Degradation of Type I Collagen by Rat Mucosal Keratinocytes

EVIDENCE FOR SECRETION OF A SPECIFIC EPITHELIAL COLLAGENASE*

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Feeder-cell-independent serially propagating keratinocytes from rat oral mucosa (tongue) dissolved reconstituted type I collagen fibrils, although rather slowly. Analysis of the conditioned medium from such cultures revealed secretion of a M₅ = 65,000 collagenase which remained almost entirely latent in the absence of exogenous protease activity. Addition of trypsin (0.1–1.0 μg/ml) or plasmin (1.0–4.0 μg/ml) resulted in substantial acceleration of the collagenolytic process in stimulated secretion of latent collagenase and, at higher concentrations, in conversion of the latent enzyme to the catalytic form. The keratinocyte collagenase was indistinguishable from interstitial, fibroblast-type collagenase by several criteria including: (i) cleavage of native type I collagen in solution at the characteristic collagenase-sensitive locus at 22 °C and dissolution of reconstituted type I collagen fibrils at 35 °C; (ii) activation by trypsin and by organomercaptan reagents and inhibition by Zn²⁺ and Ca²⁺ chelators; and (iii) cross-reaction with antibody to fibroblast-type procollagenase. Expression of collagenolytic activity in keratinocyte cultures was effectively regulated by cell density. The activity (on a per cell basis) was maximal at 10–20% confluence and was more than 95% "contact-inhibited" at subconfluent and early confluent densities (2–4 × 10⁵/cm²). Our findings show that mucosal keratinocytes possess a potent enzymatic apparatus for degradation of interstitial collagen fibrils which includes a classical vertebrate collagenase.

The biologic degradation of interstitial collagen fibrils presumably is initiated by proteolytic attack on the constituent collagen molecules by the enzyme collagenase (1, 2). In support of this concept, collagenase and its inactive precursor, procollagenase, have been identified in conditioned culture media or extracts of cells classically associated with connective tissue metabolism in healthy and inflamed tissues (3–10).

Invasion of mesenchymal domains by epithelial cells during fetal development and during carcinoma growth requires dissolution of interstitial (type I and III) collagen fibrils which form the skeletal framework of the subepithelial connective tissue. It is still unresolved whether invasive epithelial cells degrade stromal connective tissue by secretion of lytic en-

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Fig. 1. Development of feeder cell-independent keratinocyte lines from rat oral mucosa. A, rat oral mucosa from the ventral surface of the tongue was explanted in a 35-mm dish and incubated for 14 days at 32 °C with MEM, 20% fetal calf serum, 0.5% dimethyl sulfoxide. Note absence of fibroblasts around the continuous epithelial sheet. Stained with Coomassie Blue. B, clonal density subculture (REP) seeded on a bed of human skin fibroblasts and grown for 7 days at 32 °C as described above. Stained with Rhodanile Blue. C, fourth passage culture (REP) seeded at clonal density on a human fibroblast feeder layer and incubated at 37 °C for 14 days. The fibroblasts are largely displaced from the growth surface and appear as dark dotted lines around the epithelial colonies (arrows). Stained with Rhodanile Blue. D, fourth passage colony seeded without feeder cells. Multilayering and stratification (pyknotic nuclei) are evident toward the center (thick arrow). A single fibroblastic cell is seen at the edge of the colony (thin arrow). Phase contrast, bar is 50 μm. E, fifth passage cells (REP) derived by subcultivation of a culture similar to that shown in Fig. 1C. The cells display the discoid or circular shape typical of single epithelial cells. Phase contrast, bar is 100 μm. F, confluent monolayer (REP) showing cobblestone morphology. Phase contrast, bar is 100 μm. G, developing three cell colony (CC1-4). SEM, bar is 10 μm. H, subconfluent to early confluent monolayer. The cells are connected by multiple focal contacts; the intercellular gaps are still open at this time but will close as the culture becomes confluent. Scanning electron micrograph, bar is 10 μm.

20% confluency) dissolved the fibril coating after a characteristic 3 to 4-day lag period (Fig. 2). At the same time, collagenolytic activity accumulated in the medium at a rate of 0.08–0.10 unit/ml/day. During the lag period the enzyme was recovered almost entirely in latent form and by day 8, after the entire substrate layer was dissolved, still only 16% of the enzyme had been activated. We considered the possibility that the active enzyme initially bound to the collagen coating, but
media from companion cultures seeded on plastic did not contain more active enzyme (data not shown). These findings prompted us to calculate the rate of secretion and activation of collagenase actually required to dissolve the collagen layer. The result showed that generation by the cell layer of as little as 15 milliliters of active collagenase/ml/day or a total of 0.1 unit/ml over an 8-day period, which is close to that observed in Fig. 2, was sufficient to dissolve the 224-225 μg collagen fibrils.

