

Regulation of Connective Tissue Collagenase Production: Stimulators from Adult and Fetal Epidermal Cells

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ABSTRACT We have examined the ability of primary adult rabbit skin cells to regulate collagenase production *in vitro*. Dermal cells constitutively produce collagenase in culture, and enzyme production by these cells can be influenced by epithelial cells. Co-culture with skin epidermal cells resulted in more enzyme production by dermal cells, whereas co-culture with corneal epithelial cells yielded less enzyme activity. Connective tissue cells from a different source, cornea, also produced collagenase when co-cultured with skin epidermal cells, although the stromal cells alone made no enzyme.

The drug cytochalasin B had very little influence on collagenase production by dermal cells, either alone or in co-culture with epidermal cells, but did significantly potentiate enzyme production by corneal stromal cells responding to epidermal effector molecules.

Epidermal-cell-conditioned medium from both fetal and adult rabbit skin was a potent source of stimulators (apparent mol wt 20,500 and 55,000) of connective-tissue-cell collagenase production. Stimulator production by epidermal cultures was cell density dependent. Optimal production of stimulators occurred in adult cultures containing 10^6 epidermal cells/ml of medium, and in fetal cultures containing 10^5 cells/ml. Inhibitors of connective tissue cell enzyme production were not detected in conditioned medium from either adult or fetal epidermal cells.

A growing body of evidence suggests that fibroblast behavior *in situ* may be regulated by neighboring cells over relatively short distances via secreted effector molecules called cytokines. Both mononuclear cells and epidermal cells produce cytokines (1). One of these molecules has been shown to be a fibroblast mitogen when derived from either monocytes (2, 3), macrophages (4), or epithelial cells (5, 6), and another molecule, mononuclear cell factor, stimulates collagenase production by fibroblasts (7). Some of these effector molecules may be interleukin 1 (2, 3, 5-7).

We are interested in cellular interactions that regulate collagenase production, and have previously shown that production of this enzyme by stromal cells from the adult rabbit cornea can be regulated *in vitro* by cell products from the epithelium of the same tissue (8, 9). Consistent with the observation that no enzyme is present in normal adult corneal tissue (10), we found that primary cultures of stromal or epithelial cells, or the two cell types mixed, made no collagenase. Indeed, conditioned-medium experiments demonstrated that the corneal epithelium made inhibitors (apparent mol wt 19,000 and 7,000) of stromal cell enzyme production. When,

however, we added cytochalasin B (CB)¹ to corneal cell cultures, their behavior changed. Under the *in vitro* influence of CB, adult corneal epithelial cells secreted substances (apparent mol wt 19,000 and 54,000) that stimulated corneal stromal cells to produce collagenase. The stromal cells required CB in this system to respond to these effector molecules; however CB alone did not stimulate enzyme production. These stimulators and inhibitors influenced stromal cell synthesis/secretion of the enzyme, and had no effect on collagenase activity or activation of the latent form of the enzyme (9). Hence, by adding CB to mixed corneal cell cultures we were able to convert them from actively inhibited to collagenase-producing cultures, a transition involving a change in the behavior of both cell types in this interaction.

Immunohistochemical staining studies that have localized collagenase *in situ* in normal skin and cornea suggest that enzyme production in these two tissues is different. Collagen-

¹ *Abbreviations used in this paper:* CB, cytochalasin B; DME, Dulbecco's modified Eagle medium; FCS, fetal calf serum; HBSS, Hank's balanced salt solution.

ase antiserum stains only the papillary dermis, the connective tissue closest to the epidermis, of normal human skin (11). In contrast, antiserum with the same specificity does not stain any portion of normal human cornea, but does stain the entire stroma of ulcerating corneas (10). Hence, collagenase appears to be a constitutive part of the dermis of normal skin at the dermal-epidermal interface, and possibly continuously produced there. On the other hand, the enzyme is not a constituent of normal cornea, but appears during ulceration, and consequently may only be produced in this tissue during reparative processes or under pathological conditions.

The presence in normal skin of constitutive dermal collagenase at the dermal-epidermal interface (11) suggests the possibility of a continuously stimulatory cellular interaction in this tissue. Hence, in the present study we have characterized the collagenase-producing capabilities of isolated primary dermal cell, epidermal cell, and mixed cell cultures from adult rabbit skin. We have also examined epidermal cells from fetal and adult rabbit skin for their ability to produce regulators of connective-tissue-cell enzyme production and compare their function in this regard with similar corneal cell cultures.

MATERIALS AND METHODS

Preparation of Cells: New Zealand white rabbits were the source of cultured cells. Primary cell cultures were used in all experiments to examine cell populations that were as close to the original tissue state as possible.

