Regulation of corneal collagenase production: Epithelial–stromal cell interactions

(collagenolysis/cellular control/cytochalasin B)

BARBARA JOHNSON-MULLER AND JEROME GROSS

The Developmental Biology Laboratory, Department of Medicine, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts 02114

ABSTRACT   Mixtures of epithelial and stromal cells isolated from normal adult rabbit cornea, when cocultured in the presence of cytochalasin B, produced latent collagenase, whereas neither cell type alone, nor the mixture in the absence of this agent, did so. The enzyme, a characteristic animal collagenase, required proteolytic activation. The relative concentrations of epithelial and stromal cells had a profound effect on collagenase production, the enzyme activity being directly proportional to the number of stromal cells but inversely proportional to the number of epithelial cells. The amount of enzyme released into the medium was also directly proportional to cytochalasin B concentration. Media conditioned by cytochalasin B-treated epithelial or stromal cells did not stimulate collagenase secretion by the other cell type. The data suggest direct cell contact or close proximity as the mode of productive interaction and tentatively identify the stromal cell as the source of enzyme and the epithelial cell as a stimulator.

Degradation of collagen in the development, remodeling, and repair of mesenchymal tissues requires the controlled action of a group of specific enzymes capable of selectively cleaving this fibrous protein (1, 2).

The possibility that interactions between two different cell types might significantly modulate the production or activation of collagenase was suggested by Grillo and Gross (3), who described collagenolytic activity in the edge of healing skin wounds in the guinea pig. They noted that sheets of epithelium isolated from the wound edge, when recombined with granulation tissue from the same region, resulted in significantly more activity than that produced by either tissue alone; epithelium and wound mesenchyme cultured separately were poor or erratic producers of collagenase. More recently, the ability of blood monocytic cell populations to induce or stimulate collagenase production by macrophages (4) and human rheumatoid synovial cells (5, 6) has been described. Soluble secreted factors appear to be the mediating agent here rather than cell-to-cell contact. Various biological and chemical agents such as neutral proteases (7), prostaglandins (8), bacterial endotoxins (9), colchicine (10), phorbol myristic acid (C. E. Brinckerhoff and E. D. Harris, Jr., personal communication), and cytochalasin B (11) are also known to stimulate collagenase production by a number of cultured cell types. The enzyme secreted into the medium in these cell cultures is usually in an inactive form that can be activated by exposure to proteases such as trypsin (12–15) or plasmin (16) and by certain organic mercurial compounds (17).

The rabbit cornea proved to be a particularly useful tissue for the study of collagenase regulation by epithelial–mesenchymal cell interaction because the two populations may be readily separated with essentially no cross contamination and

both will spread and proliferate rapidly in culture. Neither epithelial nor stromal cells from normal corneas nor mixtures thereof secrete detectable collagenase into the medium. However, if either population was derived from rabbit corneas healing after severe alkali burns, collagenase production by mixed cultures was readily obtained (D. Newsome and J. Gross, unpublished data).

We describe here the production* of collagenase by mixed cultures of primary epithelial and stromal cells derived by enzymatic separation from normal adult rabbit corneas and the role played by cytochalsin B in potentiating this function.

MATERIALS AND METHODS

Preparation of Corneal Epithelial and Stromal Cells. Corneas were obtained from adult male albino New Zealand rabbits weighing 2 kg. Rabbits were killed by intravenous injection of sodium pentobarbitol, and corneas were excised by cutting around the corneal periphery just inside the limbus.

To obtain pure populations of epithelial cells, excised corneas were "split" with trypsin by modification of the method of Szabo (18). Each pair of corneas was incubated in 5 ml of Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution plus 0.25% trypsin (GIBCO) for 12–16 hr at 4°C. After incubation the epithelium was gently pushed from the stromal surface with the edge of a scalpel blade. The isolated epithelial cells and cell clumps were collected, suspended in fresh medium, sedimented at 1000 × g, resuspended in the same culture medium, and plated at the desired cell density. Medium consisted of Dulbecco's modified Eagle medium (GIBCO) containing 5% trypsin-treated fetal calf serum (FCS) and antibiotics (penicillin at 100 units/ml, streptomycin at 100 μg/ml; GIBCO).

Because of the problem of measuring cell numbers in clumped cell suspensions, epithelial cell concentration was expressed as a known fraction of the epithelial population of the whole cornea.