Although the level of fibroblast contamination at this stage was below the detection limit of 0.1%, we proceeded to clone the epithelial cells by limiting dilution to avoid ambiguity as to the origin of the enzyme. Fifteen out of eighteen clones harbored secreting clones detectable levels of latent collagenase activity (0.05-0.35 unit/ml) (Table I).

Keratinocyte Collagenase—The latent collagenase harvested from unstimulated mucosal keratinocyte cultures was activated to approximately the same level by trypsin and by organomercurials. Optimal activity was achieved at 5-20 μg/ml of trypsin (10 min, 22°C) and at 0.5-1.0 mM 4-amino-phenylmercuric acetate (present throughout the assay). Trypsin-activated harvest medium protein made a single initial cleavage of rat tail tendon type I molecules as evidenced by generation of 1/4 and 1/2 length fragments (Fig. 3, A and B). With longer incubation times an additional, shorter fragment accounting for approximately 63% of the a-chain size was formed by excision of 120-130 amino acids from the COOH-terminal end of the native 75% fragment. Generation of this 63% fragments (Fig. 3B, arrows), which is characteristic of harvest media from cells and tissues of the rat (45-47), was blocked by addition of the serine-protease inhibitor PMSF2, and therefore in all likelihood was catalyzed by a protease distinct from collagenase.

The inhibition pattern (Table II) was consistent with that of a metallo-protease and closely resembled those previously reported for mesenchymal collagenases (1, 2), with the possible exception of a rather surprising 65% inhibition by 1 mM 3-nitrophenyl-p'-guanidino benzoate, an active-site titrant for trypsin-like serine proteases. The inhibitory property resides with the intact compound as neither of its two hydrolysis products, 3-nitrophenol and guanidinobenzoate, had any effect. No inhibition was observed with diisopropylfluorophosphate. Of the macromolecular protease inhibitors only α2-macroglobulin blocked the activity of the enzyme.

The electrophoretic migration of the enzyme was studied by zymography under nondenaturing conditions. Concentrated harvest medium was resolved by SDS-PAGE and the resultant electrophoretogram incubated in contact with a native collagen fibril film after removal of SDS by repeated washing with Triton X-100. The epithelial harvest medium produced a major lytic band at M, = 65,000 daltons which co-migrated (within a few kilodaltons) with the human fibroblast procollagenase isozymes (M, = 65,000, 60,000). In addition, unidentified minor trailing bands were seen at M, = 68,000 and 92,000 (Fig. 4A). The M, of the procollagenase standard, and presumably of epithelial collagenase as well, determined by zymography was 5,000-10,000 higher than that previously determined with the completely unfolded proteins (5). The specificity of the reaction was shown by inclusion of parallel lanes with trypsin (1 μg) and thermolysin (1 μg) which did not produce visible lysis of the substrate under the same conditions (Fig. 4B, lanes 2 and 3). Substitution of gelatin for native collagen in the zymogram showed that the mass = 65 kDa collagenolytic enzyme had no measurable activity against gelatin and was electrophoretically distinct from the major M, 105,000 gelatin-cleaving protease secreted by rat mucosal keratinocytes (data not shown).

The results summarized above strongly suggested that the collagen-cleaving epithelial protease was a genuine vertebrate collagenase. This was further supported by immunoperoxidase staining of Western blots prepared from epithelial culture media as shown in Fig. 5. A polyclonal antibody raised in rabbits against human fibroblast procollagenase recognized a major double band (60,000, 65,000) which co-migrated with sulfate-polyacrylamide gel electrophoresis; TLCK, tosyl-lysyl-chloromethyl ketone; TPCK, tosyl-phenyl-chloromethyl ketone; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; TBS, Tris-buffered saline.