Fetal skin epidermal cells were taken from 28-d fetuses (30-d term). These were obtained by administering an overdose (390 mg) of sodium pentobarbital (Lemmon Co., Sellersville, PA; 65 mg/ml) to a timed-pregnant rabbit, surgically removing the uterus, and dissecting out the fetuses in a tissue culture hood by sterile technique. Typically, 8–12 fetuses were obtained per pregnant rabbit. The body skin, which was very sparsely haired, was removed from the whole torso of each fetus, cut into 1-cm-wide strips, and separated into epithelium and dermis by incubation in Ca^{++} - Mg^{++} -free Hanks' balanced salt solution (HBSS) plus 0.25% trypsin (Gibco Laboratories, Grand Island, NY) for 36 h at 4°C. The enzymatically loosened epidermis was removed cleanly from the underlying dermis with sterile forceps, and came off as a continuous sheet including hair follicles. The epidermal sheet was cut into fragments with a sterile scalpel blade, and the fragments were pipetted vigorously in culture medium (see *Culture Conditions*) to release the more basal cells. The remaining keratinized pieces were separated from free epidermal cells and clumps by passage through sterile gauze, and were discarded. Isolated epidermal cells and clumps were sedimented at 100 g, resuspended in 20 ml of fresh culture medium per fetal skin, and plated at determined densities.

Skin epidermal cells were also obtained from young adult rabbits (5 lb, either sex). Adult skin was dissected as 2 × 3-cm patches from the relatively hairless portion of the inner surface of the ear. Skin patches were washed by vigorous shaking in three changes of HBSS containing antibiotics, and separated into epidermis and dermis by trypsin treatment as already described. However, adult ear skin, being much thinner than fetal torso skin, was incubated in cold trypsin for only 12 h. In addition, the epidermal sheet was not cut up, but simply shaken in culture medium to release cells, and the keratinized portion discarded. Isolated, washed epidermal cells from eight patches of ear skin were pooled and suspended in 50 ml of culture medium prior to plating.

The isolated dermis from the preceding procedure was used as the source of skin dermal cells. Isolated dermal patches were washed in Ca^{++} - Mg^{++} -free HBSS, scraped gently to remove adherent epidermal cells, and cut into 2–3-mm wide strips. The dermal strips were then digested in bacterial collagenase to obtain all resident cells as described previously for corneal stroma (9). The dermal cell population obtained in this way contained some epidermal cells from hair follicles that were not completely removed by the cold trypsin separation. Typically, the dermal cells from three skin patches were pooled and used per experiment.

Isolated populations of corneal epithelial and stromal cells from young adult rabbits (5 lb, either sex) were obtained as described (9). Briefly, incubation in Ca^{++} - Mg^{++} -free HBSS plus 0.25% trypsin (Gibco Laboratories) and antibiotics at 4°C overnight allowed epithelial and endothelial cells to be removed separately and completely from the cornea. The whole stroma was then washed in two changes of Ca^{++} - Mg^{++} -free HBSS plus antibiotics and digested (37°C for 4 h) with bacterial collagenase (CLS II, 125–200 U/mg, 4 mg/ml Worthington Biochemical Corp., Freehold, NJ) in culture medium to yield a stromal cell suspension. Both populations of cells were washed and plated at the desired

cell density in fresh culture medium. For any given experiment the corneal epithelial and stromal cells, respectively, from 5–15 animals were pooled.

Fetal and adult skin epidermal cells had 50 and 75–80% attachment efficiencies, respectively; adult corneal epithelial and stromal cells, and adult skin dermal cells had a 90% attachment efficiency. Attachment efficiency was determined by comparing the number of cells added to the culture dish at the time of plating with the number of cells that had attached after 24 h.

Determination of Cell Number: The concentration of freshly isolated corneal stromal and skin dermal cells was determined with a hemocytometer or Coulter counter. Both skin and corneal epithelial cells were isolated as mixtures of individual cells and clumps, making routine determination of cell number by counting unfeasible. Therefore, the concentration of epithelial cells was expressed at the time of plating in terms of milliliters of starting cell suspension. The isolated epidermal cells from one fetal torso or eight patches (as described) of adult ear skin were routinely suspended in 20 and 50 ml, respectively, of culture medium, and the epithelial cells from 10 corneas were suspended in 40 ml of medium.

To convert 1 ml of epithelial cell suspension to actual in vitro cell number for each experiment, four replicate 1-ml aliquots of freshly isolated cells were plated in 16-mm diam Costar wells in culture medium and were allowed to attach for 24 h. Adherent cells were then fixed in the culture well in 10% neutral formalin buffered with 0.1 M sodium borate at pH 8, washed with 15% isopropanol at pH 8, and dried in the same culture dish at 50°C for 1 h. The exact concentration of adherent epithelial cells in 1 ml of suspension for each preparation was determined subsequently by a fluorescence DNA assay (12) performed directly on the dried cells in each well for which we have previously determined that each diploid rabbit cell nucleus contains 7 pg of DNA. The concentration of potentially adherent cells in 1 ml of starting suspension was typically $0.5\text{--}1 \times 10^6$ skin epidermal cells, and $2\text{--}4 \times 10^5$ corneal epithelial cells.

Culture Conditions: Culture medium for both isolating and incubating cells consisted of low glucose (1 mg/ml) Dulbecco's modified Eagle medium (DME; Gibco Laboratories) containing 5% fetal calf serum (FCS) and antibiotics (penicillin at 100 U/ml, streptomycin at 100 µg/ml; Gibco Laboratories). Since epidermal cells were sensitive to the batch of FCS used, some lots causing them to produce a conditioned medium that was cytotoxic to connective tissue cells, sera had to be screened to identify lots that did not produce this effect. Flow Laboratories (McLean, VA) fetal bovine serum lot number 29101608 was suitable and used in all experiments in this paper.

