Enhanced Synthesis of Collagenase by Human Keratinocytes Cultured on Type I or Type IV Collagen

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Human keratinocytes in culture are known to produce collagenase. As part of studies to ascertain the physiologic stimuli for collagenase production by keratinocytes, we wanted to determine whether extracellular matrix could modulate the production of collagenase in vitro. Immunoprecipitable collagenase from the conditioned medium of cells grown on different types of matrix was measured. Metabolically labeled human keratinocytes were cultured in 0.1 mM calcium in serum-free medium on colloidal gold-coated coverslips plus type IV collagen, type I collagen, or laminin or in the absence of matrix. Immunoprecipitation of the conditioned medium with anti-collagenase antiserum was performed and the immunoprecipitates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, fluorography, and densitometry. The keratinocytes cultured on type IV or type I collagen produced more collagenase than did those cultured on laminin or in the absence of matrix. This effect did not reflect a general increase in secreted proteins, because the production of tissue inhibitor of metalloproteinase, or TIMP, did not increase under the same conditions. Phagocytosis of the gold salts by the keratinocytes migrating on types I or IV collagen did not account for the increased collagenase produced by these cells since the effect persisted in the absence of the colloidal gold and phagocytosis of latex beads did not augment collagenase production. J Invest Dermatol 94:341–346, 1990

We have recently shown that cultured human keratinocytes synthesize and secrete procollagenase [1,2], the metalloproteinase which initiates the degradation of native collagen. However, the physiologic significance of the production of collagenase by keratinocytes in vivo remains unknown. One situation in which keratinocyte-derived collagenase may play a role is in wound healing, when the cells are no longer stationary but migrating over a wound bed of fibronectin, fibrin, and collagen [3,4]. This hypothesis is supported by early wound healing studies [5–7], which suggested the presence of collagenase activity in wound-edge epithelium and, more recently, by the findings of Woodley et al, who demonstrated type I and type IV collagenase activity in the conditioned medium from explants of human epithelium cultured on nonviable pig dermis [8].

Studies using rabbit synovial fibroblasts have shown that collagenase gene expression correlates with changes in cell shape [9]. Clearly, epidermal cells undergo marked phenotypic changes during wound healing [10], which may increase the production of collagenase by these cells. In addition, the interstitial collagens (types I–III) have been shown to stimulate collagenase activity in cultured human fibroblasts [11]. Under normal physiologic conditions, basal keratinocytes are stationary and in contact with the lamina lucida, a laminin-rich structure which separates the cells from the lamina densa, a structure rich in type IV collagen, and the extracellular matrix of the dermis, composed in large part of types I and III collagen. During wound healing, keratinocytes migrate over type IV collagen, in epidermal wounds, or type I collagen, in dermal wounds. Thus, the interaction between the keratinocytes and these collagen molecules may stimulate collagenase production, similar to their effect on dermal fibroblasts.

Recent studies by O'Keefe et al [12] and by Woodley et al [13] have provided an in vitro model with which to study collagenase production by keratinocytes in contact with extracellular matrix. In these studies, keratinocytes were plated on colloidal gold-coated coverslips, as described by Albrecht-Buehler [14], which were subsequently coated with matrix molecules. The colloidal gold allows rapid assessment of keratinocyte behavior on the different matrices. In this system, keratinocyte migration is promoted by types I and IV collagen and inhibited by laminin [13].

The purpose of this study was to determine if extracellular matrix affected collagenase synthesis by keratinocytes. We assessed collagenase production by keratinocytes plated on colloidal gold-coated coverslips plus types I or IV collagen or laminin using immunoprecipitation.

MATERIALS AND METHODS

Cell Culture Human neonatal keratinocytes were obtained from neonatal foreskins [15] and subcultured in serum-free MCDB 153 medium with 0.1 mM calcium and supplements: hydrocortisone,
0.4 μg/ml; insulin, 5 μg/ml; phosphoethanolamine, 0.1 mM; ethanolamine, 0.1 mM; epidermal growth factor, 5 ng/ml; bovine pituitary extract, 150 μg/ml; histidine, 0.24 mM; isoleucine, 0.75 mM; methionine, 0.09 mM; phenylalanine, 0.09 mM; tryptophan, 0.045 mM; tyrosine, 0.075 mM [16]. The cells were passaged at least twice in the above medium, which renders them free of contaminating fibroblasts [1].