### Table I

| Collagenase activity | Number of clones |
|----------------------|-----------------|
| units/ml             |                 |
| less than detectable | 3               |
| 0.06-0.15            | 8               |
| 0.16-0.25            | 4               |
| 0.26-0.35            | 3               |
| 0.35-0.5             | 18              |

1 The abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; APMA, 4-aminophenylmercuric acetate; BB, bovine serum albumin; DMSO (in Miniprint), dimethyl sulfoxide; ECTPA, ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetracacetate; MEM, Eagle's minimum essential medium with Earle's salts; NPGB, p-nitrophenyl-p'-guanidino benzoate; PBS, phosphate-buffered saline without Ca++ and Mg++; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, tosyl-lysyl-chloromethyl ketone; TPCK, tosyl-phenyl-chloromethyl ketone; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; TBS, Tris-buffered saline.
human fibroblast procollagenase and two minor bands (45,000, 48,000), presumably activated forms of collagenase (Fig. 5, lane 2). Pre-immune IgG and immune IgG absorbed out by passage over a procollagenase-Sepharose column showed no reactivity (Fig. 5, lanes 3 and 4). On the other hand, each of nine murine monoclonal antibodies raised against human fibroblast procollagenase, including three which strongly inhibit the human enzyme, failed to cross-react with the rat enzyme.

**Stimulation of Collagen Breakdown by Proteases**—The rather high proportion of latent collagenase activity recovered in medium conditioned by rat mucosal keratinocytes (Fig. 2) suggested that the cells failed to activate the latent enzyme to any significant degree and that addition of proteases capable of converting procollagenase to the catalytic form (trypsin, plasmin) might substantially increase the rate of collagen breakdown. We therefore examined the effect of addition of P-dioxane to 50% v/v and harvested by centrifugation. The precipitate was dissolved in 50 mM Tris-HCl buffer, pH 7.5, 0.2 M NaCl, 0.5 M glucose was incubated at 22 °C with concentrated trypsin—activator harvest medium containing 0.8 unit/ml of keratinocyte collagenase. Aliquots of the reaction mixture were withdrawn at various time intervals and mixed with inhibitor (10 mM EDTA for incubation with 1 unit/ml of human fibroblast collagenase; 1 mM PMSF for trypsin). Native helical fragments and uncleaved collagen were precipitated by addition of P-dioxane to 50%

### Table II

*Inhibition of keratinocyte collagenase activity in epithelial harvest media*

| Inhibitor                  | Concentration | Fibre lysis | Inhibition |
|----------------------------|---------------|-------------|------------|
| None                       | 100           | 94.8        | 14.2       |
| Dimethyl sulfoxide         | 10.0          | 81.4        | 14.2       |
| EDTA                       | 10.0          | 8.0         | 99.2       |
| EGTA                       | 10.0          | 1.5         | 98.4       |
| 1,10-Phenanthroline        | 1.0           | 1.1         | 99.8       |
| TPCK*                      | 1.0           | 87.9        | 7.3        |
| TLCK                       | 1.0           | 90.0        | 5.1        |
| PMSF                       | 1.0           | 90.3        | 4.7        |
| NPGB*                      | 1.0           | 32.6        | 65.6       |
| p-Nitrophenol              | 1.0           | 88.1        | 16.0       |
| Guanidinobenzoate          | 1.0           | 93.9        | 1.0        |
| Dithiothreitol             | 1.0           | 91.3        | 3.7        |
| Cysteine                   | 10.0          | 100         |            |
| N-Ethylmaleimide           | 1.0           | 93.1        | 1.8        |
| HgCl₂                      | 1.0           | 13.8        | 85.4       |
| APMA                       | 5.0           | 25.5        | 75.2       |
| Leupeptin                  | 1.0           | 90.1        | 5.0        |
| Pepstatin*                 | 1.0           | 70.9        | 25.2       |
| Antipain                   | 1.0           | 83.7        | 11.7       |
| α2-Macroglobulin           | 100           | 5.1         | 94.6       |
| α1-Antitrypsin             | 100           | 92.1        | 2.8        |
| Soybean trypsin inhibitor  | 100           | 92.9        | 2.0        |
| Trasylol                   | 100           | 81.5        | 14.0       |

*For example, the lowest concentration of trypsin (0.1 μg/ml) gave an almost 5-fold stimulation of collagenase secretion but only a 2.5-fold increase in the rate of fibril dissolution, as most of the collagenase remained latent. Raising the trypsin concentration to 0.3 or 1.0 μg/ml (Fig. 6, C and D) gave no further increase in (total) medium collagenase activity but **TABLE II**