All cell culture was carried out at 37°C in a moist atmosphere of 4% CO_2 /96% air. The CO_2 level was lower than the conventional 5% to slightly raise the pH of the culture medium since connective tissue cells produce optimal amounts of collagenase in vitro under mildly alkaline conditions (13).

Skin and corneal cells isolated for evaluation of inherent collagenase production, or for use as a target cell population in a bioassay, were plated in 7-mm diam wells of Costar (Cambridge, MA) 96-well culture plates in 0.2 ml of culture medium. Both dermal and stromal cells were plated at 6.0×10^4 cells per well, and epithelial cells from both tissues at $10^4\text{--}1.5 \times 10^5$ cells per well. The same cell densities were used in individual and mixed cell cultures.

In those experiments designed to characterize collagenase production by individual and mixed skin cell cultures, and corneal-skin cell co-cultures, four to eight replicates of each experimental condition were run. Culture medium with or without CB was added to cells 3 d after they were plated, and was harvested after 6 d of incubation. Harvested media were stored at 4°C and assayed for collagenase the same of the following day.

Preparation of Conditioned Medium: To prepare conditioned medium, freshly isolated skin epidermal cells, were plated at known densities in 16-mm diam wells of 24-well cluster tissue culture dishes (Costar). Cells were plated in DME plus 5% FCS and antibiotics, and allowed to attach for 24 h, after which their medium was replaced with 1 ml/well of the same fresh medium. Conditioned medium from such cultures was harvested after 2–4 d and stored frozen at -20°C until used.

Before application to cells, conditioned media were dialyzed (Spectrapore membrane tubing; 3,000-mol-wt cut off) extensively at 4°C against DME, supplemented with 5% FCS, and in some cases with CB (Aldrich Chemical Co., Milwaukee, WI; batches PH/3452/57E and PH/3452/72H; final concentration of 5 µg/ml) in dimethyl sulfoxide (final concentration of 0.5%), sterilized by passage through a Millipore filter (pore size = 0.45 µm; Millipore Corp., Bedford, MA), and added directly to recipient corneal cells.

Chromatography of Conditioned Medium: Conditioned medium from epidermal cells was filtered prior to chromatography to remove debris and dialyzed extensively against DME inorganic salts (constituents in mg/liter: anhydrous $\text{CaCl}_2 = 200$, $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O} = 0.1$, $\text{KCl} = 400$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} = 200$, $\text{NaCl} = 6400$, $\text{NaHCO}_3 = 3,700$, $\text{Na}_2\text{H}_2\text{PO}_4 \cdot \text{H}_2\text{O} = 125$) plus antibiotics. 10 ml of dialyzed conditioned medium was chromatographed at 4°C on a 2 × 80-cm Ultrogel Aca54 (LKB, Rockville, MD) column equilibrated with DME inorganic salts plus antibiotics. 40 fractions, 7 ml each, were collected for each sample and stored frozen until assayed for stimulators of collagenase

production by corneal stromal cells. Just prior to addition to cells, each column fraction was supplemented with FCS to 5% as well as minimal essential medium amino acids (50 \times concentrate with L-glutamine; Gibco Laboratories), minimal essential medium vitamin solution (100 \times concentrate; Gibco Laboratories), minimal essential medium sodium pyruvate solution (100 \times concentrate; 100 mM; Gibco Laboratories), and D-glucose (100 \times concentrate; 100 mg/ml) to a final 1 \times concentration of each to reconstitute a complete medium, with CB (final concentration 5 μ g/ml), and was sterilized by passage through a Millipore filter (pore size = 0.45 μ m; Millipore Corp.). The gel filtration column was calibrated with blue dextran, hemoglobin, cytochrome c, and tritiated water. The protein content of each fraction was determined by the Lowry method (14) using BSA as the standard.

Bioassay for Stimulators and Inhibitors: The ability of skin epidermal cell preparations to stimulate collagenase production by a connective tissue was tested on corneal stromal cells in the presence and absence of CB. Skin dermal cells were not used because they were constitutively producing collagenase, as will be described, and consequently were less useful as a target cell population. The inhibitory activity of epidermal cell conditioned medium was assayed on mixed corneal epithelial-stromal cell cultures plus CB in which epithelial cell concentration was optimal for collagenase production by the stromal cells (9).

For each experimental condition, three or four replicate cultures were run simultaneously. Control medium, conditioned media, and column fractions were added to cells 2 d after they were plated and were collected from cells after 4 additional days of incubation. Such media were stored at 4°C and were assayed for collagenase activity the same or the following day. Control medium consisted of DME plus 5% FCS and antibiotics with or without CB at 5 μ g/ml in dimethyl sulfoxide (0.5% final concentration).