Cell Culture on Extracellular Matrix In order to assess the effect of extracellular matrix on collagenase production, the keratinocytes were subcultured into dishes coated with pepsinized human placental type IV collagen (Sigma Chemical, St. Louis, MO), laminin isolated from the Engelbreth-Holm-Swarm tumor (BRL Laboratories, Bethesda, MD), or pepsin-solubilized bovine dermal type I collagen (Vitrogen 100, Collagen Corp., Palo Alto, CA). Extracellular matrix proteins (0–90 μg) were added to 35-mm culture dishes in 1 ml of Hank’s buffered salt solution (HBSS). After incubation for 2 h at 37°C, the protein solution was aspirated from the dish, the dish was rinsed with HBSS, and 100,000 keratinocytes were plated in 1 ml of MCDB media as detailed above. In most experiments, cell behavior on the various matrices served as an internal control, allowing assessment of matrix binding and keratinocyte function. In order to monitor the behavior of cells plated on matrix proteins, a coverslip coated with colloidal gold [14] was placed in the petri dishes and the matrix molecules were subsequently added. In other experiments, the matrix molecules were added directly to the dishes in the absence of the coverslips.

Immunoprecipitation Collagenase and TIMP were assessed by immunoprecipitation of metabolically labeled protein from the conditioned supernatants with specific antiserum as previously described [1,2]. One hundred thousand keratinocytes were subcultured in 35-mm dishes coated with extracellular matrix. The cells were cultured in methionine-free MCDB without amino acid supplements and without hydrocortisone, which suppresses collagenase production by human skin fibroblasts [17]. L-[35S]-methionine (Amersham Corp., Arlington Heights, IL), 50 μCi/ml, was added to the cultures and the labeled conditioned medium collected after 36 h. Preabsorption with normal rabbit serum [18] and immunoprecipitation with rabbit antisera to collagenase or TIMP were performed. Equal volumes of immunoprecipitation samples (50–70 μl) were analyzed on 10% SDS-polyacrylamide gels according to Laemmli [19]. The gels were lightly stained with Coomassie Blue, impregnated with Fluor-o-Hance (Research Products International, Mount Prospect, IL), dried, and incubated with Kodak X-Omat XR5 x-ray film (Eastman Kodak, Rochester, NY) for 5–7 d at −70°C. Densitometry tracings of the resultant fluorograms were performed with a Hoefer GS 300 Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA).

Phagocytosis of Latex Beads In order to assess the effect of phagocytosis on collagenase production by the keratinocytes, 0.9-μm polystyrene latex beads (Sigma Chemical, St. Louis, MO) were added to metabolically labeled keratinocytes and immunoprecipitation of the conditioned medium performed after incubation with the beads for 48 h. The latex beads were coated with fibronectin (250 μg/ml) or BSA (250 μg/ml) as described by Takashima and Grinnell [20]. Indirect immunofluorescence studies were performed to demonstrate phagocytosis of the beads by the keratinocytes. Larger beads (2.02 μm, Duke Scientific Corp., Palo Alto, CA), which were more easily visualized with indirect immunofluorescence, were coated with fibronectin or BSA and incubated with plated keratinocytes for 24 h. The cells were lightly treated with 0.25% trypsin (GIBCO Labs, Grand Island, NY) to remove the beads adherent to the cell surface, fixed with 3% formaldehyde in PBS, and permeabilized with 0.1% Triton-X in PBS. Indirect immunofluorescence with rabbit antisera to fibronectin or BSA (Organon Teknika-Cappel, West Chester, PA) and fluorescein-conjugated goat anti-rabbit IgG (Organon Teknika-Cappel) was then performed.

