Rat Lens Cultures: MIP Expression and Domains of Intercellular Coupling

Paul G. FitzGerald and Daniel A. Goodenough

A simple means of establishing a differentiating rat lens culture system is presented, which exhibits a high degree of both morphologic and biochemical differentiation along lens-specific lines. Morphological differentiation includes cell enlargement, displacement from the cell substratum, and the loss of intracellular organelles. Biochemical differentiation includes the de novo synthesis of the Main Intrinsic Polypeptide (MIP), a putative fiber cell junctional polypeptide, as well as the synthesis of the beta and gamma crystallins. Mapping of domains of intercellular communication as defined by the spread of intracellularly injected Lucifer Yellow CH and electrical coupling, are compared to the distribution of biochemically and morphologically differentiated cells. Invest Ophthalmol Vis Sci 27:755–771, 1986

The ocular lens has become a popular subject for the study of membrane and communication biology, particularly from a developmental point of view. The terminally differentiated fiber cells are extensively coupled, both metabolically and electrically.1,2 The presumed mediators of this coupling are the extraordinary number of intercellular contacts that the fiber cells synthesize during their differentiation.2 These contacts bear a superficial morphological resemblance to the well-studied gap junction of the hepatocyte; however, biochemical, immunocytochemical and molecular biological evidence supports the belief that these two structures are unique gene products, and, at best, are analogous in function only.3–11

Extensive efforts have been directed at defining the protein components of the fiber cell junctions. Hepatocyte gap junctions occupy a small percentage of the hepatocyte plasma membrane, and their unique resistance to specific detergents permits an extraordinary degree of purification to apparent morphological homogeneity.12 Upon SDS PAGE analysis, purified hepatocyte junctional preparations are dominated by a single protein with a relative molecular weight of approximately 27 kd.8,12,13 During lens cell differentiation a lens-specific integral membrane protein of slightly greater than 28 kd (the Main Intrinsic Polypeptide, or MIP) is synthesized in great quantity, as are the fiber cell junctions.14–17 The coincidence in both the abundance and the developmental appearance of the MIP and fiber cell junctions, combined with the similarity in the relative molecular weights of the MIP and the hepatocyte junctional protein, has led to the hypothesis that MIP is the fiber cell junctional protein. Numerous investigations have explored this relationship, attempting to unequivocally link the two.5,8,11,18–22 However, despite extensive efforts, ambiguity in the functional role of the MIP remains.

Unlike the hepatocyte junctions, the lens junctions cannot be purified to morphological homogeneity,11,23 and so a compelling structural/biochemical association cannot be made. In an effort to circumvent the inability to establish such an association, anti-MIP immunocytochemistry has been performed at both the light microscope17,24 and electron microscope levels, in the latter case on isolated membranes18 and in situ on frozen thin sections.3 These localizations have been repeated by others25,26 and, with one exception,23 the results have shown that the MIP is present both junctionally and nonjunctionally in the fiber cell membrane, and is apparently absent, or undetectable in the epithelial cell plasma membranes and junctional complexes.3 These results contrast with those from the hepatocyte where immunolabeling with antibodies to the hepatocyte junctional protein have resulted in junction-specific labeling, and immunonegativity in the nonjunctional plasma membrane.27,28

From the Department of Anatomy, Harvard Medical School, Boston, Massachusetts. Supported by USPHS National Research Service Award EY05642 and USPHS Grant EY 02430. Submitted for publication: June 11, 1985. Reprint requests: Paul G. FitzGerald, PhD, Department of Human Anatomy, School of Medicine, University of California, Davis, CA 95616.
Recent physiological studies, however, in which MIP has been incorporated into synthetic liposomes, suggest that the MIP exhibits gating and channel properties expected of a gap junction protein. Thus the role of the MIP, which represents better than 30% of the fiber cell integral membrane protein, remains equivocal. The cytoarchitecture of the ocular lens, particularly the extraordinary length and unusual degree of coupling of the fiber cells, combined with the presence of a thick collagenous capsule, has made it a difficult subject for experimental manipulation in organ culture, at least with respect to the study of the MIP. For these reasons, we have developed a differentiating rat lens culture system, and characterized it with respect to the expression and distribution of the MIP, intercellular junctions, and to the communication competence of the different lens cells that co-exist in this system. This work has been presented in preliminary form.

Materials and Methods

Sources

Rats were obtained from Charles River Breeding Laboratories; Wilmington, MA; trypsin, collagenase, medium 199, penicillin/streptomycin from Grand Island Biological; Grand Island, NY; fetal calf serum from Hy-Clone; Salt Lake City, Utah; 35S-methionine from New England Nuclear, Boston, MA; peroxidase-conjugated- and rhodamine-conjugated goat anti-rabbit immunoglobulin from Cappel Labs; Cochranville, PA; Lucifer Yellow, CH from Polysciences, Inc.; Warrington, PA. All procedures involving animals were performed in accordance with the ARVO Resolution on the Use of Animals in Research.

Lens Cultures

Thirty-six 21-day old CD rats were killed by decapitation, and the heads collected on ice. Intact eyes were isolated, transferred to a sterile hood, rinsed for 3–5 sec in 95% ethanol, and stored in Hanks-buffered Medium 199 (M199). Using a dissecting microscope the posterior of the eye was opened and the lens removed to fresh M199.