The endothelium and Descemet's membrane were peeled from the remaining corneal tissue and discarded. Pure populations of stromal cells were obtained from the remaining stromal tissues by digestion with bacterial collagenase (19). Each pair of stromata was scored extensively with a scalpel, then incubated in 2.5 ml of Dulbecco's modified Eagle medium containing antibiotics and crude bacterial collagenase (Worthington Biochemical CLS II, 175 units/mg) at 4 mg/ml for 4 hours at 37°C in a moist atmosphere of 5% CO_2/95% air. This procedure resulted in complete solubilization of the extracellular matrix and formation of a homogeneous stromal cell suspension. The cells were washed three times in Dulbecco's

Abbreviations: CB, cytochalasin B; CB_1, CB_2, two separate batches of Aldrich cytochalasin B; FCS, fetal calf serum.
* The term "production" should be understood to stand for production/secretion.

modified Eagle medium plus antibiotics, resuspended in the same medium plus 5% trypsin-treated FCS and antibiotics, counted, and plated at the desired cell density.

All experiments were performed with primary cell cultures.

Culture Conditions. Cultures consisting of epithelial cells alone, stromal cells alone, or epithelial–stromal cell mixtures were established in 16-mm diameter wells of cluster tissue culture dishes (Costar). Unless otherwise specified, in individual and mixed cell cultures stromal cells were plated at 3.0×10^5 cells per well (confluent at 4×10^5 cells per well), and epithelial cells were plated at a density equal to the epithelial population covering 1/4th of a cornea (designated here as 1X). After plating, corneal cells were left to attach for 24 hr at 37°C in a moist atmosphere of 5% CO_2/95% air, after which the culture medium was replaced by fresh complete medium at 1 ml/well. Complete culture medium consisted of filter-sterilized Dulbecco's modified Eagle medium with 5% trypsin-treated FCS and antibiotics and containing 0.5% dimethyl sulfoxide with or without various concentrations (final medium concentration 1–10 μg/ml) of cytochalasin B (CB; Aldrich). Two batches of CB were used in these experiments: Aldrich batch PH/2668/77K and PH/3452/57E (designated here as CB_1 and CB_2, respectively).

Cells were cultured at 37°C in a moist atmosphere of 5% CO_2/95% air for 9–15 days. Culture medium was collected and replaced every 3 days, and, after 9 days, experimental culture medium was replaced by medium without dimethyl sulfoxide and CB. Collected culture medium was stored at 4°C and was assayed for collagenase activity the following day. Collagenase activity of stored culture media remained stable for at least a month.

Trypsin Treatment of Fetal Calf Serum and Activation of Latent Collagenase. Both FCS and collected culture medium were treated with trypsin prior to use in order to inactivate the general protease inhibitor α_2-macroglobulin in the FCS, and to activate latent collagenase in the medium. To prepare trypsin-treated FCS or activated culture medium, FCS (Flow Laboratories) or collected culture medium was incubated with 1/10th its volume of trypsin treated with N-tosylphenylalanine chloromethyl ketone (TPCK-trypsin, Worthington) at 1 mg/ml in buffer A (0.2 M NaCl/50 mM Tris·HCl/1 mM $CaCl_2$, pH 7.4) for 7 min at 37°C. After incubation, the same amount (1/10th the original volume) of soybean trypsin inhibitor (Worthington) at 5 mg/ml in buffer A was added to the mixture of inactivate TPCK-trypsin.

Collagenase Assay. The collagenase activity of collected culture medium was measured by the reconstituted radioactive collagen fibril assay (20) with [^{14}C]acetylated rat tail tendon collagen as substrate (21). Radioactive collagen (0.2%; 2500–3000 cpm total) in 100 μl of 0.4 M NaCl/0.02 M Tris, pH 7.4, was polymerized to fibrils at 34°C for 1 hr, and 200 μl of trypsin-activated, unconcentrated culture medium was then added to the gel. The resulting reaction mixture was incubated at 34°C for 20 hr. Each assay contained a trypsin control of collagen denaturation in which substrate was incubated with 200 μl of 0.015% trypsin (Worthington) in buffer A, and radioactivity obtained was subtracted from experimental values.

Collagenase activity was expressed as units/ml of culture medium, in which 1 unit of collagenase is defined as the amount of enzyme that will degrade 1 μg of collagen fibrils per minute at 34°C.

Collagenase-characteristic TC^A and TC^B degradation products were detected in reaction mixtures by polyacrylamide gel electrophoresis in the buffer system of Nagai et al. (22).

