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Cell Biology

Regulation of stromal cell collagenase production in adult rabbit cornea: *In vitro* stimulation and inhibition by epithelial cell products

(cell–cell interactions/cytochalasin B)

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ABSTRACT **Media conditioned by epithelial cells from the adult rabbit cornea were capable of both stimulating and inhibiting production of latent collagenase by stromal cells from the same source. Cytochalasin B was required in this *in vitro* system for both secretion of stimulators by epithelial cells and production of collagenase by stromal cells in response. Optimal production of stimulators occurred in low-density epithelial cell cultures. Chromatographed conditioned medium from such cultures contained three stimulator fractions with apparent molecular weights of 19,000, 54,000, and ≥ 90,000. High-density epithelial cell cultures secreted inhibitors of stromal cell collagenase production with apparent molecular weights of 7000 and 19,000. Cytochalasin B was not required for production of inhibitors. Inhibitory conditioned medium blocked the effect of the 19,000-dalton and 54,000-dalton stimulator on stromal cells. The data suggest that epithelial cells, in ways depending on their density, may modulate collagen degradation in the integument.**

Epithelial–mesenchymal cell interactions are fundamental to morphogenesis in embryonic development (1), limb regeneration (2), and wound repair (3). Previously we described an *in vitro* interaction between primary corneal epithelial and connective tissue (stromal) cells derived from the adult rabbit that may, at least in part, regulate collagenase production, and hence collagen remodeling in this tissue (4).

We demonstrated that mixtures of corneal epithelial and stromal cells co-cultured in the presence of cytochalasin B (CB) produced latent collagenase, whereas neither cell type alone with or without CB nor the mixtures without this agent did so. This effect was due to new synthesis or secretion of enzyme or both. Collagenase activity in mixed cultures was shown to be directly proportional to stromal cell number and CB concentration, whereas enzyme levels decreased in mixed cultures as epithelial cell number was increased over the concentration range examined. Media conditioned by CB-treated epithelial or stromal cells failed to stimulate collagenase production by either cell type, suggesting that stimulation required either direct cell contact or close proximity. In these preliminary conditioned-medium experiments, however, epithelial cell medium had been obtained from high-density cultures, and had been diluted 1:2. Therefore, it may not have been optimal for stromal cell stimulation or, indeed, it may have been inhibitory.

In the present work, conditioned media from epithelial cultures of various densities are examined. Epithelial cells, depending on their density, are shown to secrete both stimulators and inhibitors of stromal cell collagenase production, and hence, be capable of both positive and negative control of enzyme release in this system.

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MATERIALS AND METHODS

Preparation of Corneal Epithelial and Stromal Cells. Pure populations of primary epithelial and stromal cells from excised adult rabbit corneas were obtained by enzymatic digestion as described (4). Complete medium used to wash and plate isolated cells consisted of Dulbecco's modified Eagle's medium (GIBCO) containing 5% fetal calf serum and antibiotics (penicillin at 100 units/ml, streptomycin at 100 μg/ml; GIBCO). This differs from the medium used previously in that fetal calf serum was not pretreated with trypsin (4).

Each experiment utilized epithelial and stromal cell populations that were pooled from the corneas of the same 5 or 10 rabbits. The concentration of freshly isolated stromal cells was determined by using a hemocytometer and was adjusted appropriately prior to plating. Freshly isolated epithelial cells existed as a mixture of individual cells and cell clumps, making determination of cell number by hemocytometer impractical. For this reason, the concentration of epithelial cells was expressed at the time of plating in terms of *X*, in which 1*X* was equal to the epithelial population covering 1/4 of a cornea. The exact concentration of epithelial cells in 1*X* for each experiment was determined subsequently by the DNA assay described below.

Primary cell cultures were used in all experiments. Both cell types had a 90% plating efficiency, which was determined by comparing the number of cells plated with the number of cells that had attached to the culture dish after 24 hr.

Determination of Epithelial Cell Number. To convert 1*X* epithelial cells to actual cell number for each experiment, four replicate 1*X* cultures of freshly isolated cells were plated in 16-mm Costar wells in complete medium and were allowed to attach for 24 hr. The cells were then washed, detached from the well with trypsin, fixed in 10% neutral formalin buffered with 0.1 M sodium borate at pH 8, and at a later time assayed individually for DNA by using the fluorometric method of Hinegardner (5). By using rabbit stromal cells that had been fixed immediately after isolation as a standard, it was possible to determine the epithelial cell number. Sea urchin (*Strongylocentrotus drobachiensis*) sperm heads were also used as a standard, after the fluorescence equivalent of sperm heads to rabbit cells was determined. The value of 1*X* was typically 2–4 $\times 10^5$ epithelial cells.