Figure 1. A: Immunoprecipitation of collagenase produced by human keratinocytes plated on type IV collagen and laminin. Keratinocytes were plated on coverslips coated with colloidal gold plus type IV collagen (T-IV, 15 μg/ml, lanes 1 and 2), laminin (LN, 90 μg/ml, lanes 3 and 4), or no matrix molecules (PL, lanes 5 and 6). The conditioned medium was immunoprecipitated with rabbit anti-collagenase antiserum (e) or normal rabbit serum (c). Equal volumes of the immunoprecipitation samples were analyzed on 10% SDS-polyacrylamide gel electrophoresis and fluorography as per Materials and Methods. This fluorogram demonstrates that more collagenase (large arrow), as reflected by the band density, was synthesized by the keratinocytes plated on type IV collagen, compared with the amount synthesized by cells plated on laminin or in the absence of matrix molecules. The protein doublet immunoprecipitated in addition to collagenase (*) represents a contaminant in the immunogen. Fibronectin (small arrow) binds non-specifically to staph protein A and can be seen in both the control and experimental lanes. The migration positions of marker proteins are shown on the left and represent 200, 97.4, 69, 53, 46, and 22.5 kDa. B: Densitometric scan of collagenase bands from fluorogram in A. The fluorogram in A was scanned with a densitometer and the relative densities of the collagenase bands are shown.
RESULTS

As demonstrated in a representative fluorogram in Fig 1A, the keratinocytes plated and cultured on type IV collagen produced more collagenase than those cultured on gold-coated coverslips in the absence of matrix or those plated on laminin. The relative densities of the collagenase bands on the various matrices were compared by densitometry, as shown in Fig 1B, in which the relative densities of the collagenase bands from the fluorogram in Fig 1A are depicted. Further augmentation of collagenase production was not seen with concentrations of type IV collagen greater than 15 μg/ml. Woodley et al [13] also reported little augmentation of keratinocyte migration with concentrations of type IV collagen greater than 15 μg/ml, presumably due to saturation of the binding sites on the petri dishes. The increased amount of collagenase produced by the keratinocytes plated on type IV collagen was not a result of differences in the number of cells in the dishes with different matrix molecules. Keratinocytes (100,000) were plated as per the immunoprecipitation experiments on type IV collagen (15 μg/ml), laminin (60 μg/ml), or uncoated gold coverslips and the attached cells trypsinized and counted in a Coulter counter after 36 h in culture. The number of attached cells were 50,560 ± 11,580 on type IV collagen; 58,220 ± 7460 on laminin; and 57,040 ± 5160 on uncoated coverslips. Similar results were obtained by Woodley et al [21], who were also unable to demonstrate differences in keratinocyte attachment to different matrix molecules when cultured under the same conditions as used in this study.

Type IV collagen was used in the initial experiments because of its marked effect on keratinocyte migration [13]. However, despite producing a less pronounced effect on keratinocyte migration [13], type I collagen (15 μg/ml) also stimulated collagenase production by the keratinocytes when compared with no matrix or BSA (Fig 2).

Cultured keratinocytes also synthesize and secrete TIMP, a 28.5 kD glycoprotein, into the medium [2]. In order to determine whether the production of collagenase was selectively increased in the presence of type IV collagen, TIMP was immunoprecipitated from the conditioned medium of keratinocytes plated on type IV collagen or laminin. In contrast to the increase in collagenase synthesis seen when the cells were plated on type IV collagen, the synthesis of TIMP did not increase (Fig 3). Thus, the enhancement of collagenase production in the presence of type IV collagen did not reflect an overall increase in secreted proteins by the keratinocytes. In the experiment shown in Fig 3, the amount of TIMP secreted by the cells cultured on laminin was greater than that secreted by the keratinocytes cultured on type IV collagen. This finding could not be confirmed in several subsequent experiments, however.