Experimental Design. To minimize variability in a particular experiment, all variables within the experiment were tested on epithelial (with the exception described below) and stromal cells from the same rabbit. In addition, five replicates of each experiment, using the cells from five rabbits, were run simultaneously and the same fresh culture medium was used for the duration of the experiment. In those experiments in which the relative concentrations of epithelial and stromal cells in epithelial–stromal cell mixtures were studied, the epithelial cells from five rabbits were pooled before being plated with the individual populations of stromal cells in each replicate.

In mixed cell cultures, epithelial cells were plated first, then 4 hr later the suspensions of stromal cells were added and both cell types were resuspended together with pipetting. During the first 4-hr period at 37°C, epithelial cells began attaching to the plastic. Essentially complete attachment of both cell types had occurred by 24 hr. Stromal cells and epithelial cells were cultured separately under identical conditions and attached in the same time periods.

RESULTS

Cytochalasin B and Collagenase Production. We evaluate here the ability of normal stromal and epithelial cells, separately and in mixtures, to produce and secrete collagenase in the presence and absence of CB.

In the absence of CB, cultures of epithelial cells, stromal cells, and epithelial–stromal cell mixtures produced no detectable collagenases over the time period studied (Fig. 1A). Similarly, cultures of epithelial cells in the presence of CB produced no enzyme (Fig. 1, Table 1).

The effect of CB on collagenase production by stromal cells alone, on the other hand, appeared to depend on the batch of CB, and the particular rabbit from which the cells were obtained. Two different batches of CB from the same commercial supplier were used. Stromal cells from 13 different rabbits in the presence of CB_1 at 5 μg/ml produced no detectable collagenase (Fig. 1A, Table 1). However, with CB_2, stromal cell populations from 40% of the rabbits produced the enzyme (Table 1). In the experiment illustrated in Fig. 1B, in which the medium contained CB_2 at 10 μg/ml, stromal cells obtained from one of five rabbits produced collagenase. All cultures of CB_2-sensitive stromal cells were maximally active by 3 days of incubation; half remained so for the duration of the culture period (9 days), and the others lost activity with time.

Collagenase was produced by epithelial-stromal cell mixtures in the presence of CB_1 and CB_2 at the appropriate ratio of epithelial to stromal cells (see below) for 97% of the 67 rabbits used in these experiments (Table 1), and in each case it continued to be produced at a constant rate for the duration of the culture period (Fig. 1). In the presence of CB_1 at 5 μg/ml or CB_2 at 10 μg/ml, there was no significant difference ($P > 0.1$) in the time, rate, or magnitude of collagenase production by mixed cultures.

Table 1.   Percentage of CB-treated corneal cell strains secreting collagenase

| Culture | CB treatment | | |
	CB_1, 5 μg/ml	CB_2, 5 μg/ml	CB_2, 10 μg/ml
Epithelial cells	0/17 (0%)	0/10 (0%)	0/25 (0%)
Stromal cells	0/13 (0%)	4/10 (40%)	13/30 (43%)
Epithelial–stromal cell mixture	26/27 (96%)	10/10 (100%)	39/40 (98%)

Data were pooled from a number of experiments. The denominator represents the number of cell strains, each from a single rabbit, and the numerator represents the number of these strains that secreted collagenase.
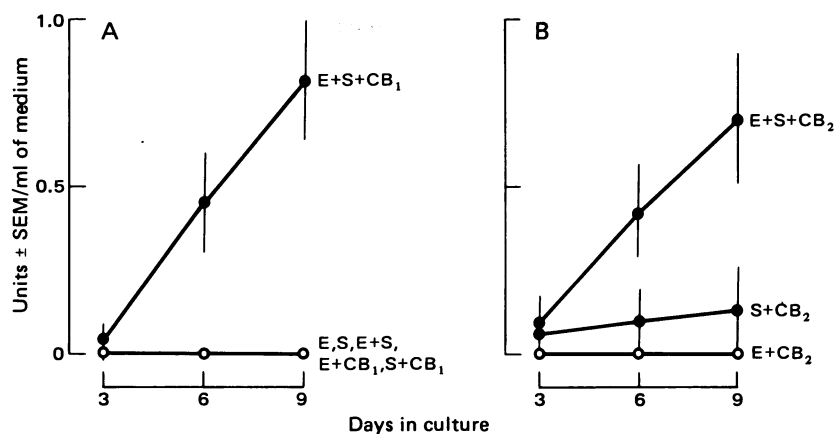
FIG. 1.   Cumulative collagenase production as a function of days in culture by normal adult rabbit corneal cells. Each data point represents the mean value and standard error of the mean of five cell strains obtained from five different rabbits. (*A*) Effects of CB_1 at 5 μg/ml. (*B*) Effects of CB_2 at 10 μg/ml. E, epithelium (1X, the number of cells obtained from 1/4th of the corneal surface, was used in all experiments); S, stromal cells (3 × 10^5 per well).