Preparation of Conditioned Medium. All cell culture was carried out at 37°C in a moist atmosphere of 5% CO_2/95% air. To prepare conditioned medium, freshly isolated epithelial cells at 0.5–1.0 $\times 10^5$ per well (low density) and 5–7 $\times 10^5$ cells per well (high density), and also freshly isolated stromal cells at 3.0 $\times 10^5$ cells per well, were cultured separately in 16-mm-diameter wells of cluster tissue culture dishes (Costar). Cells were plated in complete culture medium, and allowed to attach for

Abbreviation: CB, cytochalasin B.

24 hr, after which their medium was replaced with fresh complete medium (1 ml per well) with or without CB (Aldrich batch PH/3452/57E) in dimethyl sulfoxide at final concentrations of 5 μg/ml and 0.5%, respectively.

Culture fluid from such cultures was harvested and replaced at 3 and 6 days, and used as conditioned medium. Before use a sample of each medium was sterilized by passage through a Millipore filter (pore size = 0.45 μm), and added directly to recipient corneal cells. To eliminate nutrient depletion as the cause of observed effects, another sample of each medium was dialized extensively at 4°C against complete culture medium, supplemented with CB at 5 μg/ml and 5% fetal calf serum, sterilized by filtration, and added to cells.

Chromatography of Conditioned Medium. Medium conditioned by low-density epithelial cells (cultured with CB) was brought to 80% saturation with ammonium sulfate, left to precipitate for 15 hr at 4°C, and sedimented at 27,000 × *g* for 15 min to collect the precipitate. The resulting pellet was solubilized in 1/10 its original volume of Dulbecco's modified Eagle's medium plus antibiotics, dialyzed twice against a 50-vol change of the modified Eagle's medium plus antibiotics at 4°C, and filtered prior to chromatography to remove debris. Medium conditioned by high-density epithelial cells (cultured without CB) was simply filtered prior to chromatography to remove any cells. Some medium from high-density epithelial cells was precipitated with 80% saturated ammonium sulfate and concentrated 3-fold prior to chromatography. The results obtained with this preparation were qualitatively the same as those obtained with straight conditioned medium.

Ten milliliters of each conditioned medium was chromatographed at 4°C on a 2 × 80 cm Ultrogel AcA 54 (LKB) column equilibrated with Dulbecco's modified Eagle's medium plus antibiotics. Forty fractions, 7 ml each, were collected for each sample and stored frozen until assayed for stimulators or inhibitors of corneal collagenase production. Because corneal cultures required both CB and fetal calf serum to secrete enzyme in this system, just prior to assay each column fraction was supplemented with CB at 5 μg/ml and fetal calf serum to 5% and was sterilized by passage through a Millipore filter. The gel filtration column was calibrated with blue dextran, bovine serum albumin, ovalbumin, cytochrome *c*, and tritiated water.

Culture Conditions. Corneal cells were plated in 7-mm-diameter wells of Falcon Micro Test II tissue culture plates in 0.2 ml of culture fluid per well. In individual and mixed cell cultures, stromal cells were plated at 6.0×10^4 cells per well, and epithelial cells at 8.9×10^2 to 1.3×10^5 cells per well depending on the experiment. For each experimental condition, three to five replicate cultures were run simultaneously. Control and conditioned culture media were added to cells one day after they were plated. Control medium consisted of complete medium containing 0.5% dimethyl sulfoxide and CB at 5 μg/ml.

Control and experimental media were collected and replaced fresh every 3 days for 6 or 9 days. Media harvested at each time point were stored at 4°C and were assayed separately for collagenase activity the same or the following day. Cumulative enzyme activity was determined by adding values obtained at each time point to each other.

The ability of crude conditioned medium, or column fractions, to stimulate corneal collagenase production was always tested on stromal cells in the presence of CB. The inhibitory activity of medium or fractions was tested on mixed epithelial–stromal cell culture plus CB, where epithelial cell concentration was low, and hence, the culture conditions optimal for collagenase production.

