induced synthesis of the 57-kDa proenzyme. The results of the experiment presented in Fig. 4 show that N-linked complex oligosaccharides are present in the 47-kDa

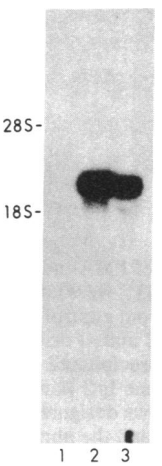


FIG. 2. RNA blot analysis of collagenase mRNA. Poly(A)$^+$ RNA was isolated (26) from control fetal (lane 1), adult (lane 3) and PMA-treated fetal (lane 2) skin fibroblasts. The blot containing 1 μg of fetal and 5 μg of adult skin fibroblast poly(A)$^+$ RNA was probed with nick-translated collagenase cDNA clone pCol 185.2. The locations of 18 and 28S rRNA are identified.

active enzyme form. Treatment of the 47-kDa proteolytically activated enzyme with endoglycosidase F (Fig. 4) results in its conversion into the 42-kDa enzyme form, indistinguishable, by $NaDodSO_4$/PAGE, from the unmodified activated collagenase. In contrast, treatment with endoglycosidase H, an enzyme specific for high-mannose type oligosaccharides (24), had no effect (Fig. 4). Digestion of the 57-kDa proenzyme form with endoglycosidase F also resulted in its conversion to the 52-kDa polypeptide (data not shown).

**Constitutive Level of Expression of Fibroblast-Type Interstitial Collagenase Is Tissue Specific.** Two distinct types of collagenase, one from human skin fibroblasts and the other from human granulocytes, capable of degrading the interstitial collagens, have been identified (2–5). We, therefore, addressed the question of whether human endothelial cells and fibroblasts from various other connective tissues secrete procollagenase identical to that of human skin fibroblasts and whether the constitutive level of enzyme expression is tissue specific. Procollagenase secreted by human endothelial cells and by fibroblasts derived from fetal and adult skin, colon, cornea, gingiva, and lung was subjected to $NaDodSO_4$/PAGE, transferred to nitrocellulose filters (25), and stained for immunoreactive enzyme with collagenase specific antibody. As shown in Fig. 5 and in Table 1, the constitutive level of collagenase synthesis and secretion by these cells varies significantly. Treatment with PMA results in the induction of enzyme secretion in all cell lines studied (Table 1). The rate of enzyme secretion by PMA-induced cells is quite similar (≈3–7 μg of enzyme per $10^6$ cells per 24 hr) and the degree of stimulation is, therefore, defined by the basal level of collagenase secretion.

Endothelial cells as well as the various fibroblast cell strains secrete procollagenase in two forms indistinguishable from those of adult skin fibroblasts. Treatment with PMA results in the coordinate induction of the synthesis and secretion of both forms of the proenzyme (Fig. 5). Proteolytic activation of skin fibroblast collagenase by limited digestion with trypsin results in the cleavage of 81 amino acids from the amino-terminal end of the proenzyme molecule (9). The results presented in Fig. 6 show that trypsin activation of procollagenase secreted by endothelial cells, fetal skin, and corneal fibroblasts produces mature active enzyme forms identical to those of adult skin fibroblasts by $NaDodSO_4$/PAGE. Finally, the enzymes secreted by endothelial cells and by different tissue-derived fibroblast cells are immunologically cross-reactive with the adult skin fibroblast enzyme (data not shown). These results indicate that the constitutive level of collagenase expression is tissue specific, and furthermore, endothelial cells and fibroblasts derived from different human connective tissues secrete two proenzyme forms of collagenase that are most likely structurally identical to the human skin fibroblast enzyme.

