STIMULATION OF CORNEAL DIFFERENTIATION BY INTERACTION BETWEEN CELL SURFACE AND EXTRACELLULAR MATRIX

I. Morphometric Analysis of Transfilter "Induction"

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ABSTRACT

The present study was undertaken to determine whether or not physical contact with the substratum is essential for the stimulatory effect of extracellular matrix (ECM) on corneal epithelial collagen synthesis. Previous studies showed that collagenous substrata stimulate isolated epithelia to produce three times as much collagen as they produce on noncollagenous substrate; killed collagenous substrata (e.g., lens capsule) are just as effective as living substrata (e.g., living lens) in promoting the production of new corneal stroma in vitro. In the experiments to be reported here, corneal epithelia were placed on one side of Nucleopore filters of different pore sizes and killed lens capsule on the other, with the expectation that contact of the reacting cells with the lens ECM should be limited by the number and size of the cell processes that can traverse the pores. Transfilter cultures were grown for 24 h in [^{3}H]proline-containing media and incorporation of isotope into hot trichloroacetic acid-soluble protein was used to measure corneal epithelial collagen production. Epithelial collagen synthesis increases directly as the size of the pores in the interposed filter increases and decreases as the thickness of the filter layer increases. Cell processes within Nucleopore filters were identified with the transmission electron microscope with difficulty; with the scanning electron microscope, however, the processes could easily be seen emerging from the undersurface of even 0.1-μm pore size filters. Morphometric techniques were used to show that cell surface area thus exposed to the underlying ECM is linearly correlated with enhancement of collagen synthesis. Epithelial cell processes did not pass through ultrathin (25-μm thick) 0.45-μm pore size Millipore filters nor did "induction" occur across them. The results are discussed in relation to current theories of embryonic tissue interaction.

Recent investigations of embryonic tissue interaction in vitro have called renewed attention to Grobstein's longstanding hypothesis that extracellular matrix (ECM) produced by the "inducer" either is or at least contains the material which stimulates or stabilizes the differentiation of the reacting tissue. In investigations of notochord-somite tissue recombinations, Kosher et al (20) have found that chondroitin sulfate proteoglycan added to the culture medium effectively substitutes

for notochord in promoting somite chondrogenesis, recalling the demonstration by Nevo and Dorfman (28) that chondromucoprotein added to the culture medium of suspended chondrocytes has a positive feedback on its own synthesis. Matrix molecules produced by embryonic chick notochord and neural tube at the time of notochord-somite interaction include chondroitin sulfate as well as collagen (6, 34, 15, 20). During embryonic chick development in vivo, cephalic neural crest mesenchyme which migrates to a position under the pigmented epithelium differentiates into scleral cartilage. Newsome (29) has shown that clones of unexpressed neural crest cells undergo chondrogenesis when cultured on Millipore filter containing ECM secreted by pigment epithelium.

In investigations of the enhancement of corneal differentiation by lens in vitro, we found that the killed collagenous lens capsule is as effective as living lens in stimulating corneal epithelial synthesis of corneal matrix molecules and overt differentiation of corneal stroma (7, 8, 14, 24, 25). Enzymatically isolated epithelia, when placed directly on a dead lens capsule, produce a morphologically identifiable stroma at the lens-epithelium interface and within the epithelium itself, in intercellular clefts between adjacent eipthelial cells (25). Lens capsule extracted in NaOH to remove noncollagenous protein, collagen-rich vitreous humor, frozen-killed corneal stroma, killed cartilage matrix, rat tail tendon collagen gels, and pure cartilage (chondrosarcoma) collagen are almost equally effective in stimulating the corneal epithelium to produce a corneal stroma similar in biochemical composition and in ulstructure to the primary stroma synthesized by corneal epithelium in vivo. Corneal epithelia cultured on noncollagenous substrata such as glass, plastic, albumin, keratin, or Millipore filter, synthesize a base-line level of collagen and glycosaminoglycan (GAG) which is only one-third of the level achieved by cultures grown on collagenous substrata and produce no overt corneal stroma.

Since highly purified chondrosarcoma collagen was as effective as the collagenous lens capsule in promoting stroma production and since none of the noncollagenous substrata stimulated corneal differentiation, we concluded that the collagen in the adjacent ECM is one of the active factors controlling differentiation of the corneal epithelium in vitro and perhaps in vivo as well (25). This work supports the idea that collagen can have a direct effect on cell differentiation, as proposed originally by Konigsberg and Hauschka (18) who demonstrated that collagen is required in vitro as a substratum for the development of differentiated muscle from isolated embryonic chick myoblasts. That GAG also plays a role in corneal development is suggested by the fact that heparan and chondroitin sulfates, when added to the culture medium, double GAG synthesis by epithelia; however, the addition of polysaccharides does not influence collagen synthesis nor promote visible stroma differentiation (26).

The availability of thin Nucleopore filters (32) that are readily penetrated by cell processes (35) allowed us to modify our in vitro system to study the role of cell-ECM contact in the stimulation of collagen synthesis by lens extracellular matrix. Nucleopore filters of various pore sizes were interposed between corneal epithelia and killed lens capsules (Fig. 1) and both the cell surface area exposed by cytoplasmic processes traversing the

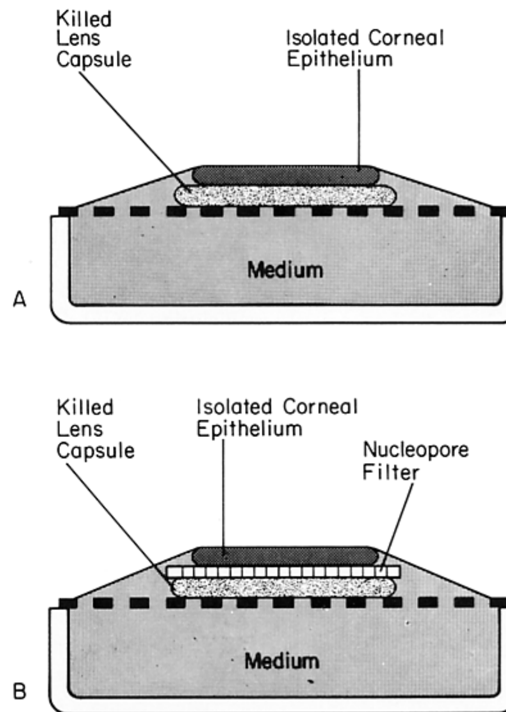

FIGURE 1 Diagrams of the cultures. (*a*) Epithelium cultured directly on killed lens capsule. (*b*) Epithelium separated from the killed lens capsule by a Nucleopore filter barrier to evaluate the role of cell contact with ECM in stimulating corneal differentiation.

filter and epithelial collagen production were measured. The resulting data show that direct cell contact with ECM is responsible for the stimulatory effect of collagen on stroma production by corneal epithelium.

MATERIALS AND METHODS

Organ Culture

Corneas were dissected from 5-day old White Leghorn chick embryos (Spafas, Norwich, Conn.) and corneal epithelia were isolated by trypsin-collagenase digestion as previously described (25). Individual epithelia were cultured basal-side down on one of the following substrata:(*a*) 0.1-μm, 0.2-μm, 0.4-μm, or 0.8-μm pore size (8-μm thick) Nucleopore filters which were washed and autoclaved (Arthur H. Thomas Co., Philadelphia, Pa.); (*b*) ultrathin (25-μm thick) Millipore filters (type HA, pore size 0.45 μm, Millipore Corp., Bedford, Mass.) which were washed and either autoclaved or sterilized by UV light: *c*) 12-day old embryonic chick lens, killed by autoclaving or by repeated (10 times) freeze-thawing in distilled water. In the latter case, most of the lens cell debris was removed because the capsules broke; these substrata will be referred to as lens capsule preparations. Epithelia were also cultured transfilter from the lens substratum. In this case, an epithelium was placed on a Nucleopore or Millipore filter (either side), which in turn was placed on a frozen-killed lens; the epithelium and lens thus were separated by a filter barrier (Fig. 1). Epithelia, covered by a meniscus of medium, were grown on the various substrata at the air-liquid interface on metal grids in Falcon dishes (No.3010, BioQuest Div, Becton, Dickinson & Co., Cockeysville, Md.). The medium was Ham's F-12 stock supplemented with 10% fetal calf serum (Grand Island Biological Corp., Grand Island, N.Y.), 0.25% whole embryo extract (5), and antibiotics 100 U'ml pencillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone (E. R. Squibb & Sons, Princeton, N.J.). Cultures were incubated for 24 h (unless otherwise specified) at 38°C in a humidified gas mixture (95% air, 5% CO_2).