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| Pepstatin | 1.0           | 70.9        | 25.2       |
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| α2-Macroglobulin | 100        | 5.1         | 94.6       |
| α1-Antitrypsin | 100        | 92.1        | 2.8        |
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| Trasylol  | 100           | 81.5        | 14.0       |
stimulated fibril dissolution 8–10-fold as virtually all of the enzyme was converted to the active form (Fig. 6, B versus C and D). At 1.0 μg/ml of trypsin the cells failed to reattach after dissolution of the collagen coating (arrow) and apparently stopped secreting. Taken together, these findings suggest, somewhat surprisingly, that even subactivating concentrations of trypsin stimulated fibril dissolution (Fig. 6, A versus B), although not nearly as much as higher concentrations (0.3 and 1.0 μg/ml) which activated the enzyme (Fig. 6, B versus C). These findings suggest that exogenous protease activity stimulated collagen breakdown in keratinocyte cultures by two independent but synergistic effects, namely by stimulation of secretion of latent collagenase. (Fig. 6, A versus B–D) and by activation of the latent enzyme (Fig. 6, B versus C and D).

**Role of Plasminogen Activation**—Based on work by Werb et al. (28) we considered the possibility that plasmin, which mediates tissue proteolysis in a variety of processes (48), might induce collagen breakdown in epithelial cultures. Plasminogen and plasmin (4.0 μg/ml) both stimulated collagen breakdown to the same extent (Fig. 7) but did not measurably activate the latent collagenase (Fig. 6). The ability of plasmin to substitute for plasmin (Fig. 7) suggested that the cells converted the added plasminogen to the catalytic form (plasmin) by secretion of plasminogen activator(s). Analysis of the harvest medium by fibrin zymography revealed two bands of plasminogen-dependent fibrinolytic activity, a major Mr = 48,000 band, presumably u-PA, and a minor, barely visible Mr = 70,000 band, presumably t-PA (Fig. 7, inset). This was further analyzed using cells of clone CC1-4 seeded at densities varying from 5 to 100% confluence (10^4 to 4 x 10^5/cm²). As shown in Fig. 8, the rate of collagen breakdown was maximal at 5–10 x 10^5/cm² (10–20% confluence) and 15–20-fold lower at confluence (4 x 10^5/cm²). The rate of secretion of specific collagenase as measured by the radiofibril assay was also inversely related to cell density. Based on the total collagenase activity accumulated in the medium over the duration of the experiment, thinly spread cultures (5 x 10^5/cm²) secreted 8-fold more collagenase than subconfluent to early confluent (2 x 10^5/cm²) cultures (0.25 versus 0.03 unit/10⁶ cells/day).

**DISCUSSION**

This study has shown that clonal and parental rat mucosal keratinocytes maintained in culture are capable of dissolving native reconstituted type I collagen fibrils. Moreover, a collagenolytic protease was recovered from the harvest medium which was indistinguishable from genuine vertebrate collagenases by a number of criteria, most notably by its ability to cleave native molecules of type I collagen in solution at 22 °C in a manner characteristic of mammalian collagenases and to completely dissolve reconstituted, trypsin-resistant collagen fibrils at 35 °C. In support of this conclusion it was shown by zymography and immunostaining of Western blots that the collagenolytic activity co-migrated with (human) fibroblast procollagenase and was recognized by antibody to this enzyme. Other properties shared with vertebrate collagenases derived from mesenchymal cells include an inhibition pattern consistent with that of a metallo-protease and a requirement for exposure of the latent form to exogenous protease activity (trypsin) or to organomercurials (4-aminophenylmercuric acetate) for generation of catalytic activity. The epithelial collagenase possessed little or no activity against gelatin and in SDS-PAGE was clearly separated from the major Mr = 105,000 gelatinase also produced by these cells.