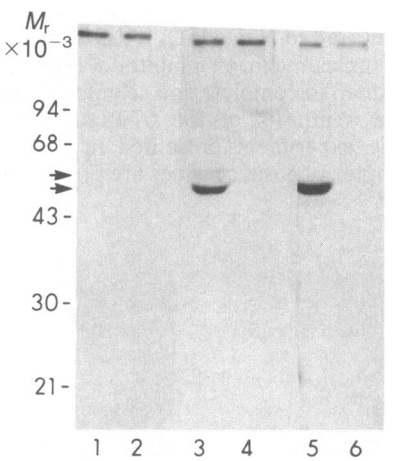


FIG. 3. Effect of tunicamycin on the synthesis of PMA-induced procollagenase. Fetal skin fibroblast cultures (AG 4431) were labeled with [$^3$H]leucine. Samples of serum-free medium from control cells (lanes 1 and 2) and cells treated with PMA (lanes 3 and 4) or PMA and tunicamycin (lanes 5 and 6) were immunoprecipitated with collagenase specific (lanes 1, 3, and 5) or nonimmune IgG (lanes 2, 4, and 6) and subjected to $NaDodSO_4$/PAGE. Arrows designate the migration positions of the purified procollagenase, and the numbers represent molecular weight markers.

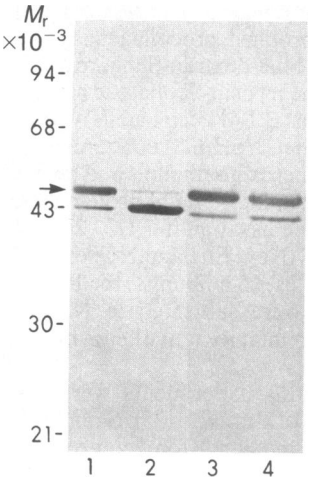


FIG. 4. Endoglycosidase digestion of proteolytically activated collagenase. A preparation containing predominantly the 47-kDa trypsin-activated enzyme form (lanes 1 and 3) was digested with endoglycosidase F (lane 2) or endoglycosidase H (lane 4). The immunoblot of control and endoglycosidase-treated enzyme (1 μg per lane) was labeled with anti-collagenase serum. The arrow designates the migration position of the 47-kDa enzyme form, and the numbers represent molecular weight markers.

The rate of collagenase secretion in these experiments was measured by immunoassay using a specific anti-collagenase antibody. However, functional enzyme assays indicate that the levels of collagenase activity in the conditioned medium of PMA-treated fetal skin, colon, corneal, and lung fibroblasts was significantly lower than expected (0.1–0.5 unit per $10^6$ cells per 24 hr) based on a specific activity of ≈0.8 unit/μg of enzyme protein reported for purified skin fibroblast procollagenase after proteolytic activation (2). We, therefore, assayed the levels of immunoreactive 28-kDa metalloendoprotease inhibitor that binds stoichiometrically to activated collagenase resulting in the formation of an enzyme-inhibitor complex (14, 31). As seen in Table 1, the constitutive level of inhibitor expression did not correlate with the level of enzyme expression. Treatment of various fibroblast cell strains with PMA also results in an increase in the synthesis and secretion of the metallo-endoprotease inhibitor

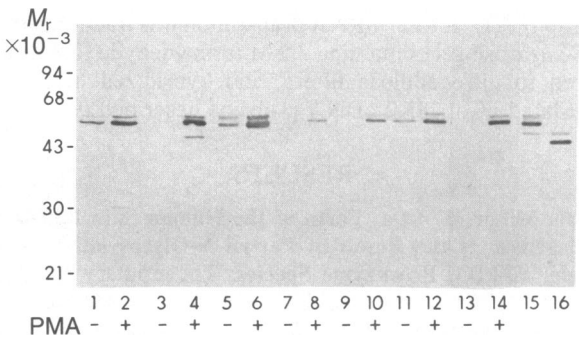


FIG. 5. Comparison of procollagenase secreted by human endothelial cells and fibroblasts derived from different tissues. Samples of serum-free conditioned media from control (−) and PMA-treated (+) cell cultures were collected as described in Table 1. The immunoblot was stained for immunoreactive collagenase secreted by human endothelial cells (lanes 1 and 2) fetal skin (lanes 3 and 4), adult skin (lanes 5 and 6), colon (lanes 7 and 8), corneal (lanes 9 and 10), gingival (lanes 11 and 12), and lung (lanes 13 and 14) fibroblasts. Immunostaining of purified proenzyme (lane 15) and trypsin-activated collagenase (lane 16) is shown for comparison. The numbers indicate migration position of molecular weight markers.