Collagenase Assay. Culture medium was assayed unconcentrated for collagenase activity after exposure to trypsin (4) to activate the latent form of the enzyme. Two assay methods were used: The older reconstituted radioactive collagen fibril gel assay (4), and the more recently described [^{14}C]collagen film assay (6). Assays were at 37°C. In both cases 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. One unit of collagenase was defined as the amount of enzyme that degraded 1 μg of collagen fibrils per minute at 37°C.

RESULTS

Effect of Epithelial Cell Concentration in Mixed Cell Culture. Previous examination of the effect of epithelial cell concentration on collagenase production by epithelial–stromal cell cultures plus CB yielded an unexpected negative correlation between increasing epithelial cell number and collagenase production (4). However, the optimal epithelial cell density for enzyme stimulation in these experiments was not determined. Therefore, a complete range of concentrations has been examined.

In mixed corneal cell cultures there was a sharp optimal epithelial cell concentration for stimulation of collagenase production (Fig. 1). Enzyme activity in the mixed cultures containing CB was directly proportional to epithelial cell number when 3×10^5 stromal cells per ml were cocultured with 5×10^3 to 5×10^4 epithelial cells per ml. However, collagenase production became inversely proportional to epithelial cell number at densities of 5×10^4 to 6.8×10^5 epithelial cells per ml. The optimal epithelial cell concentration for collagenase production under these culture conditions was 5×10^4 cells per ml.

Conditioned Medium and Collagenase Production. Determination of an optimal epithelial cell concentration for maximal collagenase activity by mixed corneal cultures prompted reexamination of the effect of conditioned medium from each cell type on the other, because earlier experiments had only utilized conditioned medium from relatively high-density epithelial cell cultures (4).

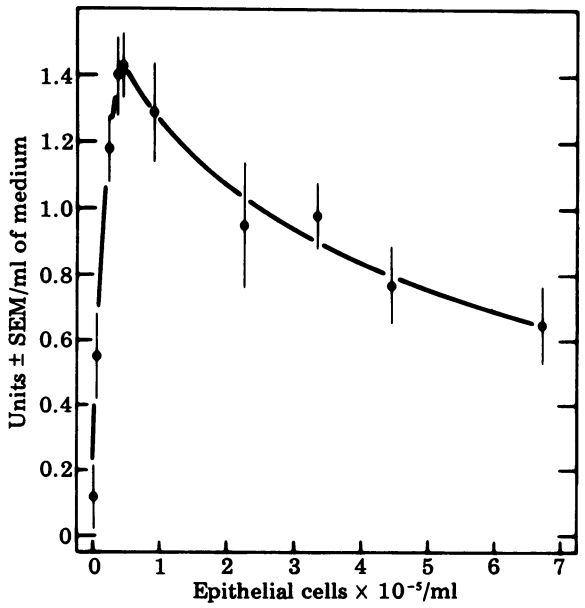

FIG. 1. Effect of epithelial cell concentration on cumulative collagenase production by mixed epithelial–stromal cell cultures plus CB at 9 days. Stromal cells = 3×10^5 cells per ml; CB = 5 μg/ml; *n* = 5 replicate cultures for each data point.

Medium conditioned by CB-treated epithelial cells stimulated collagenase production by stromal cells, whereas medium from CB-treated stomal cells never stimulated collagenase production by epithelial cells (data not shown). In addition, as shown in Fig. 2, conditioned medium from CB-treated low-density epithelial cell cultures (1×10^5 cells per ml of medium) was 2 times more stimulatory than medium from CB-treated high-density epithelial cell cultures (5.2×10^5 per ml of medium), reflecting the same effect of epithelial cell concentration on stimulation of collagenase production as was seen in the mixed cell experiment (Fig. 1). These reciprocal conditioned media experiments indicate that in mixed cultures epithelial cells release agent(s) that stimulate stromal cells to secrete collagenase.

The enzyme produced by medium-stimulated stromal cells was always latent. Therefore this epithelial effect was not one of activation of an inactive enzyme.