Isotope Incorporation

Collagen synthesis was estimated by measuring the amount of [^{3}H]proline (G, 6 Ci/mmol, New England Nuclear, Boston, Ma., 5μCi/ml medium) that was incorporated during the 24-h culture period into hot trichloroacetic acid (TCA)-soluble material. Eight epithelia were set up in one organ culture dish and each biochemical determination of collagen synthesis is the result of their pooled effort. Isotopically labeled tissues plus substrata were rinsed in regular Hanks' solution, sonicated in ice-cold 5% TCA, and the insoluble material was collected by centrifugation at 4°C. The precipitate was resuspended in 5% TCA and heated at 90°C for 45 min. After cooling to room temperature, the samples were centifuged and the radioactivity in the neutralized supernate was measured in 10 ml of Aquasol (USV Pharmaceutical Corp.) using a Beckman LS-150 liquid scintillation counter (Beckman Instruments Inc., Fullerton, Calif.). It has been previously shown (25) that at least 85% of the radioactivity in the hot TCA-soluble material is sensitive to a highly purified, protease-free collagenase (30). Since there is little degradation of newly produced collagen during the 24-h culture period (25), the measurement of newly produced collagen reflects fairly accurately the amount synthesized in this period.

In order to determine what proportion of the [^{3}H]-proline in the hot TCA extract was converted to [^{3}H]hydroxyproline, cultures were labeled with 10 μCi/ml [2, 3-^{3}H]proline (N, 25 Ci/mmol, New England Nuclear) for 24 h. The cultures were pooled (16–24 epithelia plus substrata) and collagen was extracted with hot TCA as described above. The TCA was removed with ether and the dried precipitate was hydrolyzed overnight in 6 N HCl. The dried hydrolyzate was dissolved in distilled water containing 0.5 mg/ml proline and hydroxyproline as carrier. Tritiated proline was converted to [^{3}H]pyrrole according to the method of Switzer and Summer (33), extracted in toluene, and counted in 10 ml Aquasol. The remaining aqueous portion of the sample was boiled, and [^{3}H]hydroxyproline extracted in toluene and counted. For epithelia cultured on filters (without lens), the hypro-to-pro ratio was 0.63 ± 0.02 (*n*, 4), while the hypro-to-pro ratio of epithelia cultured on lens was 0.74 ± 0.04 (*n*, 3).

Although each experiment consists of eight epithelia, the results are expressed as collagen synthesis per epithelium in Table II, because the exposed surface area was calculated on a per epithelium basis. 5-day old corneal epithelium behaves consistently in culture; DNA accumulates at a level only slightly less than that which would have occurred in the same 24-h period in vivo (25). We conclude that there is relatively little cell death in this in vitro system and that cell numbers are comparable from epithelium to epithelium in the time period studied (25). Moreover, 5-day corneal epithelia cultured for 24 h on noncollagenous substrata, such as filters, accumulate DNA, RNA, and noncollagenous protein at approximately the same level (25).

Electron Microscopy

For transmission electron microscopy, cultures were fixed in gluteraldehyde-formaldehyde followed by osmium tetroxide, stained en bloc with uranyl acetate, and embedded in Araldite as described previously (16). For scanning electron microscopy, epithelium plus filter were first removed from the underlying lens and then processed as above, except that after fixation in osmium tetroxide and dehydration in ethanol, they were critical-point dried in a Samdri apparatus by use of liquid CO_2. The undersurface of filters not in contact with lens was

also studied. Dried specimens were glued epithelium-side down on aluminum chucks and coated with a palladium-gold (60:40) alloy (Ladd Research Industries, Inc., Burlington, Vt.). Specimens were scanned at 25 kV in a JEOL SMU-3 scanning electron microscope.

Morphometric Methods

Examination of the filter undersurface revealed cell processes emerging from pores in the filter in an area restricted roughly by the size of the epithelium found on the opposite side of the filter. After a reconnaissance scan, the specimen was removed from the scope, and a 400-mesh copper grid (Ted Pella Co., Tustin, Calif.) was placed on the filter over the cell processes. An area three grid squares across and three grid squares down (nine grid squares total, total area 25,186 μm^2, open area 10,712 μm^2) exceeded the area of the filter covered by an individual epithelium, as outlined by underlying cell processes. A systematic random sample area of the lower left-hand corner of each of the nine grid squares was photographed at a magnification of 10,000. Since the smallest cell process was about 0.2 mm wide at this magnification, a graph grid was constructed with vertical and horizontal lines 0.2-mm apart. Therefore, the smallest cell process could not span two consecutive intersections of lines in the graph in any direction. The graph grid was placed over each photograph and the number of times that a line intersection fell on a cell process was recorded as a "hit." A hit was assigned the area of one graph square (0.04 mm^2). Thus, using a sterological point-counting technique (3), we analyzed equivalent test areas of the undersurface of filters. The thickness of Nucleopore filters was determined from thick sections by direct measurement employing a calibrated stage micrometer.

RESULTS

Collagen Synthesis in Nucleopore Transfilter Cultures

In the initial experiment, enzymatically isolated corneal epithelia were grown on killed lens capsule either in direct contact with or separated from the capsule by Nucleopore filters with 0.8-μm size holes. Collagen synthesis during the 24-h culture period was determined by measuring the incorporation of [^{3}H]proline into hot TCA-soluble protein. It was found that an epithelium separated from the lens by the large pore-size Nucleopore filter produced almost 75% as much collagen as when the epithelium was in direct contact with the lens. Epithelium cultured directly on Nucleopore filter alone synthesized collagen at the base-line level (Fig. 2) common to all other noncollagenous substrata previously examined (25).

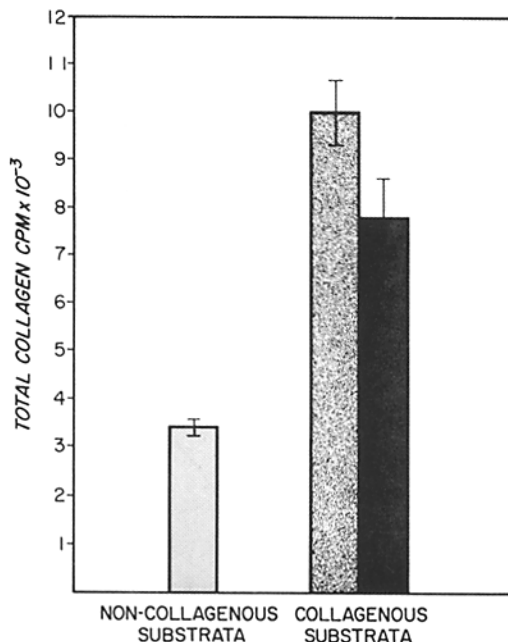

FIGURE 2 Epithelium separated from the killed lens capsule by a Nucleopore filter (black) produces about 75% of the amount of collagen produced by epithelium in direct contact with lens ECM (stippled). Epithelia cultured on collagenous substrata such as lens capsule, concentrated chondrosarcoma collagen gels, and rat tail tendon gels synthesize nearly three times as much collagen in the 24-h culture period as do epithelia cultured on noncollagenous substrata such as Millipore filter, glass, and plastic (light) (25). Since epithelial collagen production on a variety of noncollagenous substrata is consistently lower, we refer to this as the base-line level. Epithelia cultured directly on Nucleopore filter without an underlying lens produce collagen at this base-line level.

