1. The tensile strength of the glue is too low and cannot withstand the forces induced by the muscle.
2. The adherence force of the glue to the tissue is insufficient to hold the reattached muscle in place.
3. The glue is too elastic and, therefore, allows slippage of the reattached muscle.

It is possible to adjust the coagulation time of the glue by controlling the thrombin concentration. Rapid coagulation shortens the time needed to hold the muscle in place and may decrease the chances for muscle slippage.

Histologic examination revealed similar findings in both groups. No difference was found in the amount of fibrous tissue that developed in either group. In a previous study, 10 extraocular muscles were glued with Tissel; and two muscles slipped and retracted to the orbital space. In that and another study, no measurements of the muscle location were taken; therefore, the efficacy of the glue could not have been precisely tested.

In the current study, endophthalmitis developed in 18.8% of the sutured eyes. This was probably caused by globe perforation by the needle or during muscle disinsertion. Surprisingly, even in the glue group, endophthalmitis developed, although only in 2.4% of the eyes. Inadvertent microperforation during muscle disinsertion might have been the cause of endophthalmitis in the glue group because no sutures were passed in the sclera. The statistically significant difference between the occurrence of endophthalmitis in the two groups (P = 0.013) indicates that biologic glue is safer to use than suturing. Gluing the extraocular muscles can offer the strabismus surgeon an alternative to suturing the muscle to the globe and can prevent the complications of globe perforation. However, better control and handling of the glue is necessary for improved effectiveness at the muscle reattachment site before it can be used clinically. An improved glue with higher strength will help to achieve better results.

Key Words
extraocular muscles, fibrin glue, strabismus surgery

References

Regulation of Paracrine Cytokine Balance Controlling Collagenase Synthesis by Corneal Cells

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Supported by National Institutes of Health grants RO1-EY06408 (MEF), RO1-EY09828 (MEF), T32-AR07098 (KJS), and F32-EY06719 (KJS), and by an agreement between Massachusetts General Hospital and the Shiseido Company of Japan. MEF is a Japan and Davis Stain Research Tumor Blindsness Professor. Submitted for publication May 22, 1996; revised August 26, 1996; accepted September 30, 1996.

Proprietary interest category: N.
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Purpose. Classic studies have demonstrated that corneal epithelial cell density in culture can alter the balance of stimulatory and inhibitory cytokines controlling the elaboration of collagenolytic activity by co-cultured stromal cells. The current study attempts to bring the understanding of this mechanism to a molecular level.

Methods. A rabbit primary corneal cell culture model was used.

Results. Using molecular probes that bind to and neutralize specific cytokines, a major stimulator for stromal cell collagenase synthesis released by corneal epithelial cells into culture medium was identified as interleukin-1 alpha (IL-1alpha), and a secondary stimulator was characterized as a heparin-binding cytokine. An inverse relationship between net collagenase stimulatory activity and epithelial cell plating density was demonstrated. In contrast, the release of inhibitory activity for IL-1-stimulated collagenase synthesis was not subject to the cell density effect. Direct measurement of IL-1alpha protein

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levels revealed that this cytokine was released much more efficiently on a per cell basis when cells were plated at low density than when they were plated at high density. The effect was not caused by greater cell lysis at low cell density and was mediated only partially by changes at the IL-1α synthesis level.

Conclusions. These data provide evidence that epithelial cells release stimulatory cytokines for collagenase expression more efficiently when they have limited contact with their neighbors and that this has important consequences for the overall paracrine cytokine balance controlling collagenase synthesis. Alteration of the paracrine cytokine balance by changes in cell contact may be an important means for regulating epithelial–stromal interactions involved in corneal development and repair. Invest Ophthalmol Vis Sci. 1997; 38:546–552.