Typically, in response to CB, corneal cell mixtures from two to five rabbits produced collagenase by 3 days in culture, and all five rabbits did so at both 6 and 9 days in culture.

We have analyzed the variability in level of enzyme production in five replicate cultures from cells of a single animal. For such cultures the standard error was 4% of the mean enzyme production at 3, 6, and 9 days, as compared with a standard error for five replicate cultures obtained from the cells of five different rabbits of 100% of the mean at 3 days, 33% at 6 days, and 33% at 9 days. Thus, the major source of variation is individual animal differences rather than the assay system and culture conditions.

The effect of CB on collagenase production by all responding corneal cell cultures was reversible. Removal of CB from the medium of collagenase-producing cultures on day 9 reduced enzyme production by 50% (of day 9 level) at day 12 and by 100% at day 15.

All collagenase detected in these cell cultures required trypsin activation even though the serum used in the medium had been pretreated with trypsin. Characteristic TC^A and TC^B collagen degradation products were produced by the collagenase obtained in the medium of cell cultures.

Effect of Relative Concentration of CB, Stromal Cells, and Epithelial Cells. The individual effects of CB concentration, stromal cell concentration, and epithelial cell concentration on collagenase production by epithelial–stromal cell cultures plus CB were evaluated. In a series of three experiments, each component of the collagenase-producing system was made the variable while the other two components were fixed. The standard plating concentrations were CB at 10 μg/ml, 3.0 × 10^5 stromal cells per well, and 1X epithelial cells per well.

Three-, six-, and nine-day culture media were assayed for collagenase activity for all three types of experiments. Because the trend in enzyme activity at all times was in the same direction for each experiment, only cumulative collagenase activities produced by 9 days of culture will be presented here.

When CB concentration was varied (1–10 μg/ml) in epithelial–stromal cell cultures, a positive correlation was found to exist between a limited range of CB concentrations and the amount of collagenase in the cell culture medium (Fig. 2). At CB concentrations between 1 and 7.5 μg/ml, a direct dose–response relationship with collagenase activity was found. Saturation was obtained between 5 and 10 μg/ml.

The effective concentration range of CB for inducing collagenase production also caused morphological changes in both epithelial and stromal cells.

When stromal cell number was varied (2.0–3.5 × 10^5 cells per well) in CB-stimulated epithelial–stromal cell mixtures, a positive correlation was found between stromal cell number and collagenase production (Fig. 3A). Over the range 2.0–3.0 × 10^5 cells per well, collagenase levels increased, and there was no further increase at 3.5 × 10^5 cells per well.

Unexpectedly, a negative correlation between increasing epithelial cell number and collagenase production was observed in mixed cell cultures plus CB (Fig. 3B). With increasing epithelial cell concentration from 0.5X to 1.5X, collagenase levels decreased, and there was no further fall at 2.0X epithelial cells.

Effect of Conditioned Medium of Each Cell Type on the Other. In order to determine if collagenase production by epithelial–stromal cell mixtures plus CB involved a one-step interaction between epithelial cells and stromal cells via medium-borne factor(s) induced by CB, epithelial cells and stromal
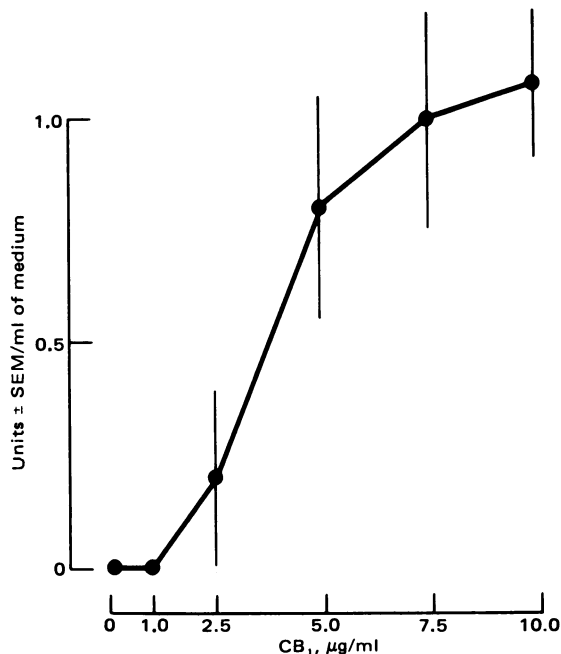