Medium from CB-treated high-density epithelial cell cultures not only stimulated less collagenase production by stromal cells alone but also blocked collagenase production by mixed corneal cell cultures. Mixed cell cultures coincubated with medium from CB-treated high-density epithelial cell cultures produced only 25% as much collagenase as control cultures (Fig. 3). The inhibitory effect of this conditioned medium was not due to (*i*) nutrient depletion from the medium, because it was extensively dialyzed against whole medium and supplemented with serum before addition to mixed cell cultures; (*ii*) direct inhibition of collagenase activity itself (data not shown); or (*iii*) inhibition of trypsin activation of collagenase (data not shown).

CB in this system appeared to be required both for production of stimulator(s) by low-density epithelial cells and for production of detectable collagenase by stromal cells in response to the epithelial stimulators. Medium conditioned by low-density epithelial cells in the absence of CB did not stimulate stromal cell collagenase production even if such medium was subsequently supplemented with CB. Likewise, stromal cells failed to respond to "stimulatory" epithelial medium when the CB had been dialyzed away, but would respond to the same dialyzed conditioned medium when CB was added back (data not presented).

CB was not required for release of inhibitors by high-density epithelial cell cultures. Such media prepared in the absence of CB, in fact, completely blocked enzyme production by CB-supplemented mixed cell cultures (Fig. 3).

The ability of conditioned medium from high-density epithelial cell cultures to inhibit collagenase production by mixed cell cultures plus CB was directly dependent on the concentration of inhibitory medium (Fig. 4). A 1:10 dilution of this medium was still inhibitory; such medium did not become stimulatory upon dilution.

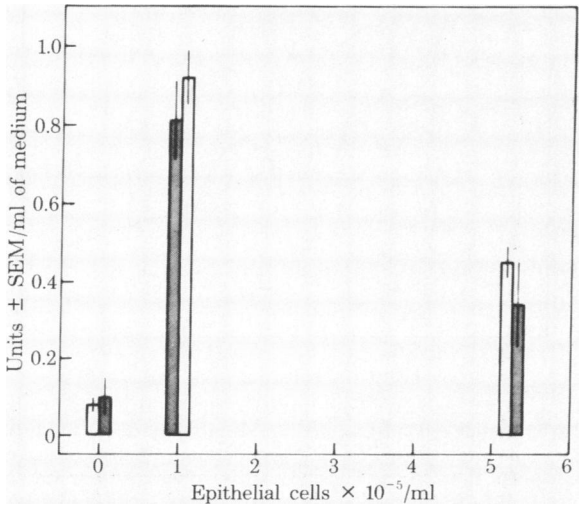

FIG. 2. Effect of conditioned medium from CB-treated epithelial cell cultures of different densities on cumulative collagenase production by stromal cell cultures at 6 days. Stromal cells = 3×10^5 cells per ml; CB = 5 μg/ml; n = 5 replicate cultures for each data point. Open bars, experiment 1; shaded bars, experiment 2.

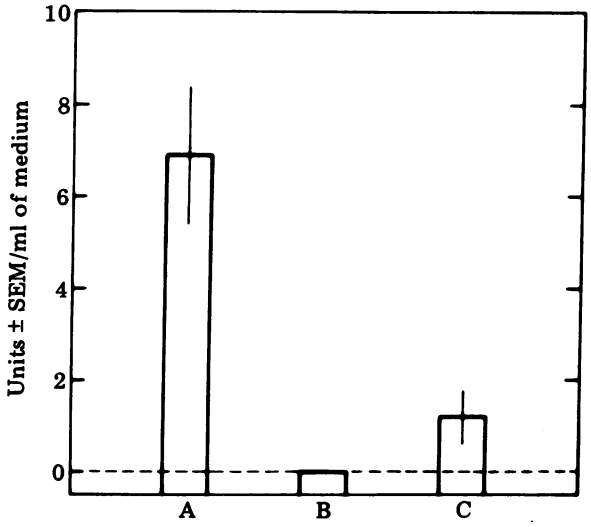

FIG. 3. Inhibitory effect of high-density epithelial cell culture medium on cumulative collagenase production by mixed corneal cell cultures at 9 days. Stromal cells = 3×10^5 cells per ml; epithelial cells = 0.5×10^5 cells per ml; CB = 5 μg/ml; n = 4 replicate cultures for each data point. Bar A, mixed cultures with fresh complete medium plus CB; bar B, mixed cultures plus medium conditioned by 6×10^5 epithelial cells per ml, then supplemented with CB; bar C, mixed cultures plus medium conditioned by 6×10^5 epithelial cells per ml in the presence of CB.