Collagenase Assay: Culture medium was assayed directly for collagenase activity by the [¹⁴C]collagen fibril film method (15) after trypsin activation of the latent form of the enzyme (in which form it is always found in this system). Collagen degradation was calculated by subtracting buffer blank values from experimental values. Substrates were always demonstrated to be native by resistance to 0.01% trypsin. 1 U of collagenase was defined as the amount of enzyme that degraded 1 μ g of collagen fibrils/min at 37°C.

RESULTS

Collagenase Production by Skin Cell Cultures

Isolated dermal cells produced collagenase in primary culture (Fig. 1*a*, hatched bar). The amount of enzyme obtained from 3 \times 10⁵ cells/ml of culture medium for a 6-d period was 0.78 \pm 0.02 U/ml. Addition of 1.1 \times 10⁶ epidermal cells to the dermal cell cultures resulted in a significant ($p = 0.001$) increase in enzyme production to 1.12 \pm 0.06 U/ml, a stimulation of 0.34 U/ml of collagenase over that produced by dermal cells alone (Fig. 1*b*, hatched bar). Epidermal cells by themselves did not make detectable amounts of collagenase (Fig. 1*d*, hatched bar).

Addition of CB to epidermal cells alone or epidermal-dermal cell mixtures did not significantly ($p = 0.05$) change the collagenase-producing characteristics of either type of culture (Fig. 1, *b* and *d*; open vs. hatched bars). Addition of CB to dermal cells alone, however, resulted in a small but significant ($p = 0.01$) increase in collagenase production by the cells (Fig. 1*a*; open vs. hatched bars). Also, even with this increase, mixed cultures with CB made significantly ($p = 0.01$) more collagenase (an additional 0.26 U/ml) than dermal cell cultures with CB (Fig. 1, *a* and *b*, open bars).

The results presented above represent the values obtained in one experiment from the pooled cells of 10 rabbits. However, each experimental condition has been repeated with comparable results in three to five separate experiments (data not shown).

Collagenase Production by Mixed Skin-Corneal Cell Cultures

Corneal epithelial cells significantly ($p = 0.001$) inhibited constitutive collagenase production by skin dermal cells (com-

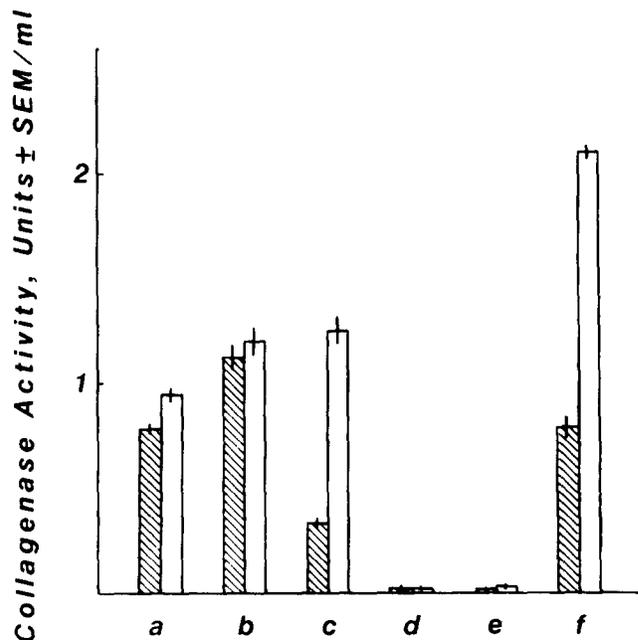


FIGURE 1 Cumulative collagenase production by skin cells and skin-corneal cell mixtures cultured for 6 d. Enzyme activity in the culture medium of (a) skin dermal cells, (b) skin dermal-epidermal cell mixture, (c) skin dermal-corneal epithelial cell mixture, (d) skin epidermal cells, (e) corneal stromal cells, and (f) corneal stromal-skin epidermal cell mixture. Cells were cultured in the absence (cross-hatched bars) and presence (open bars) of CB. Dermal and stromal cells = 3 \times 10⁵ cells/ml; epidermal cells = 1.1 \times 10⁶ cells/ml; corneal epithelial cells = 3.8 \times 10⁵ cells/ml; CB = 5 μ g/ml; $n =$ eight replicate cultures for each data point.

pare Fig. 1*a* with 1*c*, hatched bars). Only 0.33 \pm 0.03 U/ml of collagenase was produced by the mixed cell cultures in 6 d compared with 0.78 \pm 0.02 U/ml of enzyme by the dermal cells alone. Addition of CB to this mixed culture resulted in a significant ($p = 0.01$) increase in collagenase production by the culture, rather than inhibition, when enzyme levels were compared with those of dermal cells alone plus CB (Fig. 1, *a* and *c*, open bars).

Skin epidermal cells made no detectable enzyme but significantly ($p = 0.001$) stimulated collagenase production by corneal stromal cells. (Fig. 1, *e* and *f*, hatched bars). Cocultures of 1.1 \times 10⁶ epidermal cells/ml plus 3 \times 10⁵ stromal cells/ml produced 0.79 \pm 0.05 U/ml of collagenase, whereas the stromal cells alone were making essentially no enzyme (0.01 \pm 0.003 U/ml) and hence were stimulated in co-culture to produce 0.78 U/ml of enzyme.