FIG. 2.   Relationship between concentration of CB_1 and cumulative collagenase secreted into the medium at 9 days by epithelial–stromal cell mixtures. Five replicates; each well contained 3.0 × 10^5 stromal cells and 1X epithelial cells.
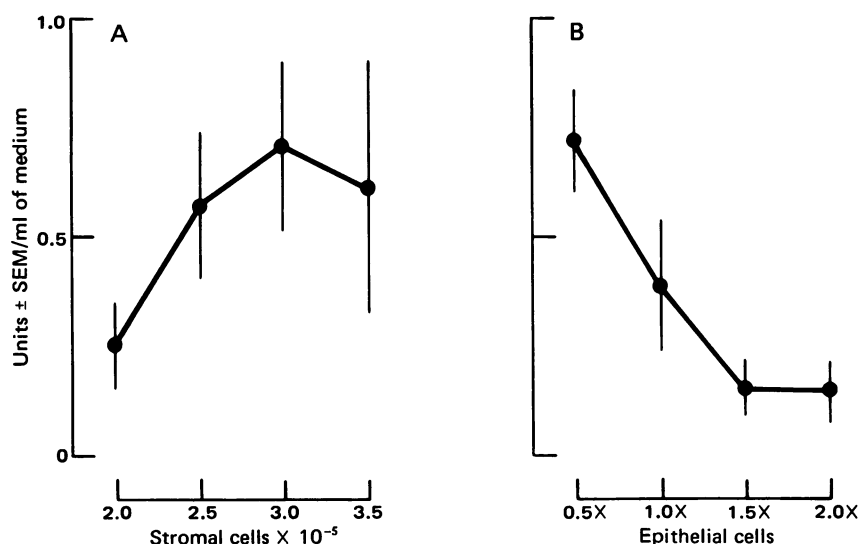
FIG. 3. Cumulative collagenase production at 9 days of culture. Five replicates. (A) Effect of increasing concentrations of stromal cells in the presence of 1X epithelial cells per well and CB_2 at 10 μg/ml. (B) Effect of increasing concentrations of epithelial cells on 3×10^5 stromal cells per well and CB_2 at 10 μg/ml.

cells were cultured separately in the presence of medium conditioned by the opposite cell type and containing added CB.

Conditioned media were obtained from either stromal cells or epithelial cells cultured separately in the presence of CB at 5 μg/ml. Three-, six-, and nine-day media were filter sterilized after harvesting and diluted 1:2 with fresh culture medium plus CB at 5 μg/ml. Conditioned media from both stromal and epithelial cells were then added to cultures of the opposite cell type. Media were collected from the resulting cultures after 3, 6, and 9 days and assayed for collagenase activity. Neither epithelial cells cultured with stromal cell-conditioned medium nor stromal cells cultured with epithelial cell-conditioned medium secreted collagenase.

DISCUSSION

Our data indicate that epithelial and stromal cells from the normal rabbit cornea interact in the presence of a soluble factor, CB in this instance, to stimulate the production of collagenase by the stromal cell.

Our results are in agreement with previous studies reporting the absence of collagenase production by normal rabbit stromal cells (23) and whole rabbit corneal stroma stripped of epithelium (24). Epithelial cells released enzymatically from other tissues, such as tadpole tail fin (25), on the other hand, secreted active collagenase in culture, suggesting significant species or tissue differences. Perhaps of more interest, there are also reports of active collagenase production by intact normal rabbit corneal tissue containing epithelium and superficial stroma (24), as well as other whole tissue explants with epithelial and connective tissue cells intact, such as skin from human (26), guinea pig and rabbit (3), and tadpole (25). These observations, when compared with our culture data, suggest that at least in the case of the rabbit cornea, coculture of normal stromal and epithelial cells requires an additional stimulus for collagenase production. In our mixed cell experiments, CB provided the necessary additional element. Intact tissue fragments in culture apparently include all the essential components for collagenase secretion.