We reasoned that if cell processes were penetrating the Nucleopore filter (35) to contact the underlying lens, then increasing the distance between the lens and the epithelium should interfere with the ability of the lens to stimulate collagen synthesis. Since Nucleopore filters are made in only one thickness (9 $\mu m \pm 1.2$ μm), we increased the distance between the lens and the epithelium by stacking 0.8-μm pore size filters. Again, collagen production was measured over a 24-h period by isotope incorporation into hot TCA-soluble protein. As can be seen in Fig. 3, total collagen production during the 24-h culture period decreases as the distance between the lens and the epithelium increases, falling to 75% of the direct

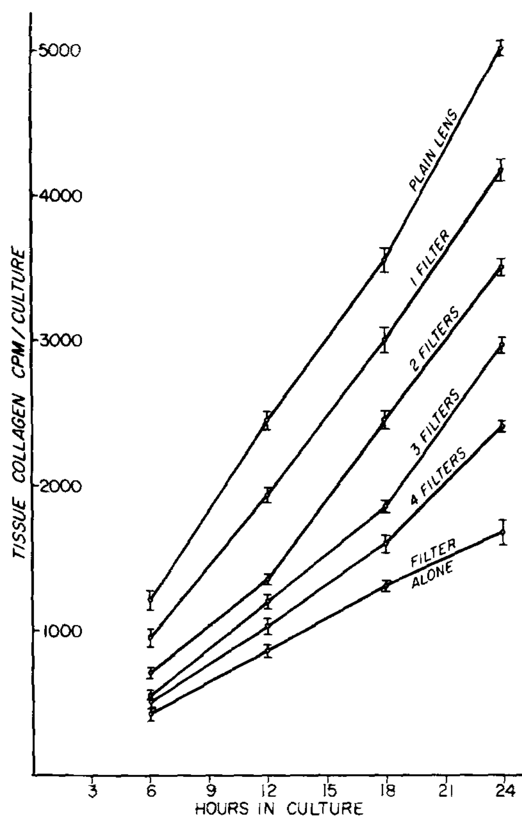

FIGURE 3 Corneal epithelium was grown on one plain Nucleopore filter, 0.8-μm pore size (bottom curve), on stacks of 0.8-μm pore size filters containing lens capsule on the side opposite the epithelium, and directly on lens capsule (top curve). The bottom curve corresponds to the base-line level synthetic activity and the top curve to the maximal "induced state." Since the stimulation of synthetic activity decreases with increasing filter thickness, the results are consistent with the idea that cell processes traverse the filter to contact the lens capsule on the other side. Cultures were grown continuously in the presence of 5μCi/ml [^{3}H]proline and harvested at various times up to 24 h in vitro. Vertical bars indicate the standard deviation about the mean for four determinations.

contact level when one filter (9-μm thick) is interposed, and to only 25% more than the baseline level when four filters (together 36 μm thick) are interposed. The size of direct channels available to cellular processes will be a function of the degree of overlap of holes in each filter. By cutting out the holes in photographs of single filters and arranging the pictures in random stacks, we were able to determine the size of direct channel space through a variety of combinations of stacked filters. We found that many channel sizes were created, some as large as 0.4 μm across.

In order to investigate the relation of channel size to transfilter stimulation of collagen synthesis by ECM, we cultured corneal epithelium on individual 0.8-μm, 0.4-μm, 0.2-μm, and 0.1-μm pore size Nucleopore filters. We expected the decreasing size of the pores would so decrease penetration of cell processes through the filters that epithelial cell contact with the extracellular matrix of the lens on the other side of the filter would be limited. If the level of collagen synthesis depends on epithelial cell contact with the lens capsule, then total synthesis should decrease as the size of the pores in the interposed filter decreases. This proved to be the case (Fig. 4). When a 0.1μm pore size filter is placed between an epithelium and the lens, collagen synthesis is greatly reduced, being only 20% greater than the nonstimulated (baseline) level. The level achieved is much greater with larger pores. In all cases where the level of collagen synthesis was greater than the nonstimulated (base-line) level, we were able to identify with the electron microscope a visible stroma of collagen fibrils deposited in clefts within the epithelium (Fig. 5) and on the epithelial undersurface next to the filter.

Electron Microscopy of Nucleopore Cultures

In order to establish that epithelial cell processes do traverse the filter to contact the lens "inductor," transfilter cultures were fixed after 24 h and thin sections were examined with the transmission electron microscope (TEM). Fig. 6 shows a cell process penetrating a channel in a 0.8-μm pore size filter. The process enters on the upper surface of the filter and, filling the cylindrical hole from edge to edge with cytoplasm, seems to probe along the channel walls in the direction of least resistance. The channels themselves pass straight through the filters, but they are not always perpendicular to the filter surface. Occasionally, the channels cross each other if the penetration angle is sufficiently oblique. The cell process illustrated in Fig. 6 seems to have coursed upward into a chamber made available by such a crisscrossing of channels in the filter. Those cell processes which emerge onto the other side of the filter may spread on the filter undersurface (Fig. 7) or along the lens capsule if they contact it. Seen in thin section, they are truly cell processes, since they are bound by a unit

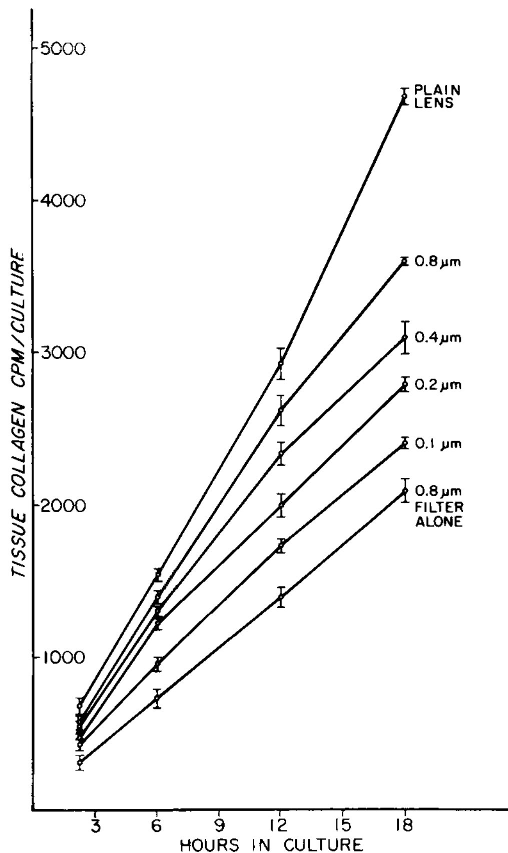

FIGURE 4 Experiment similar to that illustrated in Fig. 3, but in this case single filters of progressively smaller pore size were interposed between the lens capsule and corneal epithelium. Again, vertical bars indicate the standard deviation about the mean for four determinations. Since the stimulation of epithelial collagen synthesis decreases as the size of the pores in the interposed filter decreases, it is likely that smaller pore size filters limit epithelial-ECM contact.

membrane and contain cytoplasm filled with microfilaments (Fig. 7).

It proved difficult to quantitate the amount of transfilter cell surface from the small sample of thin sections that was practical to examine with the TEM. Therefore, we decided to examine the whole undersurface of the filter with the scanning electron microscope (SEM). As seen with the SEM, cytoplasmic processes emerging from the pores onto the undersurface of a 0.8-μm pore size filter appear to be large bulbous protrusions (Fig. 8). These processes can even be seen penetrating the small 0.1-μm pore size filter (fig. 9). If a lens capsule is present under the filter, the cell processes tend to be long and slender rather than bulbous.