Interaction between the epithelium and the stroma is important for controlling corneal morphogenesis during development and plays an important part in corneal repair. An interesting model for studying this interaction was first described by Johnson–Wint and Gross in 1978. These investigators showed that stromal cells freshly isolated from the cornea of young rabbits and plated in primary culture did not produce activity necessary for the degradation of collagen. However, this activity could be detected in a latent form in the culture medium when stromal cells were cocultured with corneal epithelial cells. In another study, it was shown that culture medium conditioned by epithelial cells could substitute for epithelial cells as the stimulator. This demonstrated that cell interaction is mediated at least in part by a substance released by the cells rather than strictly by cell-to-cell contact. In addition to stimulatory factors, corneal epithelial cells were found simultaneously to release factors that antagonize the elaboration of latent collagenolytic activity by stromal cells. Interestingly, net stimulatory activity was found to be inversely related to epithelial cell plating density. Thus, on a per cell basis, epithelial cells plated at low density stimulated the elaboration of latent collagenolytic activity in co-plated fibroblasts to a much greater degree than epithelial cells plated at high density. Such an unusual regulation of cytokine release seemed important to understand in more detail. Unfortunately, further studies along these lines were precluded by a lack of information on the molecular nature of cytokines at the time the studies were performed and on the lack of availability of cytokines in pure form.

The past two decades have seen substantial progress in the purification and characterization of cytokines and other factors from cell cultures that mediate signaling between tissues, and a number of these cytokines have now been demonstrated to be expressed in cornea. However, it remains to be determined which of these are of primary importance for determining the morphogenetic consequences of epithelial–stromal interaction and how their expression and release are regulated. In light of the new molecular information on cytokines, cell culture models such as those of Johnson–Wint and Gross offer new opportunity for understanding the mechanisms controlling epithelial–stromal cell interactions and their morphogenetic consequences. In this article, we have taken advantage of the new reagents and information available to try to bring understanding of this mechanism to a molecular level.

METHODS. Animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Nine-millimeter central corneal disks were obtained by trephination from young New Zealand White rabbits weighing 3 kg, and the endothelium was removed with forceps. Epithelium was split away from the corneas by trypsin treatment, and stromal cells were then released by collagenase treatment as described.

To prepare the epithelial cell-conditioned medium, epithelial cells collected from six corneas were dispersed by pipetting and then were plated to 100 mm culture dishes in minimal essential medium (Life Technologies, Gaithersburg, MD) containing 10% supplemented calf serum to promote cell attachment and spreading. The next day, the medium was removed, the cells were washed, and the medium was replaced with 8 ml of serum-free medium. Cells were allowed to condition this medium for 48 hours before collection. When required, this conditioned medium was absorbed for heparin-binding substances by incubation with 0.5 ml of heparin sepharose (Pharmacia, Piscataway, NJ) for 1 hour. Conditioned medium also was prepared from epithelial cells plated at twofold serially decreasing dilutions into six-well plastic culture plates. In one experiment, 35S methionine (New England Nuclear, Boston, MA) was added to the medium at 80 μCi/ml to label proteins released into the culture medium. To quantitate the number of epithelial cells per well, a parallel plate was prepared, and the DNA content of each well was measured using a fluorimetry assay; DNA content was compared to a standard curve. After conditioned medium was removed, the adherent cells were washed once with phosphate-buffered saline and then lysed by freeze-thaw three times in 500 μl of phosphate-buffered saline with phenylmethylsulfonyl fluoride (0.4 mM). Cell extracts were centrifuged to pellet debris, and the supernatants were frozen for later analysis.

To measure the stimulatory effects of epithelial cell-conditioned medium or pure cytokines on the synthesis of collagenase by stromal cells, we used a standard laboratory bioassay adapted from the Johnson–Wint and Gross protocol for measuring collagenolytic
activity. Freshly isolated cells from six corneas were plated directly into a 24-well culture dish in equal numbers (2.0 × 10^5 per well) in culture medium containing 10% supplemented calf serum. The next day, the cells were washed three times, and wells were brought to 300 µl by adding either serum-free medium or one of the epithelial cell-conditioned media. Human recombinant interleukin-1 receptor antagonist (IL-1ra) was added to cultures in some experiments at 100 ng/ml (R&D Systems, Minneapolis, MN). When the inhibitory effects of epithelial cell-conditioned medium were to be examined, human recombinant interleukin-1α (IL-1α) at 1 ng/ml was added to the cultures to stimulate collagenase synthesis. Pure cytokines tested for bioactivity in this assay included human recombinant IL-1β, epidermal growth factor, IL-6, IL-8, and hepatocyte growth factor in doses ranging from 100 pg/ml to 10 ng/ml (R&D Systems). All treatments were performed in duplicate to ensure reproducibility. 35S-methionine was included in the wells at 80 µCi/ml to label newly synthesized proteins. After 24 hours of treatment, the medium containing radiolabeled newly synthesized and secreted proteins was removed and centrifuged briefly. The total labeled proteins in an equal volume sample (15 µl) from each culture well were displayed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. Results of a representative experiment are shown in Figure 1. Untreated stromal cell cultures synthesized and secreted the typical complex profile of proteins (Fig. 1A, untreated), but none of these was the predicted size of procollagenase (53 kDa). Immunoprecipitation analysis further revealed a collagenase protein level that was barely above background in these untreated cultures (Fig. 1B). Treatment with epithelial cell-conditioned medium, however, selectively stimulated synthesis of a 53 to 51 kDa doublet of the correct size to be the latent, proenzyme form of collagenase and the related metalloproteinase, stromelysin (Fig. 1A, +CM). Immunoprecipitation with anti-collagenase serum demonstrated that the induced 53 kDa protein was procollagenase (Fig. 1B, +CM).