By performing each experiment with cells from different rabbits, we were able to establish a range of variability of enzyme production, dependent upon the individual animal source of cells. Cytochalasin B nearly always stimulated collagenase production by mixtures of normal stromal and epithelial cells. However, there was a considerable difference in amounts of enzyme produced by cells from different rabbits. This variability is only partially inherent in the assay system and culture conditions, because, in a control experiment, five replicates from cells of a single rabbit were considerably more constant in collagenase output at each time point of incubation.

A significant difference in effect between two batches of CB was also evident, in that one of the two batches (CB_2) stimulated collagenase secretion by some stromal cells alone. This raises the question of an active contaminant of CB_2, which, however, would not explain the fact that both batches of CB stimulated the mixed cultures in a comparable manner. These observations plus the earlier report of stimulation of collagenase release from a variety of fibroblastic cell types of exposure to CB (11) suggest that the stromal cell is the source of collagenase. Because enzyme production by the epithelium was never observed in our mixed cultures, it would appear that the epithelial cell functioned as the inducer.

Medium conditioned by CB-treated epithelial cells did not stimulate stromal cells; therefore, we are left with the likelihood that either direct cell contact or close proximity is required. Alternatively, a multistep process involving interaction of factors secreted by both cell types might be responsible for collagenase induction in mixed cultures. It is unlikely that the epithelium is secreting an activator of an inactive stromal cell collagenase, because such activation in culture would yield the active form of the enzyme in medium containing trypsinized serum, which is not the case.

Perhaps one of the more intriguing aspects of these studies is the importance of the relative epithelial cell–stromal cell concentrations. There appears to be an optimal ratio for stimulation; increase in the proportion of epithelial cells resulted in graded diminution of collagenase production. Apparently, higher concentrations of epithelial cells have a proportionately reduced stimulatory effect in mixed cultures. Reduction of the ratio of epithelial to stromal cells, on the other hand, accomplished by increasing the number of stromal cells, is paralleled by increasing amounts of enzyme secreted into the medium. We have not as yet determined the limits of this effect.

Cell Biology: Johnson-Muller and Gross

Proc. Natl. Acad. Sci. USA 75 (1978)   4421

What then is the role of CB? This agent is thought to alter the morphology of fibroblast-like cells by interfering with polymerization of actin to microfilaments (27, 28). We know that it also alters epithelial cell morphology, although in a different pattern. In addition, CB blocks glucose transport (29, 30) and perhaps has other as yet undefined activities. Our preliminary examination of the effect of cytochalasin D (a natural analogue of CB), which causes morphologic change in fibroblasts without affecting glucose transport (31), revealed no stimulation of collagenase production, suggesting that morphologic alteration per se of both epithelial and stromal cells is not directly connected with production of the enzyme. Another preliminary observation (data not included here) that low glucose levels in the medium, or competition with glucose transport by 2-deoxy-D-glucose, also did not stimulate collagenase production leaves unresolved, at present, the mechanism whereby CB acts. Because neither of the known mechanisms seems to apply here, the report of a third receptor for CB, of unknown function (32), and the observation of increased shedding of fibronectin by CB-treated cells (33) raise the possibility of other routes of mediation of cell function in this system.

How might these phenomena relate to *in vivo* events? The epithelium is separated from mesenchymal cells by a basement membrane barrier and other matrix elements in a normally quiescent tissue such as cornea or skin. Except under conditions of active collagen turnover such as in wound repair, other injury, or tissue remodelling, minimal or no collagenolytic activity is found. However, the enzyme is secreted into the culture medium of many, but not all, normal whole tissue explants; these may well be analogous to wounds *in vivo* in that they have cut edges from which epithelial cells migrate early and fibroblasts eventually. It is quite conceivable that injury to connective tissue permits epithelial–mesenchymal cell contact in the presence of released factors with activity analogous to CB.