Examination by SEM of the undersurface of 0.1-μm, 0.2-μm, 0.4-μm, and 0.8-μm pore size filters cultured for 24 h with corneal epithelium indicates there are obvious differences in the amount of cell surface exposed by cytoplasmic process penetration through the filters (Fig. 10). More and larger cell processes seem to have passed through the large pore size filters than the small pore size filters. To support this conclusion, it was necessary to calculate objectively the relative amount of cell surface which was exposed on the undersurface of the filter for each pore size filter used in the transfilter experiments.

Morphometric Analysis of Transfilter Cell Processes

As described in Materials and Methods, we employed a stereological point counting technique to analyze equivalent test areas of the underside of various pore size filters on which corneal epithelia were grown. An initial comparison was made between cultures of epithelia grown for 24 h on 0.4-μm pore size filters alone and cultures grown on the same size filter opposite lens for 24 h. Photographs taken at the same magnification in the SEM were overlaid with the analyzing graph grid and the number of times grid intersections fell on cell processes was translated into surface area for five different epithelial cultures in each experiment. The results in Table I indicate that the same amount of cell surface area (as estimated after critical point drying) appears on the undersurface of a filter cultured with an epithelium alone as appears on the undersurface of a filter with epithelium on the upper surface and lens on the undersurface.

Since the dead lens did not apparently promote the penetration of epithelial cell processes through the filter in transfilter cultures, we grew epithelia on various pore size filters without lens for 24 h so as to be able to analyze the amount of transfilter cell surface without the necessity of removing the lens for viewing. In addition, we measured the porosity for each pore size filter, that is the actual amount of open space the filter offers for epithelial process penetration, by counting the number of grid intersections which fell on the open pores of the filters. The results in Table II indicate that the mean cytoplasmic area per epithelium on the

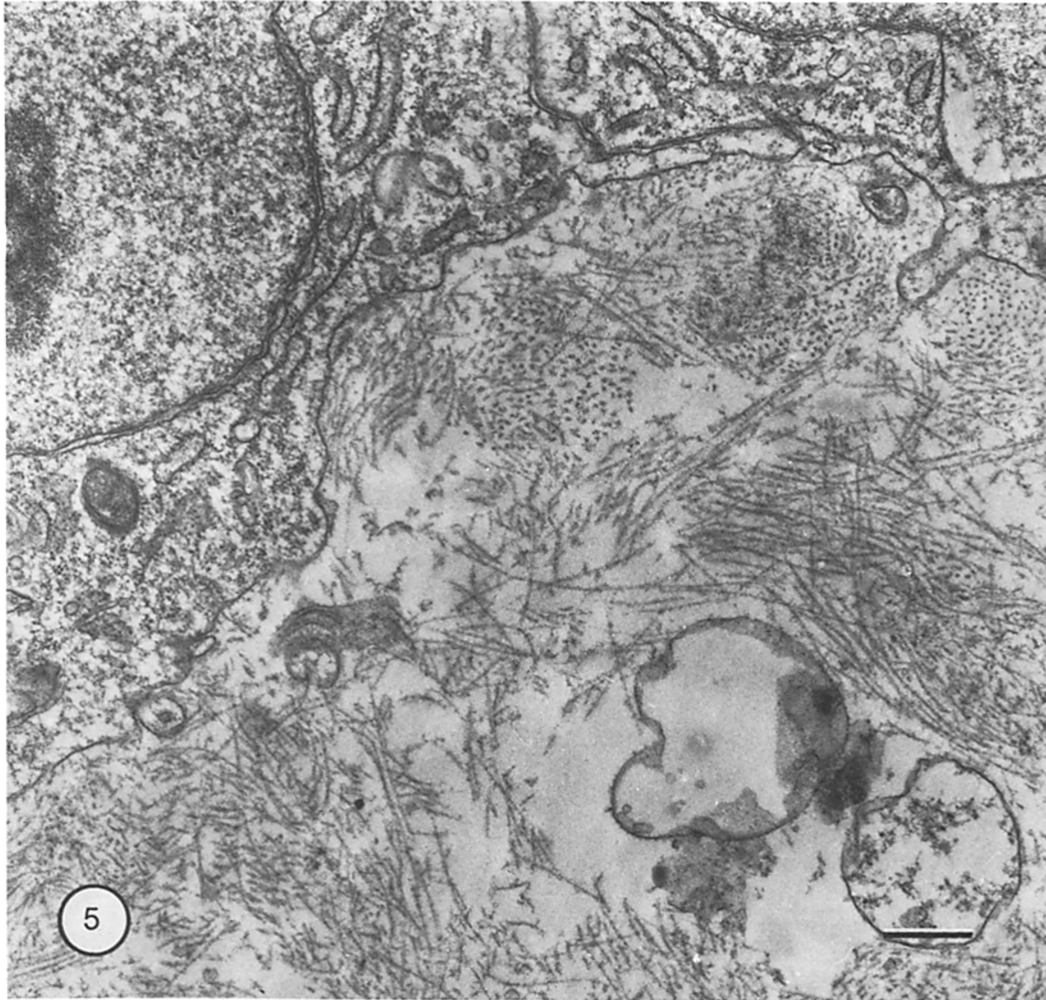

FIGURE 5 Electron micrograph showing deposition of collagen fibrils (corneal stroma) within an intercellular cleft in an epithelium grown on a 0.4-μm pore size filter for 24 h. Interestingly, the new stroma polymerizes mainly on the epithelial side of the filter, rather than next to the lens capsule on the other side. A few poorly preserved cell processes are entrapped in the new stroma. The bar represents 0.5 μm. $\times$ 23,000.