It has previously been shown that intact epithelial sheets isolated from the edges of healing wounds in guinea pigs and rabbits, and from thyroxine-induced involving anuran tail-
FIG. 6. Stimulation by trypsin and plasminogen of collagen breakdown by keratinocyte clonal line CCI-4. CCI-4 cells were seeded at a density of 200,000/cm² in companion wells coated either with labeled or with unlabeled reconstituted collagen fibrils and incubated for 4 days with MEM, with 5 mg/ml BSA in the absence of serum. Trypsin (0.1, 0.3, and 1.0 µg/ml) or plasminogen (4.0 µg/ml) was added at the beginning of the incubation period to stimulate collagen breakdown. For each set of companion cultures, the daily release of radioactivity (O, □) (as a measure of fibril dissolution) and the accumulation in the medium of trypsin-activatable and spontaneous collagenase activity (A, △) were monitored. Medium from the latter cultures was divided into two aliquots; one was immediately mixed with 300 µg/ml soybean trypsin inhibitor and assayed for catalytically active enzyme by radiofibril assay; the other was preincubated with trypsin (20 µg/ml; 10 min, 22 °C), then mixed with (300 µg/ml) soybean trypsin inhibitor, and finally assayed for resultant total collagenolytic activity. O, □, radioactivity released from the substrate coating in the presence (O) or absence (□) of cells; △, Δ, collagenolytic activity in trypsin-activated (Δ) or unactivated (Δ) medium. Cells were incubated in MEM-BSA (A); MEM-BSA with 0.1 µg/ml trypsin (B); MEM-BSA with 0.3 µg/ml trypsin (C); MEM-BSA with 1.0 µg/ml trypsin (D); and MEM-BSA with 4.0 µg/ml plasminogen (E). The susceptibility of the substrate coating to trypsin (0.1, 0.3, and 1.0 µg/ml) is shown in cell-free wells in Fig. 6, B, C, and D. Each point is mean of duplicate determinations.

FIG. 7. Stimulation of keratinocyte collagen breakdown by plasminogen and plasmin. Keratinocytes (REP) were seeded at 75,000 cells/cm² on reconstituted [3H]collagen fibrils and incubated with MEM-BSA either without (□) or with plasminogen, 4.0 µg/ml (○) or plasmin, 4.0 µg/ml (●). The release of radioactivity was monitored daily. Controls were incubated without cells either with MEM-BSA alone (▲) or with medium supplemented with 4.0 µg/ml of plasmin (▲). Each point is mean of duplicate determinations. Inset shows zymogram of conditioned (plasminogen-free) culture medium from companion culture. The medium was incubated at 22 °C with 2% SDS in electrophoretic sample buffer and resolved by electrophoresis in 11% polyacrylamide. After washing with 2.5% Triton X-100 in 50 mM Tris-HCl, pH 8.1, the electrophoretogram was overlaid with a fibrin-agar gel either with (lane 1) or without (lane 2) plasminogen (20 µg/ml) and incubated for 4 h at 37 °C. The fibrin agar gel was then stained with Coomassie Blue to visualize lytic bands. In the presence of plasminogen (lane 1), but not in its absence (lane 2), the conditioned medium produced a major lytic band at 48 kDa and a minor, barely visible band at 70 kDa.

FIG. 8. Density dependence of collagen fibril dissolution by mucosal keratinocytes. The cells (REP) were seeded on a film of reconstituted radiolabeled collagen fibrils in the presence of 1.0 µg/ml plasminogen at the densities (cells/cm²) indicated in the figure. Daily cumulated radioactivity released to the medium was monitored as a measure of collagen breakdown. The fibril dissolution data were converted to rates of collagen breakdown over the first 2 days (△), 3 days (○), and 4 days (●) and expressed as µg of collagen degraded per min per 10⁶ cells and plotted as a function of seeding density. Each point is mean of duplicate determinations.

This study, however, provides the first unequivocal evidence of elaboration of a genuine collagenase by serially propagated parental and clonal epithelial cells of any mammalian species and the first demonstration that keratinocytes are indeed capable of dissolving fibrils of type I collagen. The reactivity of antibody to human fibroblast procollagenase with Western blots of keratinocyte culture media suggests that the epithelial collagenase shares at least some antigenic determinants with skin, secretes collagenolytic activity in tissue culture (11–13).
the fibroblast enzyme, although further studies are necessary to determine the extent of homology. The maximal production of collagenase (0.25 unit/10⁶ cells/day) was several-fold lower than that obtained with dermal fibroblasts in our laboratory (1–2 units/10⁶ cells/day) but, if the smaller cell size of the keratinocyte is taken into account, the destructive potential of a subconfluent epithelial cell layer, as measured by the rate of fibril dissolution, is indeed comparable to that of subconfluent fibroblast cultures.