Using the assay described above, we performed dose–response experiments with a battery of recombinant human cytokines known to be expressed in the cornea, including IL-1α, IL-1β, epidermal growth factor, IL-6, IL-8, and hepatocyte growth factor. Of these, only the recombinant forms of bFGF or IL-1 (both the IL-1α and the IL-1β isoforms) could stimulate collagenase synthesis by corneal stromal cells. Interleukin-1 was the more potent stimulator of the two (data not shown). In our next set of experiments, we attempted to determine whether bFGF, IL-1, or both might be mediators of the collagenase stimulatory activity present in epithelial cell-conditioned medium. Basic fibroblast growth factor is a heparin-binding protein; thus, we tested the capacity of heparin-linked sepharose beads to remove stimulatory activity from the conditioned medium. For IL-1, we tested the capacity of a specific IL-1 inhibitor to interfere with stimulatory activity. For this purpose, we used IL-1ra, a naturally occurring variant of IL-1 that can compete for binding to the cell surface IL-1 receptor but that has no signal-transducing properties. Experimental results are presented in Figure 1. When conditioned medium was adsorbed with heparin–sepharose, stimulatory activity for collagenase synthesis was decreased to 60% of the unadsorbed level, as monitored by the level of immunoprecipitable collagenase (Fig. 1B; Epi+CM+H). The addition of human recombinant IL-1ra (100 ng/ml) to the conditioned medium was even more effective and completely eliminated the stimulatory activity (Fig. 1A; CM+IL-1ra). These
Figure 1. Characterization of stimulatory cytokines in epithelial cell-conditioned medium. (A) Autoradiograph of ^35S-methionine-labeled proteins synthesized and secreted during a 24-hour period by stromal cell cultures that were untreated (−), treated with epithelial cell-conditioned medium (+CM), treated with heparin-cleared conditioned medium (+CM+H) or treated with conditioned medium plus interleukin-1 receptor antagonist at 100 ng/ml (+CM+IL-1ra). The electrophoretic migration positions of molecular size standards in kilodaltons (kDa) are indicated to the left. Arrows indicate the expected migration positions for procollagenase (CL; 53 kDa) and prostromelysin (SL; 53 kDa). The doublet band representing these proteins is starred in the +CM lanes. (B) Immunoprecipitation analysis of the samples is shown (A) with immune serum raised against rabbit collagenase. The 53 kDa band representing procollagenase is precipitated specifically (arrow). Relative amounts of precipitated protein in each lane were quantitated using laser densitometry. The average value for each duplicate treatment is shown under the corresponding lanes.

Treatments were selective and did not affect the levels of other synthesized and secreted proteins in general (Fig. 1A; +CM+H, +CM+IL-1ra). These results suggest that a heparin-binding cytokine and a cytokine that signals through the IL-1 receptor contribute to the total stimulatory activity in the epithelial cell-conditioned medium. However, the latter appears to have the major activity because the heparin-binding cytokine alone is not enough to stimulate collagenase synthesis to detectable levels.

Which of the two forms of IL-1 was present in the epithelial cell-conditioned medium was examined by RIA. We found that we could detect IL-1α easily in all the different samples of conditioned media assayed; the levels of this cytokine ranged between 40 and 1000 pg/ml. Sometimes IL-1β also was detected, but the amounts were always close to the limits of detectability (<20 pg/ml), suggesting possible artifact. Similar findings were obtained when the cell layer from which the conditioned medium was obtained was assayed. In this case, IL-1α levels varied over the range found in the conditioned medium; however, IL-1β was never detectable.