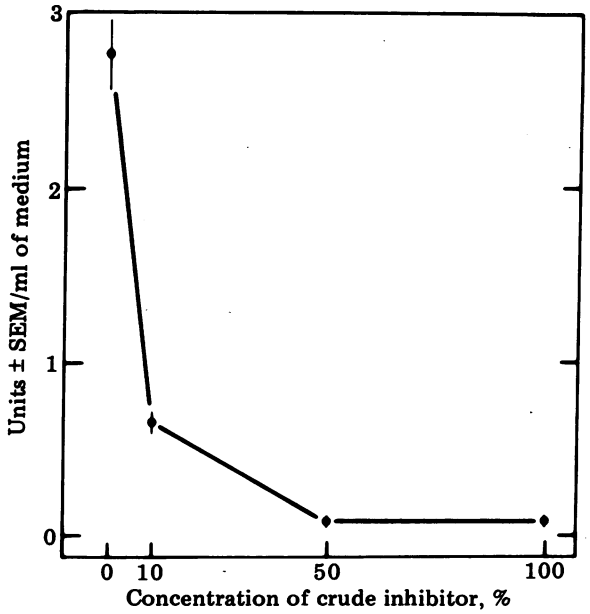

FIG. 4. Concentration dependence of inhibitory epithelial cell culture medium on cumulative collagenase production by mixed corneal cell cultures plus CB at 9 days. Crude inhibitor = medium conditioned by 6.5×10^5 cells per ml. In recipient mixed cultures, stromal cells = 3×10^5 cells per ml; epithelial cells = 0.7×10^5 cells per ml; CB = 5 μg/ml; n = 4 replicate cultures for each data point.

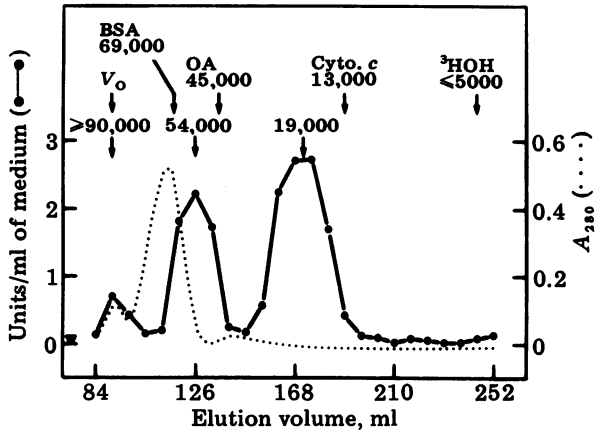

FIG. 5. Ultrogel AcA 54 gel filtration chromatography of low-density epithelial cell culture medium. Stimulatory effect of elution fractions on cumulative collagenase production by stromal cells plus CB at 6 days. The chromatographed medium was conditioned by 0.6 × 10^5 epithelial cells per ml in the presence of CB. Stromal cells = 3 × 10^5 cells per ml; CB = 5 μg/ml; n = 3 replicate cultures for each data point. ▼, Control; stromal cell cultures in complete medium plus CB. ●—●, Collagenase activity of stromal cells in response to 1 ml of eluate; ·····, A_{280} of fractions. V_o, Void volume; BSA, bovine serum albumin; OA, ovalbumin.

Chromatography of Conditioned Medium. Stimulators of collagenase production from medium conditioned by low-density epithelial cells plus CB eluted with apparent molecular weights of ≥90,000, 54,000, and 19,000 when chromatographed on Ultrogel AcA 54 (Fig. 5). This fractionation profile was fully reproduced with 10 different preparations. The response of stromal cells to the M_r 54,000 and 19,000 stimulators was dependent on the concentration of stimulator added, and became saturated at high concentrations (Fig. 6). Stimulation of stromal cells by the M_r 19,000 species could be prevented by adding crude inhibitory culture medium (Fig. 7), as could stimulation by the M_r 54,000 species (data not shown).

Conditioned medium from high-density epithelial cell cultures contained inhibitors with apparent molecular weights of 19,000 and 7000 (Fig. 8).

Control fractions of concentrated fetal calf serum that had been chromatographed on AcA 54 failed to either stimulate or inhibit collagenase production by corneal cultures. All column fractions of conditioned medium were assayed for collagenase activity prior to their addition to cells, and none were found to have any endogenous enzyme.