Previous studies by Johnson-Wint and collaborators (11, 12, 19, 20) have shown that low passage cultures of rabbit epidermal and corneal epithelial cells do not produce collagenase but induce secretion of this enzyme in cocultured fibroblasts. Together with the independent finding by Bauer et al. (14) that stromal fibroblasts in human basal cell carcinomas contain immunoreactive collagenase protein, whereas the epithelial tumor cells do not, these studies have led to the suggestion that epithelial invasion of mesenchymal domains requires induction of degradative enzymes in fibroblasts across the epithelio-mesenchymal interface. Our demonstration of a genuine vertebrate collagenase in media conditioned by keratinocytes provides evidence for existence of an alternative pathway utilizing an endogenous epithelial collagenase.

It does not yet prove, however, that this pathway is actually used in vivo nor does it exclude the possibility that epithelial cells enhance their invasive potential by induction of adjacent mesenchymal cells. Still another degradative mechanism deserves further consideration. Studies by Birek et al. (49) have shown that epithelial cells from porcine periodontal ligament are capable of phagocytizing collagen fibrils in culture. This process clearly bears resemblance to the phagocytic uptake of fibril fragments by fibroblasts which has been well documented in vivo and in vitro (50, 51). The role of collagenase in this process, if any, remains unclear. It is still uncertain whether collagenase-mediated and phagocytic collagen breakdown are two sequential stages of a single pathway (i.e. initial extracellular fragmentation of fibrils by collagenase followed by phagocytic uptake and further degradation in phagolysosomes) or represent two altogether distinct and alternative degradative mechanisms. The biological role of either mechanism in keratinocyte-mediated collagen breakdown in vivo remains to be established.

The observation that rat mucosal keratinocytes activated only a small fraction (<20%) of the latent collagenase released to the culture medium raises the question whether exogenous components are required for activation of the enzyme in vivo or, alternatively, whether secretion and activation of procollagenase are independently regulated and therefore not necessarily coordinately expressed in culture. However, this question cannot be answered with any degree of certainty on the basis of current knowledge inasmuch as the biologic pathway for procollagenase activation remains obscure. At least four mechanisms have been proposed involving: (i) an endogenous procollagenase-activating protease (52); (ii) an apparently nontactylating enzyme found in the skin and in the involving uterus (53); (iii) a nonenzymatic thiol-exchange reaction (54); and (iv) an exogenous protease such as plasmin (28). A number of studies have shown that plasmin can activate procollagenase in test tube experiments but it is uncertain to what extent this reaction takes place in vivo or even in vitro in cell culture systems. Studies by Werb et al. (28) have shown that human synovial fibroblasts possess far more activity against collagen substrates when grown in the presence of plasminogen than in its absence, although plasmin by itself has little or no lytic activity against collagen. Further studies (55, 56) have indicated that the mechanism in all likelihood is more complex than merely a conversion of procollagenase to the catalytic form since many proteolytic enzymes, including plasmin, stimulate secretion of procollagenase under conditions which yield little or no activation of the zymogen. In the present study, we have also found elevated secretion, but little or no activation, of latent collagenase in the presence of plasminogen/plasmin. Trypsin had a similar effect at or below 0.1 μg/ml but did activate the latent collagenase at higher concentrations. Although the dissolution of collagen fibrils by the cells was somewhat stimulated even at subactivating protease concentrations, it was apparent that the rate of collagen breakdown was closely linked to the generation of active enzyme.

The observation made in previous studies (16, 17) that epithelium derived from the edges of dermal wounds secretes collagenolytic activity in organ culture, whereas that from uninjured skin does not, suggests that secretion of collagenase by epithelial cells may be a wound healing response. We propose that enzymatic dissociation and plating of epithelial cells in culture at low densities in many ways simulate wound healing conditions and induce a similar response (i.e. secretion of collagenase) which is eventually turned off as the cells reach confluence. This offers a rational explanation of the apparent enigma that the enzyme is secreted in culture but not widely expressed in vivo. The inverse relationship between cell density and collagenolytic activity ("contact inhibition") which resulted in a 90–95% reduction of activity per cell in subconfluent and confluent cultures shows that two different phenotypes are expressed in sparse and densely populated cultures and that secretion of collagenase is predominately associated with the low density phenotype. Johnson-Wint (11) has previously shown that secretion by rabbit corneal epithelial cells of two distinct sets of regulatory factors, one which stimulates and one which inhibits stromal cell collagenase production, is also highly density regulated. The stimulating factors secreted at low cell density and the inhibitory components released at higher densities presumably serve the same overall purpose, namely to regulate the collagenolytic activity in the immediate environment. Although the culture conditions are not quite comparable, it is of note that Johnson-Wint's study (11) as well as ours showed maximal (collagenolytic) activity at 25–50 X 10⁶ cells/cm² which represents 5–10% of confluent densities. The dependence of collagenolytic activity on cell density represents a radical departure from the behavior of dermal fibroblasts which are rather insensitive to changes of cell density in vitro and continue to secrete collagenase at confluency (57). It will be of considerable interest to determine whether the collagenolytic activity of rat mucosal keratinocytes is regulated by a set of (endogenous) factors similar to those described by Johnson-Wint (11).