We next asked whether epithelial cells released enough IL-1α into their medium to account for the observed stimulatory activity for collagenase synthesis. To do this, we determined the level of collagenase synthesis stimulated by a sample of epithelial cell-conditioned medium and the levels of IL-1α in the conditioned medium. We then compared this to the level of collagenase synthesis stimulated by increasing doses of rabbit recombinant IL-1α plotted on a standard curve (data not shown). Conditioned medium from our sample of corneal epithelial cells contained approximately as much stimulatory activity as 7.5 pg/ml of IL-1α. By RIA, the IL-1α level was measured at 40 pg/ml. This result indicated that corneal epithelial cells release more than enough IL-1α to account for the stimulatory activity of their conditioned media, suggesting that much of this activity is masked, possibly by the presence of inhibitors of collagenase synthesis in the medium as previously demonstrated by Johnson-Wint and Gross.

Our next experiments addressed the previous finding that the net stimulatory activity for elaboration of collagenolytic activity in epithelial cell-stromal cell co-cultures is inversely related to the epithelial cell plating density. To extend the previous findings, we asked whether this inverse activity was reflected in the net stimulatory activity released in the culture medium. For this experiment, samples of conditioned medium prepared from epithelial cells plated at two-fold serial dilutions was collected and added to stromal cells for assay of the effect on synthesis of collagenase. Data are plotted in Figure 2 (top). The graph indicates the same inverse relationship between stimulatory activity and epithelial cell plating density as described by Johnson-Wint and Gross for co-culture.

Net stimulatory activity in conditioned medium is caused by the effect of total stimulators subtracted from the inhibitory effect of total inhibitors; changes in either type or both types of cytokines might deter-
mine the inverse relationship between cell plating density as documented above. Previously, it was not possible to measure the relative levels of inhibitory and stimulatory activity separately from net activity because of the lack of availability of pure stimulatory or inhibitory cytokines that could be added to culture medium to override the effects of the varying levels of endogenous cytokines. In this study, we were able to conduct this experiment: We made a relative measurement of the total inhibitory activity released into culture medium separately from stimulatory activity by testing the same conditioned medium samples described in Figure 2 (top) on stromal cells that were prestimulated with purified IL-1α. Results are presented as a graph in Figure 2 (middle). Inhibitory activity for IL-1-stimulated collagenase synthesis released into culture medium was found to be correlated directly to the epithelial cell plating density, in contrast to the net stimulatory activity. For example, medium derived from epithelial cells plated at 1 corneal equivalent (CE) depressed IL-1-stimulated collagenase synthesis by approximately 4.8-fold, whereas medium derived from the cells plated at 0.25 CE depressed IL-1-stimulated collagenase synthesis by only approximately 1.5-fold. This result suggests that it is the regulation of stimulator release that predominantly determines the inverse effect of cell density on stimulatory activity for collagenase synthesis.

To determine how IL-1α might contribute spe-
specifically to the inverse relationship between epithelial cell plating density and stimulatory activity, we measured the amount of IL-1α in each of the samples described in Figure 2 (top) by RIA. Data are graphed in Figure 2 (bottom). The levels of IL-1α in each of the samples was strikingly similar in every case and was only slightly affected by the number of cells available to release IL-1α. Thus, medium prepared from 1 CE of epithelial cells contained nearly the same amount of IL-1α as medium prepared from cells plated at 0.062 CE. When considered on a per cell basis, this translates into a much greater release from cells plated at low density than at high density.

In contrast to the case for released IL-1α, the total amount of IL-1α associated with the cell layer increased as the number of cells increased (Fig. 2, bottom). This increase was not entirely proportional to the increase in cell number. Thus, medium derived from 0.062 CE of cells produced approximately 800 pg of IL-1α, but medium derived from 1 CE of cells produced approximately four times more IL-1α (3300 pg). Nevertheless, these results suggest that the cell density effect influencing the levels of released IL-1α can be explained only partially by an effect of cell density on the rate of IL-1α synthesis.