Addition of CB greatly enhanced ($p = 0.001$) collagenase production by these mixed cell cultures (Fig. 1*f*, open vs. hatched bar). They produced 2.10 \pm 0.03 U/ml of enzyme in the presence of the drug, as compared with 0.79 \pm 0.05 U/ml in its absence. In addition, although corneal stromal cells alone plus CB produced no enzyme (0.02 \pm 0.003 U/ml; Fig. 1*e*, open bar), they were stimulated to produce 2.08 U/ml of collagenase in co-culture with skin epidermal cells plus CB. The corneal-skin co-culture experiments presented here were part of the same experiment described in the previous section, and have been repeated twice (data not presented) with the same results.

We have shown that primary skin dermal cells were constitutively producing collagenase in culture, and could only be stimulated by epidermal cells to increase collagenase produc-

tion by an additional 0.34 U/ml in the absence of CB, or 0.26 U/ml in the presence of CB. The same number of corneal stromal cells, on the other hand, could be stimulated by skin epidermal cells to increase enzyme production by an additional 0.78 U/ml without CB, or 2.08 U/ml with CB. Consequently, corneal stromal cells represent a better connective tissue target cell population than that of the skin for assaying epidermal cell effector molecules and, therefore, have been used as such throughout this study.

Conditioned Medium from Skin Epidermal Cells

Epidermal cells from both fetal and adult rabbit skin produced conditioned culture medium containing stimulator(s) of stromal cell collagenase production (Fig. 2, hatched bars). Addition of CB to the target stromal cells greatly enhanced their production of collagenase in response to the same conditioned media (Fig. 2, open bars).

Epidermal Cell Number In Vitro

We planned to examine stimulator production as a function of fetal and adult epidermal cell density in vitro. However, epidermal cells terminally differentiate in vivo and in vitro, a process characterized by loss of the cell nucleus and formation of a cross-linked envelope (16). The culture conditions employed here to obtain conditioned medium supported differentiation, as evidenced by the formation of squames, but not growth of epidermal cells (17). Therefore, to determine the numbers of cells actually contributing to the conditioned medium, we examined the rate of loss of epidermal cells from the resident population with time in culture as a function of starting cell density.

Freshly isolated adult skin epidermal cells were plated at high (1.1×10^6 cells/well) and low (1.3×10^5 cells/well) density in Costar 24-well plates. The medium, 1 ml/well, was

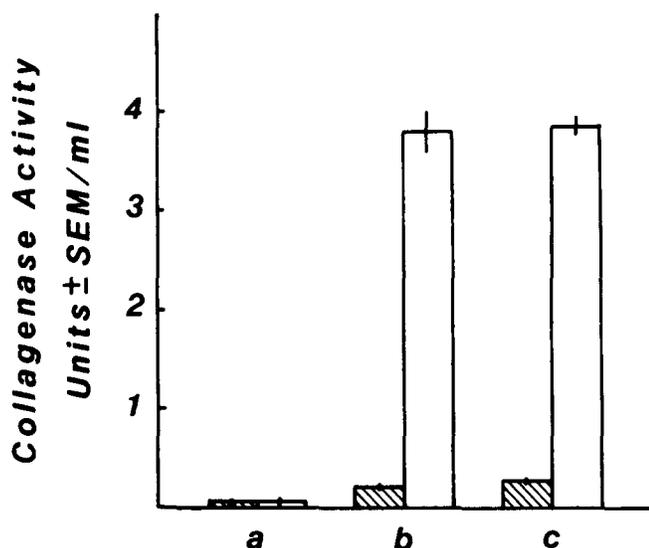


FIGURE 2 Effect of conditioned medium from skin epidermal cells on cumulative collagenase production by corneal stromal cells cultured for 6 d. Stromal cells received (a) fresh culture medium, (b) medium conditioned for 6 d by 5×10^5 fetal epidermal cells/ml, or (c) medium conditioned for 6 d by 10^6 adult epidermal cells/ml. Stromal cells were exposed to media in the absence (cross hatched bars) and presence (open bars) of CB. Stromal cells = 3×10^5 cells/ml; CB = 5 μ g/ml; n = four replicate cultures for each data point.

changed every day and four replicate samples of each experimental group fixed for DNA determination of cell number on days 0, 2, 3, and 6.

Under the culture conditions employed to generate conditioned medium in this study epidermal cell number declined steadily for the 6 d examined. By 2, 3, and 6 d of incubation high density cultures contained 62, 45, and 32%, and low density cultures 65, 51, and 38% of their original cell density, values identical to that described for nongrowing, differentiating, human epidermal cells in methocel suspension culture (16). Hence, of importance to this study, initial cell density had very little influence on the rate of epidermal cell loss in culture as measured by DNA.