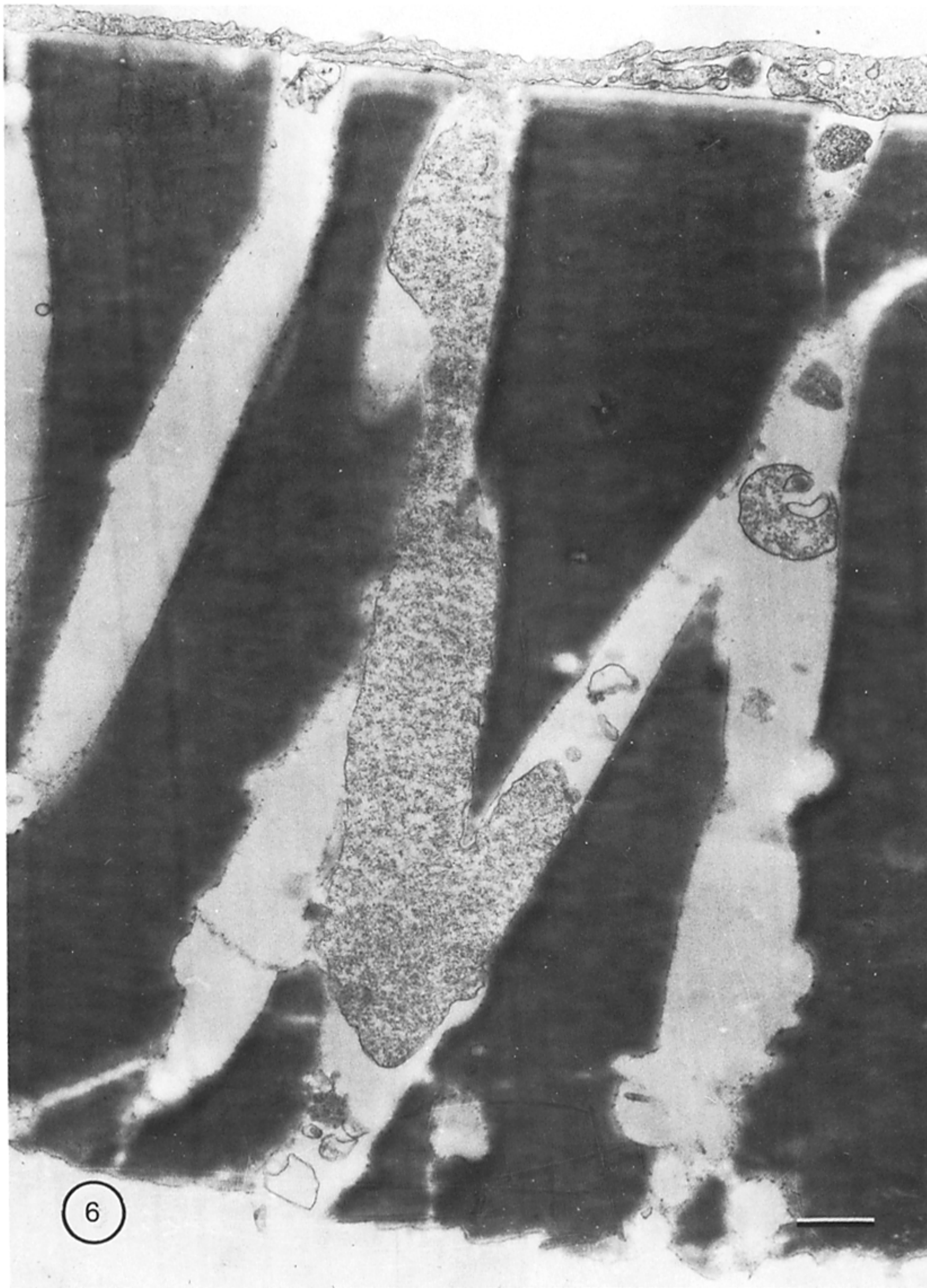

FIGURE 6 Transmission electron micrograph showin a corneal epithelial cell process extending into a channel in a 0.8-μm pore size Nucleopore filter. The bar represents 0.5 μm. $\times$ 23,000.

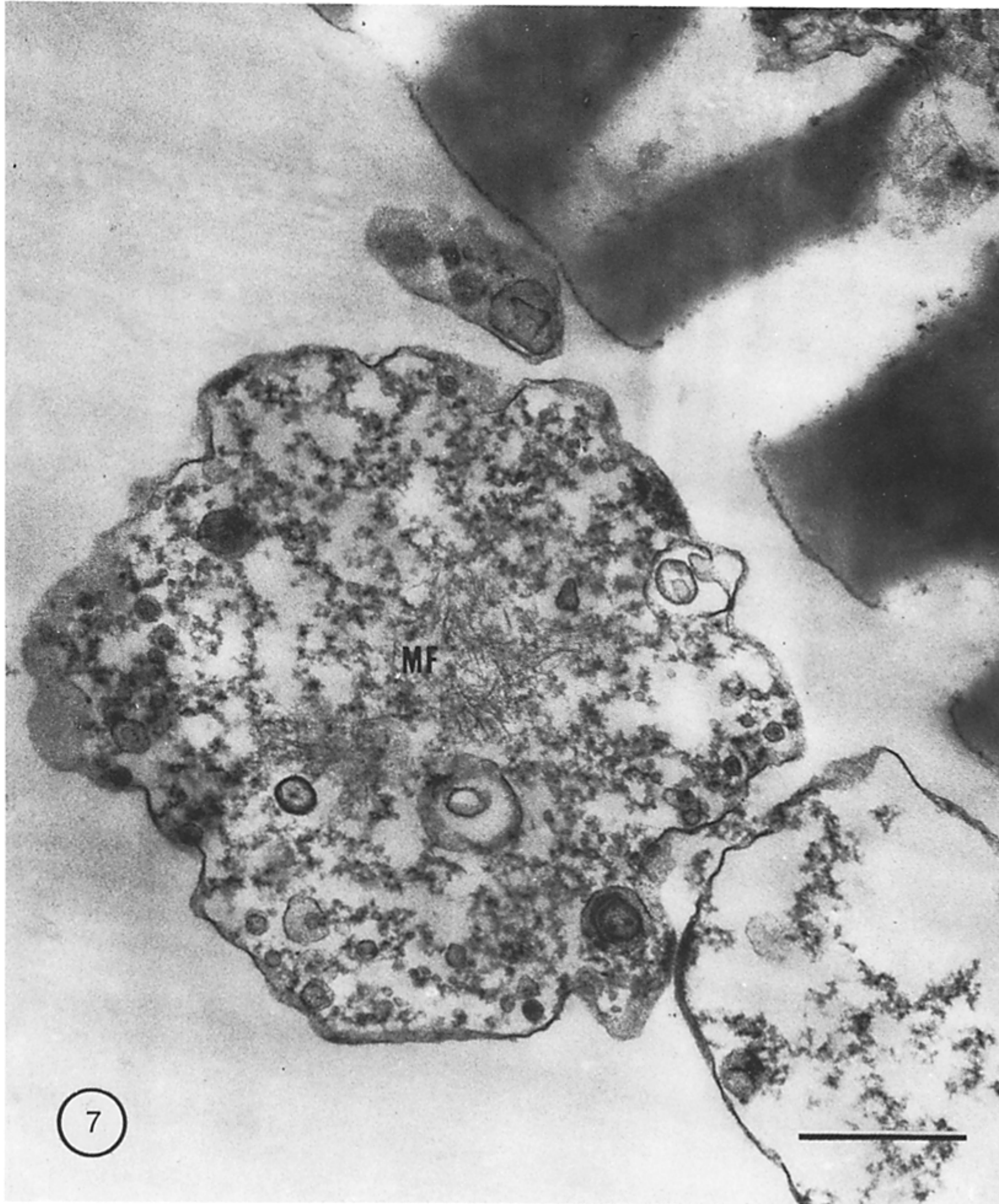

FIGURE 7 Transmission electron micrograph showing an epithelial cell process which has emerged onto the undersurface of 0.8-μm pore size Nucleopore filter. These processes typically are bound by a unit membrane and they contain microfilaments (MF). The bar represents 0.5 μm. $\times$ 47,000.