The use of colloidal gold-coated coverslips enabled us to monitor keratinocyte behavior on the matrix proteins, which served as a control for matrix binding. When the keratinocytes migrate on the gold salts and collagen-coated coverslips, phagocytosis of the gold particles occurs and can be visualized with light microscopy. It is also likely that the cells phagocytose the matrix (i.e., type IV collagen) when migrating, since tracts cleared of matrix can be seen with indirect immunofluorescence using anti-matrix antibody and fluorescent-conjugated second antibody [13]. In order to determine if the colloidal gold was influencing the effect of type IV collagen on collagenase synthesis, we evaluated collagenase production by keratinocytes in the absence of colloidal gold. When type IV collagen was allowed to bind to plates without colloidal gold-coated coverslips, similar results, specifically, increased collagenase production in the presence of type IV collagen, was seen (data not shown). However, since phagocytosis has been shown to augment collagenase production by other cell types [22,23], experiments were performed to assess directly whether phagocytosis alone could enhance collagenase synthesis by the keratinocytes. Because keratinocytes may phagocytose matrix in the absence of gold particles, latex beads were used to assess the effect of phagocytosis on collagenase production. Keratinocytes have been shown to phagocytose fibronectin-coated latex beads [20]; therefore, the cells were incubated with fibronectin-coated beads, BSA-coated beads, or uncoated beads or in the absence of beads, and the newly synthesized collagenase assessed by immunoprecipitation. Indirect immunofluorescence studies demonstrated that the keratinocytes phagocytosed fibronectin-coated beads, as shown in Fig 4, as well as the uncoated and BSA-coated beads, although they appeared to phagocytose more of the fibronectin beads. No differences in collagenase synthesis were seen by cells actively phagocytosing latex beads (Fig 5, lanes 1–3), however, compared with cells incubated in the absence of beads (Fig 5, lane 4). These studies demonstrate that, although phagocytosis occurs when cells are plated on gold particles with matrix, the
augmentation of collagenase production by type IV or type I collagen could not be accounted for by phagocytosis.

DISCUSSION

These studies indicate that collagenase synthesis and secretion by keratinocytes are stimulated by either type I or type IV collagen. Laminin, a large extracellular matrix protein localized to the basement membrane in skin, did not stimulate collagenase synthesis. These results do not reflect increased cell attachment to the various matrix proteins, since there were no significant differences in keratinocyte attachment to the matrix proteins or plastic. The increase in collagenase production by collagen was also not a result of a general increase in protein synthesis, since TIMP expression did not increase in the presence of collagen.

The experimental system used in these studies did not allow quantitation of collagenase synthesis, since the level of production by the small number of cells in each culture dish was below the level of sensitivity of either functional collagenase assays or ELISA. Similarly, although the relative amount of newly synthesized collagenase can be qualitatively compared with densitometry by comparing the relative areas under the curves, the areas under the curves cannot be compared quantitatively since the relationship between the absorbance of the fluorographic image (and the resultant densitometry

Figure 3. Effect of extracellular matrix on TIMP production. To evaluate the specificity of the response of collagenase synthesis to type IV collagen, production of another secreted protein, TIMP, was evaluated with immunoprecipitation. Type IV collagen did not increase the production of TIMP (arrow) in the keratinocytes over the production by those cells plated on laminin (lane 2) or in the absence of matrix molecules (lane 3). The corresponding control samples, immunoprecipitated with normal rabbit serum, are shown in lanes 4–6.

Figure 4. Phagocytosis of fibronectin-coated latex beads by keratinocytes. Latex beads (2 μm) were coated with fibronectin as described in Materials and Methods and incubated with cultured keratinocytes. After 24 h, the cells were washed and lightly treated with trypsin to remove cell surface-associated beads. The cells were then fixed and permeabilized and indirect immunofluorescent staining performed.

Figure 5. Effect of phagocytosis on collagenase production by keratinocytes. Metabolically labeled keratinocytes were incubated with fibronectin-coated (lanes 1 and 5), BSA-coated (lanes 2 and 6), or uncoated latex beads (lanes 3 and 7), or in the absence of beads (lanes 4 and 8) and immunoprecipitation with anti-collagenase antiserum (lanes 1–4) or normal rabbit serum (lanes 5–8) performed. Phagocytosis of the beads did not increase collagenase production since the amount of immunoprecipitated collagenase (arrow) in lanes 1–3 was not greater than the collagenase synthesized by the non-phagocytosing cells (lane 4).
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REFERENCES