Effect of Epidermal Cell Density

Epidermal cells were cultured at different densities and their conditioned media applied to stromal cells plus CB to assess stimulator production. For adult epidermal cells a direct relationship existed between the stimulator content of the conditioned medium and the log of the epidermal cell number that conditioned the medium (Fig. 3, closed circles). Measurable quantities of stimulator were produced by cultures at starting densities of 3×10^5 cells/ml to 1.3×10^6 cells/ml. Above 1.3×10^6 cells/ml the response of stromal cells reached a plateau. Dilution of medium conditioned by 1.3×10^6 cells/ml and 2.2×10^6 cells/ml indicated that the plateau was due partially to stromal cell saturation and partially to a diminished stimulatory capacity on a per epidermal cell basis of the conditioned medium (data not shown).

For fetal epidermal cell conditioned medium, a full epidermal cell concentration range has not been defined (Fig. 3, open circles). Measurable stimulator was produced by cultures containing 4×10^4 cells/ml to 6×10^5 cells/ml, with those containing $1-2 \times 10^5$ cells/ml being optimal for stimulator production. Above $1-2 \times 10^5$ cells/ml the stimulatory capacity of conditioned medium per epidermal cell diminished.

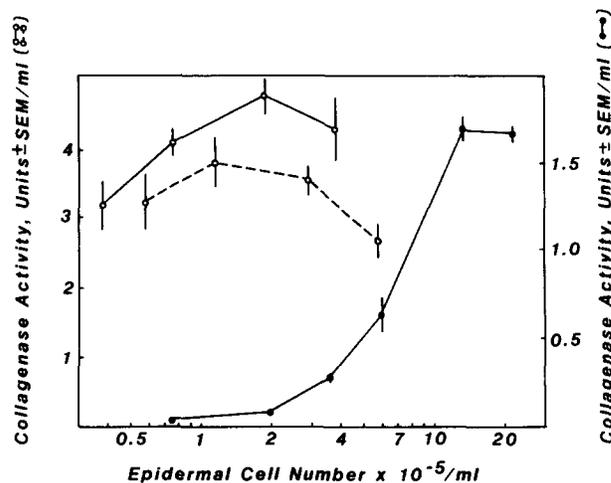


FIGURE 3 Effect of conditioned medium from skin epidermal cells cultured at different densities on cumulative collagenase production by corneal stromal cells plus CB cultured for 4 d. ●, 0-2-d conditioned medium from adult epidermal cells; ○, 0-3-d conditioned medium from fetal epidermal cells from two siblings. Data presented as a function of initial epidermal cell density. Stromal cells = 3×10^5 cells/ml; CB = 5 μ g/ml; n = four replicate cell cultures for each data point. Adult and fetal conditioned medium curves were separate experiments.

Control stromal cells in culture medium containing CB made no enzyme in this experiment (data not presented).

Dose-response Curve

The shape of the dose response curve for both fetal and adult epidermal cell conditioned medium was biphasic with the beginning of a plateau region in the middle of the curve (Fig. 4). This discontinuity in the response of stromal cells to crude and semipurified stimulator has also been observed in four other experiments (data not presented). The total titratable portion of the biphasic dose-response curve typically occurred over a 10-fold concentration range, and could be plotted as semilog functions. In addition, at these stimulator levels CB was necessary for stromal cells to respond with measurable amounts of collagenase to any concentration of conditioned medium (compare Fig. 4, *a* and *b* with 4, *c* and *d*). The collagenase response of stromal cells to such medium became saturated at higher concentrations (data not presented).

Chromatography of Conditioned Medium

Stimulators of collagenase production in medium conditioned by both fetal and adult skin epidermal cells eluted with apparent molecular weights of 20,500 and 55,000 when chromatographed on Ultrogel AcA54 (Fig. 5). The 20,500-mol-wt stimulator was always the main species produced, and the relative amount of 55,000-dalton stimulator present would vary from zero (data not presented). Stromal cells required

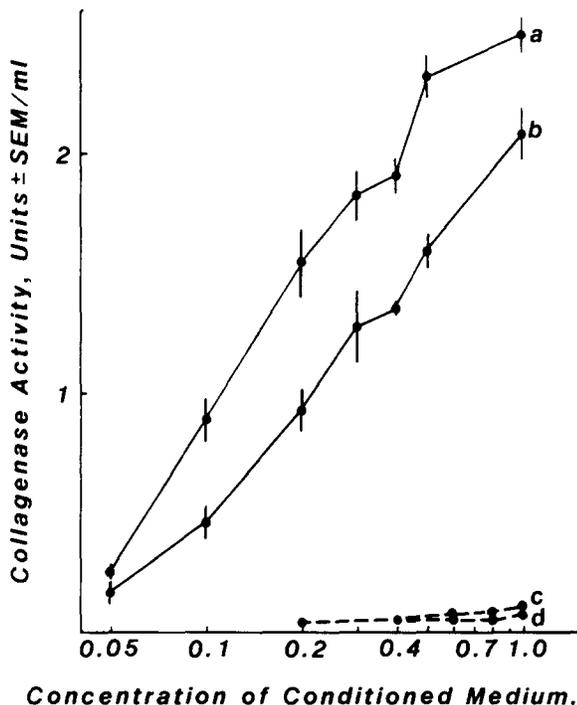


FIGURE 4 Dependence of corneal stromal cell collagenase production on concentration of conditioned medium from skin epidermal cells. Stromal cells received medium conditioned for 3 d by 2.2×10^5 fetal epidermal cells/ml (*a* and *c*), or medium conditioned for 6 d by 7.2×10^5 adult epidermal cells/ml (*b* and *d*). Stromal cells exposed to conditioned medium for 4 d in the presence (*a* and *b*) and absence (*c* and *d*) of CB. Stromal cells = 3×10^5 cells/ml; CB = $5 \mu\text{g/ml}$; $n =$ four replicates for each data point. Graph represents composite of two experiments (*a* and *c*; *b* and *d*).