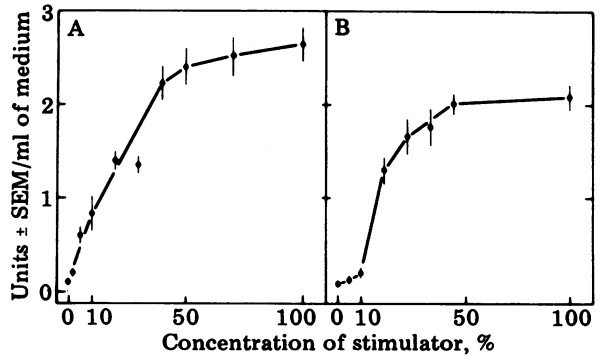

FIG. 6. Concentration dependence of stimulator fractions isolated by chromatography on cumulative collagenase production by stromal cells plus CB at 6 days. Stromal cells = 3 × 10^5 cells per ml; CB = 5 μg/ml. (*A*) Pooled M_r 19,000 stimulator fractions; n = 5 replicate cultures for each data point. (*B*) Pooled M_r 54,000 stimulator fractions; n = 4 replicate cultures for each data point.

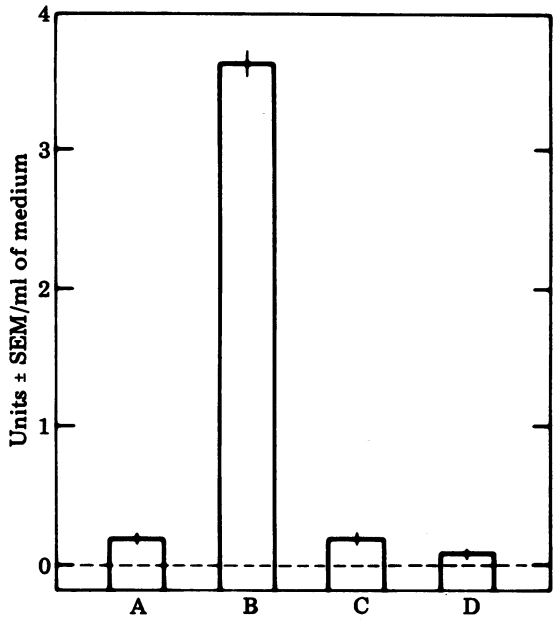

FIG. 7. Inhibitory effect of high-density epithelial cell culture medium on cumulative collagenase production by stromal cells plus M_r 19,000 stimulator at 6 days. Inhibitory medium was conditioned by 6 × 10^5 epithelial cells per ml. Stromal cells = 3 × 10^5 cells per ml; n = 4 replicate cultures for each data point. Bar A, stromal cells with fresh culture medium plus CB; bar B, stromal cells with 50% M_r 19,000 stimulator plus CB; bar C, stromal cells with 50% M_r 19,000 stimulator and 50% high-density epithelial cell culture medium plus CB; bar D, stromal cells with 50% high-density epithelial cell culture medium plus CB.

DISCUSSION

The results obtained here suggest that epithelial cells, depending on their density, are capable of both stimulating and inhibiting stromal cells with respect to collagenase production, and hence, may be capable of modulating collagen degradation in the cornea, and perhaps in all integument. Reciprocal medium exchange experiments indicate that secreted factors are responsible for these epithelial effects, and demonstrate that

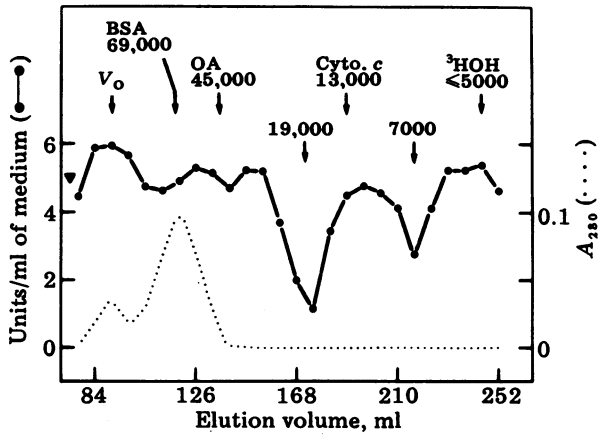

FIG. 8. Ultrogel AcA 54 gel filtration chromatography of high-density epithelial cell culture medium. Inhibitory effect of elution fractions on cumulative collagenase production by mixed corneal cell cultures plus CB at 9 days. The chromatographed medium was conditioned by 6 × 10^5 epithelial cells per ml. In mixed cultures stromal cells = 3 × 10^5 cells per ml; epithelial cells = 0.6 × 10^5 cells per ml; CB = 5 μg/ml; n = 3 replicate cultures for each data point. ▼, Control; epithelial–stromal cell mixture cultured in complete medium plus CB. ●—●, Collagenase activity of mixed cultures in response to 1 ml of eluate; ·····, A_{280} of fractions. Abbreviations as for Fig. 5.