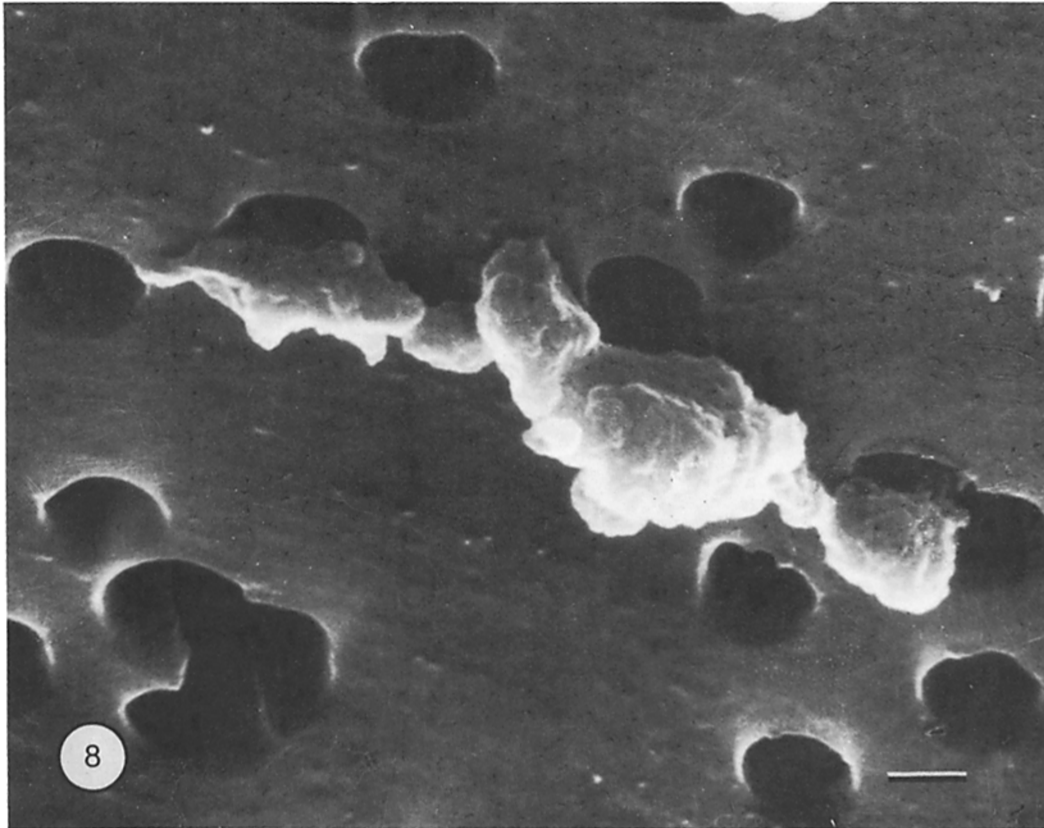

FIGURE 8 Scanning electron micrograph of the undersurface of a 0.8-μm pore size Nucleopore filter showing the emergence of cell processes derived from the corneal epithelium cultured in the other side of the filter. The bar represents 0.5 μm. × 20,000.

undersurface of the filter decreases directly as the size of the pores in the filter decreases. The porosity (open space) of the filters, on the other hand, does not differ as greatly among the various pore size filters. For example, the porosity of a 0.4-μm pore size filter is similar (due to a larger number of pores) to that of a 0.8-μm pore size filter (Table II). While little correlation exists between collagen synthesis and filter porosity, a strong correlation can be made between the degree of collagen production and the actual exposed surface area created by the transfilter cell processes. A plot of the log of the data in columns 3 and 4 of Table II indicates a linear relation between the exposed surface area and the level of increased collagen synthesis for each pore size filter examined (Fig. 11).

Examination of Millipore Transfilter Cultures

In order to compare our transfilter system to those used by Grobstein (10) and other earlier workers studying transfilter induction, we cultured corneal epithelia on ultrathin (25-μm thick) 0.45-μm pore size Millipore filters placed on dead lens capsules. As can be seen from Table III, collagen production by corneal epithelium on Millipore filter cultured transfilter to lens occurs at the base-line (nonstimulated) level. Even after 48 h on Millipore filter, corneal epithelium cultured transfilter to lens synthesizes collagen only at the base-line level, suggesting that cell processes have not crossed the filter to contact the lens.

Careful examination of the undersurface of

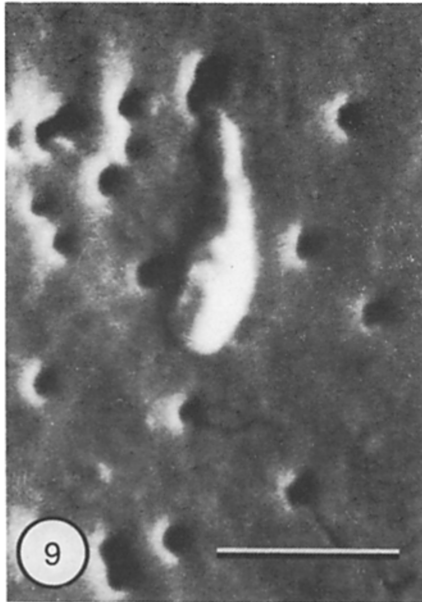

FIGURE 9 Scanning electron micrograph of the undersurface of a 0.1-μm pore size Nucleopore filter. A small cell process from the epithelium on the other side has emerged. The bar represents 0.5 μm. $\times$ 50,000.

ultrathin Millipore filters bearing epithelia on the opposite side failed to reveal penetration of cellular processes through the filters (0/40 cases). The filter undersurface appears irregularly contoured, with interconnecting strands of cellulose meshing to form holes and channels of a variety of sizes and shapes (Fig. 12). Filters which were sterilized by UV treatment or short-term autoclaving (5 min) were indistinguishable from those which were not sterilized at all. However, longer periods of autoclaving (15 min) caused the filters to ripple and shrink and the pores and channels in these filters seemed greatly reduced (Fig. 12).

DISCUSSION

The response of corneal epithelium in vitro to direct contact with extracellular matrix (ECM) is heightened collagen and GAG synthesis and the production of a biochemically and morphologically defined stroma. Isolated epithelia cultured on noncollagenous substrata synthesize collagen at a low (base-line) level and fail to produce a stroma (25). In the present work, study of the nature of the interaction of the corneal epithelium with underlying extracellular matrix was facilitated by the ready invasion across Nucleopore filters of naked cell processes extruded by isolated epithelium. By interposing filters which allowed varying degrees of contact between the lens extracellular matrix and the epithelial cells, we were able to demonstrate intermediate levels of epithelial collagen synthesis which were above the base-line level but below the level achieved on unimpeded contact with lens capsule. Variable amounts of orthogonally arranged striated collagen fibrils were produced by the epithelia that synthesized collagen at intermediate levels.

Since epithelia cultured on Nucleopore filters alone fail to produce a stroma, it seemed likely that transfilter stimulation of collagen synthesis was due to the contact of the epithelial cell processes with the lens. Therefore, we measured by morphometric techniques the amount of epithelial surface exposed by cell processes at intermediate levels of corneal stroma production. By this means, a linear relationship was demonstrated between the level of epithelial collagen synthesis and the total area of cell surface contacting lens extracellular matrix. Thus, we conclude that a major anabolic activity of corneal cells, collagen synthesis, is regulated by cell surface-ECM interaction.

It is unlikely that transfilter stimulation of epithelial collagen production by ECM could be due to the diffusion of molecules originating from the dead lens. For such a situation, a diffusion gradient would have to be maintained over a short distance (10–15 μm) through the filter for at least 24 h. This is improbable because the meniscus covering the tissue connects the epithelium with the medium below and permits the diffusion of substances in all directions and to all parts of the culture system. More importantly, there was no correlation between collagen production and filter porosity, that is the total open space in the filter. For example, since there are more pores in the 0.4-μm pore size filter than in the 0.8-μm pore size filter, the porosity was nearly the same (19.0% $\pm$ 3.2%) for both filters, yet transfilter epithelial collagens production on 0.8-μm pore size filters was 33% greater than that on 0.4-μm pore size filters. Moreover, there was no enhanced epithelial stroma synthesis by lens in transfilter experiments utilizing 0.45-μm pore size Millipore filters; here the porosity of the filters is greater than 75%. The amount of cell surface exposed by cell processes