Table 1. Quantitation of collagenase and 28-kDa metallo-endoprotease inhibitor secretion

| Cell type | Added PMA, 50 nM | Collagenase, μg per $10^6$ cells per 24 hr | Inhibitor, μg per $10^6$ cells per 24 hr |
|---|---|---|---|
| Human endothelial | − | 2.6 | ND |
| | + | 6.6 | 0.29 |
| Fetal skin fibroblast | − | 0.25 | 2.5 |
| (AG 4431) | + | 6.7 | 7.1 |
| Adult skin fibroblast | − | 3.2 | 1.2 |
| (WU 80547) | + | 7.5 | 3.2 |
| Adult colon fibroblast | − | 0.10 | 0.63 |
| (CRL 1459) | + | 2.5 | 1.9 |
| Adult corneal fibroblast | − | 0.20 | 2.8 |
| (WU 77245) | + | 3.6 | 7.6 |
| Adult gingival fibroblast | − | 1.3 | 0.82 |
| (CRL 1292) | + | 4.2 | 1.8 |
| Adult lung fibroblast | − | 0.22 | 1.8 |
| (WU 827105) | + | 4.3 | 4.0 |

ND, not detected. Confluent cell cultures were placed in serum-free medium containing 0.01% dimethyl sulfoxide with (+) or without (−) PMA. Samples of conditioned media were collected after a 24-hr incubation and assayed for immunoreactive collagenase and the 28-kDa metallo-endoprotease inhibitor. The data represent the mean values from duplicate flasks.

by 2- to 3-fold in agreement with our previous results (16). This increase in inhibitor levels could explain the unusually low levels of collagenase activity present in the conditioned medium of induced fetal skin, colon, corneal, and lung fibroblasts. Separation of the PMA-induced fetal skin proenzyme from the 28-kDa inhibitor by carboxymethylcellulose chromatography (2) yielded an enzyme preparation (≈0.8 unit/μg of immunoreactive protein) with a specific activity of 0.45 unit/μg of total protein. These results suggest that the 28-kDa metallo-endoprotease inhibitor secreted by human fibroblasts can modulate the activity of secreted collagenase and, therefore, functional enzyme assays may not accurately reflect the total amount of enzyme protein secreted by cultured cells.

## DISCUSSION

Human skin fibroblast collagenase is synthesized as a preproenzyme of $M_r$ 54,052, as predicted from analysis of the primary structure of collagenase mRNA. This is in good agreement with the molecular weight of the enzyme synthesized in a cell-free translation system (9). Cultured human skin fibroblasts secrete 57- and 52-kDa forms of the proenzyme. We have now shown that the appearance of the minor, 57-kDa, proenzyme species is due to partial N-linked glycosylation at one or both of the N-glycosylation recognition sites (asparagine-120 and -143) found in the collagenase primary structure. Induction of collagenase synthesis with PMA results in concomitant, proportional, increases in the secretion of both enzyme forms and in transcription of a single mRNA. This suggests that within a wide range of levels of collagenase synthesis a certain constant percentage of the total enzyme is subjected to posttranslation modification. It is unlikely that N-glycosylation of this enzyme is involved in the transport of collagenase through the cell membrane (32), since the unmodified form represents the bulk of the secreted protein. On the other hand, the presence of oligosaccharides apparently does not affect either the catalytic properties of the enzyme or the pathway of its proteolytic activation. Indeed, limited digestion with trypsin generates mature active enzyme forms with identical amino termini, independent of the presence of oligosaccharides. Collagenase glycosylation is apparently conserved in evolution as suggested by studies of rabbit synovial collagenase (33); however, its biological significance remains unclear. One can speculate that once secreted, glycosylated and nonglycosylated species may be targeted for different functions, through interaction with different components of the extracellular matrix *in vivo*.