It seemed unlikely that the greater release of stimulator at low as opposed to high cell densities might be caused by cell lysis at low density plating because the net release of inhibitors (as shown in Fig. 2, middle) was not subject to the cell density effect. The results of radiolabeling experiments offered additional support for this conclusion. In Figure 3, the total proteins synthesized and secreted by cells plated at the highest and the lowest density used for the experiment described in Figure 2 were compared. Not only is the protein profile comparable, the overall total protein released by high-density cells is much higher than the amount released by low-density cells. Furthermore, the pattern of proteins associated with the cell layer was different than that released into the culture medium and did not suggest any overlap of intracellular proteins with extracellular proteins that would result from cell lysis (data not shown).

**DISCUSSION.** A set of reports published almost two decades ago by Johnson–Wint and Gross demonstrated that cell density can alter the balance of stimulatory and inhibitory cytokines produced by corneal epithelial cells for elaboration of collagenolytic activity by stromal cells. However, the lack of molecular characterization of the cytokines involved and the lack of availability of cytokines in pure form precluded further investigation into the mechanism. In this study, we have identified a major stimulator produced by rabbit corneal epithelial cells in culture as IL-1α and a minor stimulator as a bFGF-like cytokine. By adding pure IL-1α to samples of epithelial cell-conditioned medium to override any differences in stimulatory cytokines released by cultures plated at different densities, we demonstrated that overall release of cytokines inhibitory for collagenase expression is not subject to the cell density effect. This suggests that regulation of the release of stimulatory cytokines must be the major factor determining the inverse correlation between cell plating density and net stimulatory cytokine activity for collagenase expression. Direct measurement of IL-1α protein provided more direct support for this conclusion because this cytokine was released more efficiently on a per cell basis when cells were plated at low density rather than at high density. The effect was not caused by greater cell lysis at low cell density and was mediated only partially by changes at the level of IL-1α synthesis. These data provide evidence that epithelial cells release stimulatory cytokines for collagease expression more efficiently when they have limited contact with their neighbors and that this has important consequences for the overall par-
acrine cytokine balance controlling collagenase synthesis.

There are two biochemically distinct forms of IL-1—IL-1α and IL-1β. Each of these molecules is encoded by separate genes only distantly related by sequence; however, both isotypes bind and signal equally well through the IL-1 receptor found on target cells. Both isotypes of IL-1 are translated as 31 kDa precursors, but the form found in culture medium is predominantly a proteolytic fragment of 17.5 kDa, the C terminal portion of the precursor. In humans, the IL-1α form has a pI of 5 and the IL-1β isotype has a pI of 7. A major collagenase stimulator produced by rabbit corneal epithelial cells was characterized biochemically by Johnson–Wint as a protein of 29 kDa with a pI of 4.7. This fits with the finding reported here identifying a major collagenase stimulator produced by corneal epithelial cells as IL-1α.

Our observation that IL-1α is released more efficiently at reduced cell density fits with findings of a previous study on release of an IL-1-like cytokine by the corneal epithelial cell line, SIRC. Skin epidermal cells also have been found to release an IL-1-like activity more efficiently when plated at low density. At present, it remains to be determined what causes this enhanced release. Neither of the IL-1 isotypes has a signal sequence at the N-terminus; thus, these cytokines cannot be secreted by cells according to the standard pathway through the endoplasmic reticulum. Recent studies suggest that release of cytokines without signal sequences occurs by a novel exocytosis mechanism. Interestingly, bFGF, a second cytokine identified in this study as a corneal cell collagenase stimulator, also lacks a signal sequence. In contrast, the major collagenase inhibitor released by epithelial cells, TGF-β2, which we identified in a previous study, does have a signal peptide and is secreted by normal routes. This suggests the intriguing idea that the key to understanding the novel mechanism controlling the paracrine cytokine balance lies in understanding how a reduction in cell:cell contact might activate exocytosis of cytokines without signal peptides. Molecules such as cadherins or integrins involved in epithelial cell adhesion, may have a potential role in such a mechanism. This seems an important area for further study.

Key Words
cell density, collagenase, cornea, epithelium, interleukin-1

Acknowledgments
The authors thank Dr. Jerome Gross (Massachusetts General Hospital) and Dr. Barbara Johnson–Wint (Northern Illinois University) for helpful and stimulating discussions about their work. The authors are also thank the reviewers for a very careful critique of the manuscript and for their useful suggestions. The authors especially thank Dr. Constance Brinckerhoff (Dartmouth Medical School) for the gift of rabbit collagenase antiserum.

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