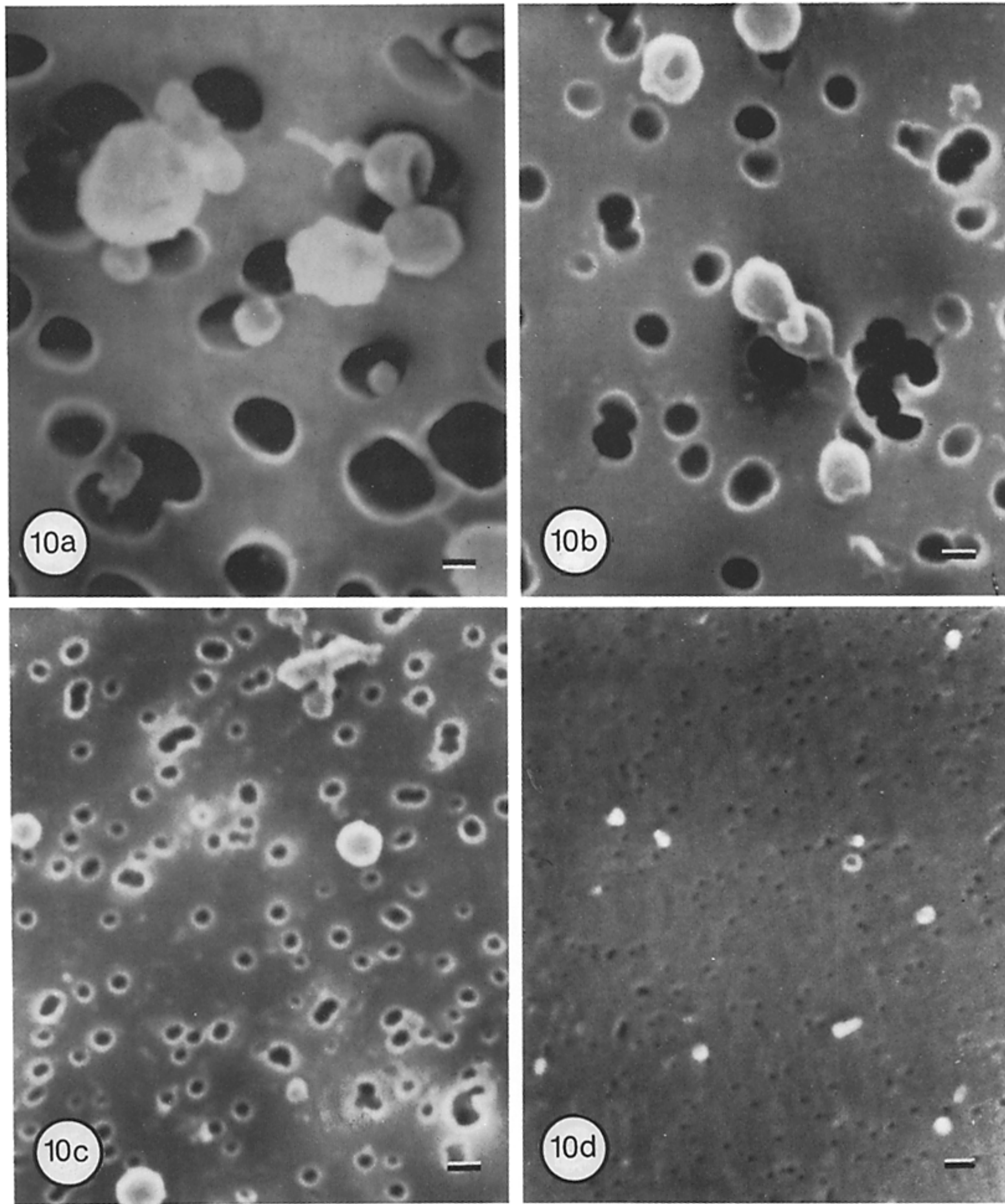

FIGURE 10 Scanning electron micrographs of cell processes emerging onto the under surface of 0.8-μm pore size (*a*) 0.4-μm pore size (*b*), 0.2-μm pore size (*c*), and 0.1μm pore size (*d*) Nucleopore filters. Photographs of this type were analyzed with the graph grid overlay to determine the amount of cell surface area created by cell processes which penetrate the filters. The bar is equivalent to 0.5 μm. $\times$ 8,500.

TABLE I

*A Comparison of the Exposed Cell Surface of Epithelia Cultured on 0.4-μm Pore Size Nucleopore Filters Alone and Transfilter to Lens**

Substratum	Exposed surface area
	μm²
0.4-μm pore size Nucleopore filter	4,447 + 1,122
0.4-μm pore size Nucleopore filter on lens capsule	4,406 ± 647

* Results are expressed per epithelium as the mean of five determinations ± the standard deviation.

TABLE II

*The Relation of Pore Size to Exposed Cell Surface in Transfilter Stimulation of Epithelial Collagen Synthesis by ECM**

Pore size	Porosity (open space)	Exposed surface area	Collagen synthesis
μm	%	*μm²*	*cpm*
0.8	21.0 ± 1.4	8,922 ± 1,842	566 ± 42
0.4	17.1 ± 1,6	4,447 ± 1,122	452 ± 19
0.2	12.3 ± 1.4	1,436 ± 406	368 ± 24
0.1	8.5 ± 1.2	350 ± 108	312 ± 11

* Results are expressed per epithelium as the mean of five determinations ± the standard deviation. Cultures were labeled for 24 h with 5 μCi/ml [³]proline.

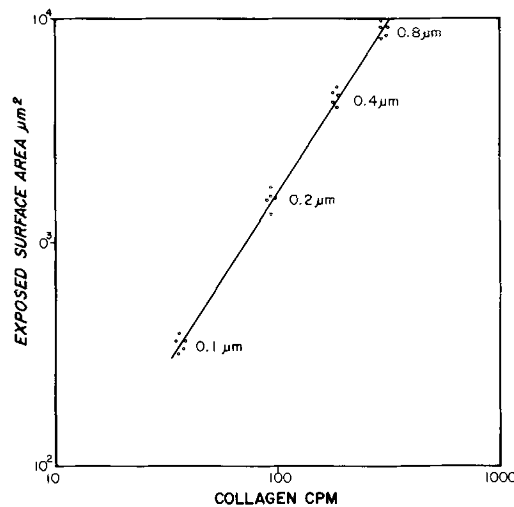

FIGURE 11 A plot on a log-log scale of the numerical data in columns 3 and 4 of Table II. The pore size of the filters is indicated. A linear relationship is shown between the exposed surface area of the emerging epithelial cell processes and the level of increased collagen synthesis. The level of increased synthesis is expressed as counts per minute above the base-line (265 cpm) level per epithelium; exposed surface area as square micrometers (Table II).

TABLE III

*The Effect of Lens Capsule on Transfilter Collagen Production by Corneal Epithelium Cultured on Ultrathin Millipore Filter**

Time in culture	Substratum	Collagen synthesis
h		*cpm*
	Millipore filter	2406 ± 264
24	Millipore filter on lens capsule	2475 ± 301
	Lens capsule	6198 ± 102
	Millipore filter	2961 ± 299
48	Millipore filter on lens capsule	3125 ± 314
	Lens capsule	12,842 ± 864

* Results are expressed as the mean of four determinations ± the standard deviation (eight epithelia per determination). Cultures were labeled for the last 24 h in vitro with 5 μCi/ml [³H]proline.

which penetrate the various pore size Nucleopore filters is linearly correlated with the level of enhanced collagen synthesis.

The quantitative relation demonstrated between pore size and exposed surface area, on the one hand, and the level of transfilter stimulation of collagen synthesis, on the other, invites a consideration of the nature of the response of the epithelium to contact with ECM. A plot of the log of the cell surface area exposed by cell processes traversing the filter versus the log of increased collagen synthesis is a straight line. This result implies that only a small amount of increased epithelial contact with lens ECM is necessary to elicit a significant increase in collagen production above the base-line level. For instance, the exposed cell surface area of epithelia cultured on 0.8-μm pore size filters is only about 30% of the total epithelial basal surface; however, collagen synthesis by 0.8-μm pore size transfilter cultures is stimulated to a level nearly 75% of the direct contact control. It is tempting to think that epithelial cell processes might contain