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Material and Methods

Materials. Eagle’s minimum essential medium with Earle’s salts (MEM), fetal bovine serum, and Pencillin G were from Flow laboratories (Rockville, Md.). Chalk embryo extract, chick liver extract, and trypsin (2.5%) were from Gibco (Grand Island, N. Y.). Dimethylsulfoxide (DMSO). Staphylococcal alpha-toxin, were prepared as previously described. 

Preparation of human skin fibroblasts (ATCC, CRL 12241). The identity of cell lines used in this study has been established by a number of criteria including: (1) formation of keratinizing colonies in non-differentiated medium, (2) production of keratin secretory proteins, (3) differentiation of keratinocytes to form keratinized, stratified squamous epithelium, (4) detachment of reactivity of antibodies raised against cell membranes from cultured, and eventually form immortal feeder-cell-independent cell lines with seemingly unlimited number of passages (122-27).

The development of epithelial cell lines is summarized in Fig. 1. Briefly, explants of whole epidermis from 18-24 h embryos were used as a source of keratinocytes. These cells were obtained by enzymatic digestion of the epidermis, and cultured at 39°C in Eagle’s minimal essential medium (MEM) with 10% fetal calf serum and 200 µg/ml of penicillin G, 20 µg/ml of streptomycin, and 100 UI/ml of fungizone. The stock was passaged as a 1:10 ratio, there being >90% of viable cells. The growth was monitored by microscopic examination of cell monolayers, and a daily count of Feeder cell inoculated with 5 x 10^4 cells per dish was made as a measure of cellular growth. The initial passage contained 5-6 days after trypsinization and seeding. Two to three passages were made without further addition of growth factors or vitamins. The culture was maintained at 37°C in an atmosphere of 10% CO2 and 5% O2. The cultures were examined for mycoplasma infection at regular intervals using the fluorescent DNA method. No contamination by mycoplasma was found in the cultures used in this study.

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Keratinocyte Collagenase

Preparation of Monospecific Antibodies to Human Fibroblast Procollagenase

Fibronectin procollagenase was purified from conditioned harvest medium by sequential chromatography on heparin-Sepharose, protein A-Sepharose and train-chelate column. Purified enzyme was blocked by incubation for 1 h at 37°C with borate buffered saline. The specific activity of the enzyme was converted to catalytic form by exposure to SDS and pepsin. The samples were then resolved by electrophoresis in 11% polyacrylamide gels. Following electrophoresis, the gels were washed for three consecutive 40 min periods in 21/2 Triton X-100 in PBS. The gels were then stained by 0.025% Coomassie blue in 70% ethanol and 10 mM Tris-HCl, pH 8.1. The samples were then incubated either with anti-procollagenase IgG (600 pg/ml) or preimmune IgG overnight at 4°C followed by 1 h at room temperature. The lanes were then washed with 0.15 M NaCl, 0.1% Tween 20 in PBS and overlaid with 5 ml X-100 in 50 mM Tris-HCl, pH 8.1, containing 0.15 M NaCl, 1% Tween 20 and 1% Triton X-100. The samples were then incubated with the antibodies overnight at 4°C followed by 1 h at room temperature. During this time, activator activity detected by the method of Tovblin et al. 1981 was visualized by a similar method 1985. Culture medium containing latent collagenase was isolated by centrifugation 37°C, and incubated with 300-500 mg of antigen entrapped in polyacrylamide gels. IgG from immune and preimmune sera were isolated by Protein A-Sepharose chromatography. Immune IgG was identified as Monospecific antibodies from outdated human plasma by the method of Tovblin et al. 1981. The samples were then resolved by electrophoresis in 11% polyacrylamide gels. Following electrophoresis, the gels were washed for three consecutive 40 min periods in 21/2 Triton X-100 in PBS. The gels were then stained by 0.025% Coomassie blue in 70% ethanol and 10 mM Tris-HCl, pH 8.1. 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