1. Harris, E. D., Jr. & Cartwright, E. C. (1977) in *Proteinases in Mammalian Cells and Tissues*, ed. Barrett, A. J. (North-Holland, New York), Vol. 2, pp. 249–283.
2. Gross, J. (1976) in *Biochemistry of Collagen*, eds. Ramachandran, G. N. & Reddi, A. H. (Plenum, New York), pp. 275–317.
3. Grillo, H. C. & Gross, J. (1967) *Dev. Biol.* 15, 300–317.
4. Wahl, L. M., Wahl, S. M., Mergenhagen, S. E. & Martin, G. E. (1975) *Science* 187, 261–263.
5. Dayer, J.-M., Russell, R. G. G. & Krane, S. M. (1977) *Science* 195, 181–183.
6. Dayer, J.-M., Robinson, D. R. & Krane, S. M. (1977) *J. Exp. Med.* 145, 1399–1404.
7. Werb, Z. & Aggeler, J. (1978) *Proc. Natl. Acad. Sci. USA* 75, 1839–1843.
8. Wahl, L. M., Olsen, C. E., Sandberg, A. L. & Mergenhagen, S. E. (1977) *Proc. Natl. Acad. Sci. USA* 74, 4955–4958.
9. Wahl, L. M., Wahl, S. M., Mergenhagen, S. E. & Martin, G. E. (1974) *Proc. Natl. Acad. Sci. USA* 71, 3598–3601.
10. Harris, E. D., Jr. & Krane, S. M., (1977) *Arthritis Rheum.* 14, 669–684.
11. Harris, E. D., Jr., Reynolds, J. J. & Werb, Z. (1975) *Nature (London)* 257, 243–244.
12. Vaes, G. & Eeckhout, Y. (1975) in *Protides of Biological Fluids*, ed. Peters, H. (Pergaman, Oxford, England), Vol. 22, pp. 391–397.
13. Bauer, E. A., Stricklin, G. P., Jeffrey, J. J. & Eisen, A. Z. (1975) *Biochem. Biophys. Res. Commun.* 64, 232–240.
14. Birkedal-Hansen, H., Cobb, C. M., Taylor, R. E. & Fullmer, H. M. (1976) *Biochim. Biophys. Acta* 438, 273–286.
15. Horwitz, A. L. & Crystal, R. C. (1976) *Biochem. Biophys. Res. Commun.* 69, 296–303.
16. Werb, Z., Mainardi, C. L., Vater, C. A. & Harris, E. D., Jr. (1977) *N. Engl. J. Med.* 296, 1017–1023.
17. Sellers, A. E., Cartwright, E., Murphy, G. & Reynolds, J. J. (1977) *Biochem. J.* 163, 303–307.
18. Szabo, G. (1955) *J. Pathol. Bacteriol.* 70, 545.
19. Dayer, J.-M., Krane, S. M., Russell, R. G. G. & Robinson, D. R. (1976) *Proc. Natl. Acad. Sci. USA* 73, 945–949.
20. Nagai, Y., Lapiere, C. M. & Gross, J. (1966) *Biochemistry* 5, 3123–3130.
21. Gisslow, J. J. & McBride, B. C. (1975) *Anal. Biochem.* 68, 70–78.
22. Nagai, Y., Gross, J. & Piez, K. A. (1964) *Ann. N. Y. Acad. Sci.* 121, 494–500.
23. Hook, R. M., Hook, C. W. & Brown, S. I. (1973) *Invest. Ophthalmol.* 12, 771–776.
24. Itoi, M., Gnadinger, M. C., Slansky, H. H., Freeman, M. I. & Dohlman, C. H. (1969) *Exp. Eye Res.* 8, 369–373.
25. Eisen, A. Z. & Gross, J. (1965) *Dev. Biol.* 12, 408–418.
26. Eisen, A. Z., Jeffrey, J. J. & Gross, J. (1968) *Biochim. Biophys. Acta* 151, 637–645.
27. Spudich, J. A. & Lin, S. (1972) *Proc. Natl. Acad. Sci. USA* 69, 442–446.
28. Hartwig, J. H. & Stossel, J. P. (1976) *J. Cell Biol.* 71, 295–303.
29. Cohn, R. H., Banerjee, S. D., Shelton, E. R. & Bernfield, M. R. (1972) *Proc. Natl. Acad. Sci. USA* 69, 2865–2869.
30. Dolberg, D. S., Bassham, J. A. & Bissell, M. J. (1975) *Exp. Cell Res.* 96, 129–137.
31. Tannenbaum, J., Tannenbaum, S. W. & Godman, G. C. (1977) *J. Cell. Physiol.* 91, 239–248.
32. Atlas, S. & Lin, S. (1978) *J. Cell Biol.* 76, 360–370.
33. Ali, I. U. & Hynes, R. O. (1977) *Biochim. Biophys. Acta* 471, 16–24.