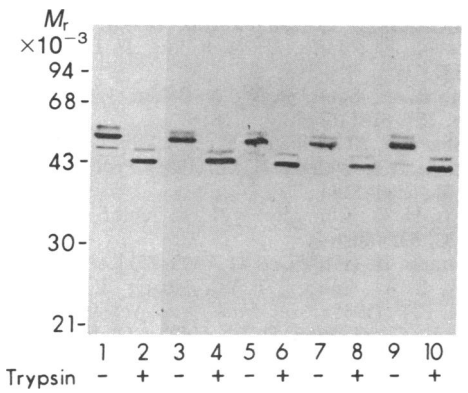


FIG. 6. Comparison of proteolytically activated collagenase secreted by human endothelial cells and fibroblasts derived from different tissues. Collagenase secretion was induced by treatment with PMA as indicated in Table 1 in all cell lines shown except adult skin fibroblast. Samples of serum-free conditioned media were treated with trypsin, where indicated (+), prior to electrophoresis. The immunoblot was stained for immunoreactive collagenase secreted by human endothelial cells (lanes 1 and 2), fetal skin (lanes 3 and 4), adult skin (lanes 5 and 6), and corneal (lanes 7 and 8) fibroblasts. Immunostaining of purified proenzyme (lane 9) and trypsin-activated skin fibroblast collagenase (lane 10) is shown for comparison. Numbers indicate the migration positions of molecular weight markers.

Human skin collagenase is apparently the most ubiquitous type of interstitial collagenase, because it is synthesized by fibroblasts derived from a variety of human connective tissues as well as by human alveolar macrophages (34) and endothelial cells (35). Our results show that the constitutive level of collagenase expression varies widely and is tissue specific. This variation could reflect tissue differences in collagen metabolism; however, the possibility that the cells derived from these tissues react differently to a transition into tissue culture cannot be excluded.

The synthesis of the 28-kDa metallo-endoprotease inhibitor does not show a direct correlation with the level of collagenase production. Our results show that collagenase activity can be modulated by this protein and thus distort the interpretation of measurements of enzyme secretion based solely on the activity assays. In an analogous system, protease nexin, a specific inhibitor of the plasminogen acti-

vator urokinase, has been reported to regulate urokinase activity present in human foreskin fibroblast conditioned medium (36). Although the mechanism(s) whereby procollagenase is activated *in vivo* is not well defined, it is clear that the interaction between collagenase and its inhibitor plays an important role in the regulation of extracellular proteolysis.

The expression of the fibroblast interstitial collagenase gene appears to be regulated developmentally as evidenced by a level of collagenase secretion 10- to 15-fold greater in adult than in fetal skin fibroblasts. Also, it has been shown that differentiated alveolar macrophages synthesize fibroblast-type collagenase and the 28-kDa inhibitor (34), while the cells from the promyelocytic leukemia cell line HL-60 secrete neither collagenase nor the 28-kDa inhibitor (37). In contrast, cells from the monocytic tumor U937, express the inhibitor but only barely detectable levels of collagenase in the basal state (38). When these cells are induced to differentiate with PMA, HL-60 cells express the 28-kDa inhibitor, but the collagenase gene remains inactive (37). On the other hand, U937 cells, which express inhibitor in the basal state, begin to synthesize large amounts of collagenase after PMA induction (38). These data suggest that agents like PMA can induce enzyme synthesis only after cells pass a commitment stage in development leading to a transition of the collagenase gene into a fraction of transcription active chromatin (39). Once the commitment occurs, collagenase expression can be regulated by agents affecting cellular proliferation, such as growth factors (40, 41), intracellular architecture (42–44) (PMA and cytochalasin B), or extracellular matrix integrity (45, 46). Finally experiments on transformation of rat fibroblasts (47) suggest that oncogene products may be involved in the regulation of a collagenase-related protein (9). Identification of the effector molecule responsible for the modulation of collagenase synthesis may, therefore, provide a further clue to understanding the underlying metabolic relationships between cell proliferation, involvement of morphogenic substances in cell communication, differentiation, and tumorigenesis.

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