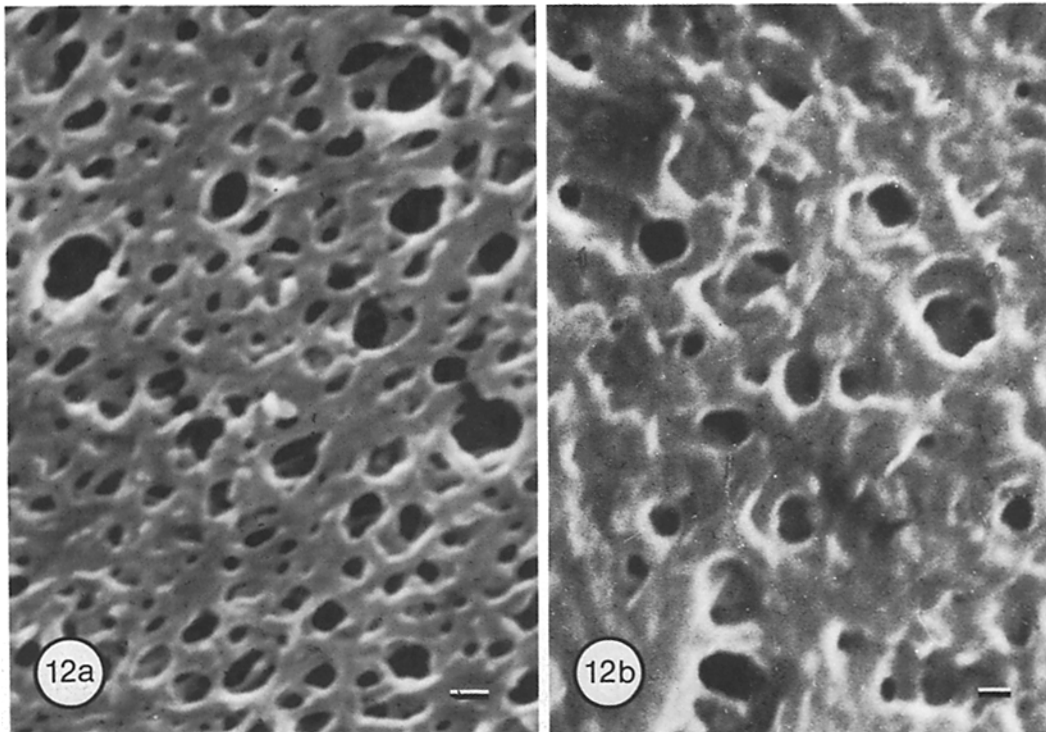

FIGURE 12 Scanning electron micrograph of the undersurface of ultrathin 0.45-μm pore size Millipore filters which bear corneal epithelia on the other side. (*a*) The undersurface of a briefly (5 min) autoclaved filter from a 48-h culture. The filter undersurface is free of epithelial cell processes. (*b*) The undersurface of a Millipore filter which was autoclaved for 15 min before being used as a substratum for transfilter corneal culture. The open spaces between interconnecting strands of cellulose are smaller. Again, there is no indication of epithelial process penetration. The bar represents 0.5 μm. (*a*) $\times$ 10,000; (*b*) $\times$ 7,000.

specific sites for collagen-ECM interaction and that the stimulatory mechanism is easily saturated. Whether enhanced collagen production can be attributed to the synthetic activity of only those epithelial cells which make contact with lens ECM or instead is the result of the pooled effort of every epithelial cell remains an open question which is difficult to answer because of the base-line synthetic activity of all of the cells.

Ancillary evidence to support the idea that it is cell surface contact with lens ECM which mediates enhanced corneal stroma production is derived from the filter-stacking experiments. We observed that the ability of the lens to promote epithelial stroma production is inversely related to the distance between the lens and the epithelium; the greater the transfilter distance, the smaller the stimulation of epithelial collagen synthesis by lens at any given time. We believe that lens-promoted epithelial collagen synthesis across stacked filters is dependent on the time it takes for a cell process to traverse the filter and thus is also related to the final amount of cell surface which emerges to contact the lens.

When corneal epithelia are isolated by treatment with trypsin-collagenase or EDTA, they are stripped of their underlying basement lamina (8). The epithelium responds to such treatment by extending blebs of cytoplasm from its basal surface. It is not surprising, then, that freshly isolated epithelium, cultured basal-side down on Nucleopore filters, can send cytoplasmic processes down into the pores and channels of the substratum. The probing of the filter by epithelial cell processes involves motile activity on the part of the living tissue. Seen in thin sections, the cell processes often contain numerous microfilaments, elements which have been implicated in cytoplasmic

motility in many tissues (for review, see reference 31). The same number of cell processes penetrate a 0.4-μm pore size filter alone as when the epithelium is cultured transfilter to lens. The dead lens, then, does not promote the cytoplasmic invasion across the filters; the invasion is inherent to the basally disrupted epithelium.

By taking advantage of the ability of the scanning electron microscope to examine total undersurface of the transfilter cultures, we were able to establish conclusively that cell processes as small as 0.1-μm in diameter are able to travel across a 10-μm thick filter. This observation would have been nearly impossible to make by analysis of thin sections with the transmission electron microscope, because the chance of finding a cell process completely traversing a 0.1-μm pore size filter in thin sections is remote. This formidable task is further complicated by poor preservation of cell structure deep within Nucleopore filters. The suggestion that 0.1-μm pore size Nucleopore filter excluded cell processes from spinal cord-metanephros transfilter cultures (35) merits re-evaluation, and the claim of Bray (4) and others that cells cannot extend long processes 0.1-μm in diameter is clearly contradicted by our study.

The demonstration here and by Wartiovaara et al. (34) that cell processes of small dimension traverse thin Nucleopore filters in no way contradicts the conclusions of Grobstein and others that cell processes do not traverse Millipore filters (12,11,17). Nucleopore filters are solid polycarbonate disks which are perforated by chemically etched, uniform-size holes (32). Although a cell process could migrate in any direction, as evidenced in cases where two channels intersect within the filter, the majority of the processes which enter the pores on the epithelial surface are forced to traverse the filter and emerge on the undersurface because most of the pores pass straight across the filter. Millipore filters, on the other hand, are composed of intersecting strands of cellulose which mesh to create a thin sponge. Cell processes readily enter on the epithelial side and may migrate into any of the numerous interconnecting spaces of Millipore filters, but usually no deeper than the first 5-μm of the filter, as determined from examination of thin sections (8). Here, we found that a 0.45-μm pore size Millipore filter (25-μm thick) interposed between the lens and corneal epithelium blocked enhanced corneal collagen synthesis by lens, and we observed no cell processes emerging from the Millipore filter undersurface, even after 48 h of culture. We conclude that Millipore filters are not conducive to the direct passage of cell processes across them.

This study provides the best evidence to date supporting Grobstein's original theory that embryonic induction, as measured in the in vitro system he introduced, may involve "interaction between intercellular materials or matrices of cells, rather than direct interaction between the cells themselves in the sense of exchange between cytoplasms or contact of their cytoplasmic boundaries" (reference 10, p. 251). In closing, however, it seems appropriate to question the continued use of the term "induction" in this context. The tissue isolation approach (27) adapted by Grobstein (9) which has been used so extensively to study so-called embryonic induction in recent years (13, 22, 2, 7, 8, 23, 25, 26) is an artificial recreation of the in vivo situation because the reacting tissues are separated by treatment with enzymes or EDTA from their own extracellular microenvironment which contains matrix molecules now known to stabilize the expression of the differentiated state. These molecules are produced by both the "inducer" and the reacting tissue in the systems which have received the most attention in recent years. The active molecules (collagen, GAG) can be products of either the inducer or the induced in the case of the cornea (8, 25, 26); in cartilage (21, 20), they are the same products as produced by chondrocytes (28). Thus, it is likely that what is being measured in these and in many other in vitro systems is the ability of the so-called reacting tissue to recover enough collagen, GAG, and other critical molecules in its glycocalyx (1) to proceed with its differentiation after the tissue isolation procedure.

In the end, the term "embryonic induction" will surely be abandoned as mechanisms of cytodifferentiation become more clearly understood. In the present study, we have shown that transfilter "embryonic induction" as studied in vitro with Nucleopore filters can be accomplished via cell processes in contact not with other cells but with the "inductor's" ECM. The real significance of the work, however, is the demonstration of a role of cell surface-ECM interaction in the stimulation of ECM synthesis by the reacting tissue. In the next paper of this series, we will examine this cell-extracellular matrix interaction in more detail.

This paper is dedicated to Professor Etienne Wolff on the occasion of his retirement.

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