stromal cells, not epithelial cells, are the producers of collagenase in this interaction. Immunocytochemical localization of collagenase in only the stromal portion of ulcerating human corneas (7) suggests that stromal cells are also a source of the enzyme under pathological conditions *in vivo*.

CB in this *in vitro* system is required both for the secretion of stimulators by epithelial cells and for the production of collagenase by stromal cells in response. The secreted collagenase was always latent, and none of the conditioned media activated the latent enzyme, influenced its activation, or affected the active product.

Optimal production of stimulators occurred in low-density epithelial cell cultures. Gel filtration chromatography of the conditioned medium of such cultures reproducibly revealed three stimulator fractions with apparent molecular weights of 19,000, 54,000, and ≥90,000. The identity of these substances is not yet known. They may represent multiples, subunits, or degradation products of the same molecule, or the same species adhering to other molecules or unrelated agents.

The 19,000-dalton stimulator may be related to mononuclear cell factor (MCF) (8, 9), a 14,000-dalton product of monocyte/macrophage cultures, which stimulates collagenase production by passaged rheumatoid synovial cells. Media conditioned by rabbit blood mononuclear cell populations are able to stimulate collagenase production by rabbit corneal cells (10). The same gene product may be used by both epithelial and blood cells for this function.

Enhancement of collagenase activity itself by a 20,000-dalton serum component has also been described (11). The 19,000-dalton stimulator does not enhance collagenase activity nor does fetal calf serum contain a component stimulatory to this system.

High-density epithelial cell cultures, on the other hand, secrete inhibitors of stromal cell collagenase production and do not require CB to produce these inhibitors. It has been reported (12) that culture medium from guinea pig corneal epithelium grown at the high densities utilized here inhibits DNA synthesis of cultured corneal stromal cells by 50%. Possibly one or both of the inhibitors discussed here is functioning through its effect on stromal cell proliferation.

The relationship of 19,000-dalton inhibitor to 19,000-dalton stimulator is not apparent. Their opposite effects on collagenase production are not simply due to different concentrations of the same agents. Stimulator is obtained from cultures containing 1/10 as many epithelial cells as those that produce inhibitors; however, a 10-fold concentration of 19,000-dalton stimulator does not convert it to inhibitor, nor does a 1:10 dilution of 19,000-dalton inhibitor yield stimulator. It is not yet clear whether crude inhibitory culture medium acts directly on stromal cells or interacts with stimulators.

The requirement for CB by both cell types suggests that both epithelial and stromal cells must undergo changes prior to productive interaction. The role of CB in facilitating their interaction in culture has not yet been determined and may not be the same for both cell types. Because this system involves both secreted products and the ability to respond to these products, it is tempting to speculate that CB increases the release of stimulators from epithelial cells and also the level of stimulator receptors on stromal cells. Supporting such roles for CB are the observations that this agent enhances the *in vitro* stimulatory effect of growth factors on fibroblasts (13) and of melanocyte-stimulating hormone on melanoma cells (14). It also increases shedding of fibronectin (15, 16) and inhibits release of other specific membrane components (17). CB may be a homologue for several different natural compounds *in vivo*, which may be present in injured tissues.

Control mechanisms for collagen degradation that depend on interaction between tissue cells, as in wound repair, may involve a finely balanced mixture of stimulatory and inhibitory substances. Epithelial cells may provide several control features of connective tissue collagenase production; blood cell elements may provide others. The relative amounts of control substances under particular conditions will determine the balance of stimulation or inhibition. Under conditions *in vitro* these secreted products, without being regionally contained, may simply diffuse into the culture medium. Such regulatory agents may function locally *in vivo*, exist in small amounts at high concentrations, and travel only short distances between cells.

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