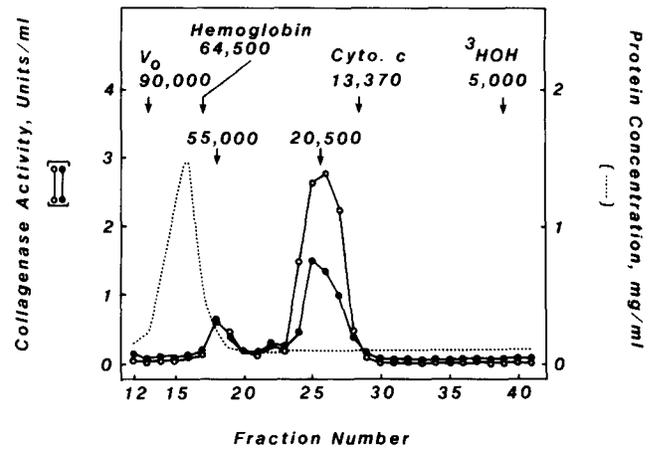


FIGURE 5 Ultrogel AcA54 gel filtration chromatography of skin epidermal cell conditioned medium. Stimulatory effect of elution fractions on cumulative collagenase production by corneal stromal cells plus CB at 4 d. Medium conditioned for 3 d by 2.6×10^5 fetal epidermal cells/ml (O) and for 6 d by 7.2×10^5 adult epidermal cells per ml (●). Stromal cells = 3×10^5 cells/ml; CB = $5 \mu\text{g/ml}$; $n =$ three replicate cell cultures for each data point. —, collagenase activity of stromal cells in response to 1 ml of eluate; - - -, protein concentration of fractions. V_0 , void volume; Cyto. c, cytochrome c.

CB to produce collagenase in response to column fractions. Column fractions were assayed for collagenase activity prior to their addition to cells, and were found to contain no endogenous enzyme.

Absence of Detectable Inhibitors

Conditioned medium from adult and fetal epidermal cells contained no detectable inhibitors of production of stromal cell collagenase. Addition of such medium to enzyme-producing mixed corneal cell cultures (9), elevated the total level of enzyme produced, rather than reducing it. In one experiment, mixed corneal cell cultures plus CB produced 1.3 ± 0.2 U of collagenase/ml of medium, whereas identical corneal cultures exposed to CB and adult epidermal cell conditioned medium produced 7.5 ± 0.3 U of enzyme/ml.

DISCUSSION

We have shown that primary cultures of rabbit dermal cells constitutively produce collagenase *in vitro* and that enzyme production by these cells can be regulated by epithelial cells. Co-culture with skin epidermal cells resulted in more enzyme production by the dermal cells whereas co-culture with corneal epithelial cells resulted in less collagenase activity. The inhibitory effect of corneal epithelial cells on collagenase production by dermal cells, a heterologous connective tissue, was identical to the effect of this epithelium on its homologous connective tissue (9).

Co-culture of skin epidermal cells with a different connective tissue cell population, that of the cornea, also resulted in stimulation of enzyme production. In this case, as we have shown before (8, 9), the stromal cells were making no detectable collagenase by themselves.

The difference in the endogenous collagenase producing behavior of primary skin and corneal connective tissue cells demonstrated here is consistent with previous observations with multipassaged skin fibroblasts (18) and corneal stromal cells (unpublished results), which do and do not produce enzyme, respectively. The reason for the endogenous differ-

ence in enzyme production by the primary connective tissue cell cultures described here may reside in the difference in their inherent cellular complexities. The dermis of mammalian skin contains at least 10 different types of cells (19): fibroblasts, fat cells, primitive mesenchymal cells, endothelial cells, smooth muscle cells, monocytes, macrophages, lymphocytes, plasma cells, and mast cells. In addition, by phase-contrast microscopic inspection we know that our dermal cell preparations are contaminated with some epidermal cells. The normal corneal stroma, on the other hand, only contains stromal fibroblasts. Hence, the dermal cell population that we isolate contains several potential collagenase generating cell-cell interactions, whereas the corneal stromal cell population does not.

Conditioned medium experiments suggest that epidermal cells can stimulate connective tissue cell collagenase production via secreted products, and hence, may be able to modulate collagen degradation in the skin by this mechanism.

Stimulator production by epidermal cells was influenced by cell density. Detectable and optimal production of stimulators per cell occurred in adult epidermal cultures plated at 3.5×10^5 – 10^6 cells/ml of culture medium, and fetal cultures plated below 4×10^4 – 10^5 cells/ml of medium. The data suggest that over these cell densities a linear relationship may exist between the stimulator content of the conditioned medium and the log of the epidermal cell density that conditioned the medium. Such semilog relationships between response and effector molecule concentration are typical of hormone-target cell interactions (20), and suggest that a hormone-like, close range of paracrine interaction may occur between epidermal cells and connective tissue cells. Above adult and fetal epidermal cell densities of 10^6 and 10^5 cells/ml of medium respectively, apparent stimulator production per cell declined. Whether this is a real decline, or reflects the simultaneous presence of an inhibitor substance, as occurs in corneal epithelium (9), is not known, although inhibitors could not be otherwise detected.

The optimal cell density for stimulator production by fetal cells was 1/10 that of adult skin cells, but the same as that for CB-treated adult corneal epithelial cells (9). Fetal rabbit skin at 28 d contains fully differentiated keratinocytes, hair follicles, and sebaceous glands (21), and consequently contains the same differentiated cell types as the adult. Fetal skin was taken from the torso, however, whereas adult skin was taken from the inner surface of the ear. As an organ, skin from the torso in rabbit contains many more appendages per unit area than inner ear skin. Whether the lower optimal density of fetal epidermal cells reflects an increased commitment to stimulator production per cell, an increase in the numbers of a relevant cell type, or some other heterogeneity, remains to be determined.

Collagenase production by corneal stromal cells was biphasic in response to increasing concentrations of stimulatory conditioned medium. Such a pattern of response results in two stable levels of enzyme production, and may be important in defining discrete limits to normal tissue degradation. We are presently pursuing this point.

The present study together with our previous one on cornea (9) suggests that skin and corneal epithelia behave differently in their overall regulation of connective tissue collagenase production. They are similar in that both can produce a 19,000–20,000- and 54,000–55,000-dalton stimulator, and both show density dependent production of stimulators. They

differ in that epidermis *in vitro* produces stimulators, whereas corneal epithelium produces inhibitors of stromal cell collagenase production and must be “induced” by CB to make stimulators. In addition, epidermal cells are completely competent to stimulate production of enzyme by stromal cells, whereas induced corneal epithelial cells only stimulate stromal cells when the stromal cells are simultaneously in the presence of CB (9). This epidermal cell competence may involve production of co-factor(s) and stimulator(s) that together act on the stromal cell. Induced corneal epithelial cells may only produce stimulators, and no co-factor(s). Therefore, CB may replace the effect of co-factors for the stromal cells in allowing them to respond to the stimulators. The presence of such epidermal co-factors is suggested by the increased requirement of target stromal cells for CB as epidermal cell effectors are purified and separated from each other. Hence, mixed epidermal-stromal cell cultures produced collagenase without CB and epidermal cell conditioned medium stimulated less, but detectable, enzyme from stromal cells without CB, but fractionated epidermal cell conditioned medium only stimulated production of collagenase by stromal cells in the presence of CB.

The precise role that CB plays in allowing corneal epithelial cells to produce stimulators, and stromal cells to respond to these and epidermal stimulators is not known. We simply use CB to facilitate these cellular effects. We have previously reported that neither the drugs’ ability to alter the morphology of cells by interfering with polymerization of actin to microfilaments, nor its ability to block glucose transport are individually responsible for its effects in this system (8). We also know from DNA determination of cell numbers that the CB concentration ($5 \mu\text{g/ml}$) that we used is not high enough to cause cell enucleation in our cultures (unpublished result), an effect that can occur at yet higher CB concentrations. We have speculated that CB may act by increasing the level of specific receptors on cells (9). This role is supported by the observations that CB enhances the *in vitro* stimulatory effect of growth factors (22) and melanocyte-stimulating hormone (23) on their specific target cells, and can inhibit the release of specific membrane components (24). Hence, via increasing the numbers of the relevant receptors, CB may enhance the stimulatory influence of a serum factor on corneal epithelial cells, resulting in production of stimulator, and similarly enhance the influence of stimulator(s) on stromal cells, resulting in production of collagenase.

The tendency of epidermis to produce stimulators *in vitro* whereas corneal epithelium produces inhibitors, is consistent with immunohistochemical localization of collagenase in normal skin and cornea, as discussed earlier, skin containing constitutive collagenase (11) and cornea containing no enzyme (10). Production of enzyme by stromal cells in pathological conditions, such as ulcerating corneas (10), may partially reflect the ability of corneal epithelial cells to produce stimulators when specifically activated, as they do *in vitro* in response to CB (9).

Rabbit corneal and skin epithelial cells have been shown to retain some of their differences in culture (25, 26). These are reflected in a different morphology, in their ability to be repeatedly passaged, and the nonidentity of some of their extractable proteins. Differences in skin and corneal epithelial cell regulatory behavior may reflect similar differences in the dynamics of collagen turnover. More active dermal remodeling may reflect the presence of epidermal appendages such as

hair follicles and sebaceous glands that within the context of the hair cycle are constantly replacing themselves. Cornea on the other hand, displays no such dynamic activity. When the demand arises, however, and the need for collagen turnover increases, as in wound healing, corneal epithelial cells may be able to utilize the same gene products to at least partially regulate stromal cell function.

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