To free the lenses of adherent retina and ciliary body, the pooled lenses were suspended in M199 containing 0.1% trypsin (M199T) in a 15-ml conical centrifuge tube. The sample was agitated by end-over-end rotation through 6–10 changes of M199T, at 3–5 min/change, at 37°C, until all adherent material was digested free and washed away. Cleaned lenses were then suspended in Ca++- and Mg++-free M199 containing 0.1% collagenase and 0.1% trypsin, at 37°C. After 5 min the capsules were mechanically disrupted, and the cortical lens cells dispersed by vigorous tituration with a Pasteur pipette. The suspension was allowed to digest for 20–30 min with occasional agitation, until the capsule was thoroughly degraded. The mixture was pelleted at 100–200 g for 10 min, resuspended in M199, with 10% fetal calf serum and penicillin/streptomycin (complete medium), and seeded at a density ranging from 10^5–10^6 cells/35 mm dish. After 24 hr the dishes were rinsed twice with complete medium to remove nonadherent debris. No subsequent medium changes were necessary over the 4–6 wk life of the cultures. Plastic dishes or glass coverslips were coated with rat tail collagen prior to use.

Electrophysiological Measurements

Either whole 35-mm cultures or glass coverslips removed from cultures were used for assessing communication competence. Cells were rinsed free of complete medium and overlain with medium 199 containing 0.01 mg/ml collagenase. Dishes were placed on an inverted Zeiss IM35 microscope (Carl Zeiss, Inc., Oberkochen, West Germany) equipped with a warming stage, and continuously gassed with 95% O2–5% CO2, to maintain appropriate pH of the medium. Intracellular dye transfer and electrical coupling were measured using techniques described previously.

Immunoreagents

The purification of MIP and the affinity purification of rabbit anti-MIP have been described previously. Anti-crystallin antisera were the generous gift of Dr. J. Zigler at the National Institutes of Health, Bethesda, MD.

Immunofluorescence

Either fresh cultures, or cultures which had been probed with microelectrodes, were fixed in freshly prepared 2% paraformaldehyde in 50 mM cacodylate buffer, pH 7.4 for 2–12 hr. Cells were rinsed in phosphate-buffered saline (PBS), and permeabilized with 0.1% Triton X-100 in PBS containing 5% normal goat serum (NGS) for 15 min. Cultures were washed with PBS-NGS, and immunolabeled with 1 ml of PBS-NGS containing either 10–50 µl antiserum (for crystallins), 0.0086 µg/ml of affinity-purified anti-MIP, 10–50 µl nonspecific rabbit serum, or 0.043 µg nonspecific rabbit IgG, for 2 hr. Cultures were washed 6X at 5 min/wash with PBS/NGS, and primary antibodies visualized with rhodamine-conjugated goat anti-rabbit immunoglob-
ulin, used at 1:500 or 1:1000 dilution of manufacturer's stock.

**Biosynthetic Labeling of Lens Cultures**

Thirty-five millimeter culture dishes were overlain with 0.8 ml of complete medium containing 100 μCi of 35S-methionine, for 8–12 hr. Cells were homogenized in 2 ml of 10 mM tris, pH 10, containing 0.5 mM diisopropylfluorophosphate, and 0.1 mM EDTA, at 0°C. An insoluble and soluble fraction were defined by centrifugation of the homogenate at 9 × 10⁶ g×min. To the soluble fraction was added 25 μl nonspecific antiserum. The insoluble pellet was extracted by stirring in the presence of 1.0% NP40 detergent in PBS. The sample was diluted to 0.5% NP40 with PBS, and centrifuged to remove insoluble material. To the supernatant (100 μl) was added an equal volume of either 0.175 μg anti-MIP or 0.875 μg nonspecific rabbit IgG in 0.5% NP40 in PBS. Primary antibody was precipitated by addition of saturating levels of Protein A-Sepharose 4B, suspended in 0.5% NP40 in PBS. After 60 min agitation, the beads were washed 2X each with 1 ml of 0.1% SDS in 50 mM tris, pH 7.6, 2X with 0.5% NP40 in PBS, and 2X with 5 mM tris. Bound proteins were extracted and resolved by electrophoresis under standard conditions. Visualization of the bands was achieved with Kodak (Rochester, NY) X-omat AR film, at -80°C for 12–72 hr.

**Electron Microscopy**

For routine electron microscopy, cultures in 35-mm plastic dishes were fixed with a combination of glutaraldehyde, tannic acid, and osmium tetraoxide. Cells were subsequently stained en bloc with uranyl acetate and processed conventionally into SPURR embedding resin. Sections were stained with lead citrate.

**Freeze Fracture**

Fracturing was performed on a Balzers BAF 301 (Balzers High Vacuum Corp.; Nashua, NH), and replicas prepared using standard techniques.

**Results**

The freeze-fracture surface morphology of cortical lens fibers in the rat lens in situ (Fig. 1) displayed regions rich in fiber cell junctions, resembling those described by Kuszak, et al in the chick.32 Figure 2, however, which is of the same surface of the same cell but some distance further along, is nearly devoid of junctions. Figures 1 and 2 suggest that junctional density varies radically along the longitudinal axis of the cell. This variability in junctional density was found in both the superficial and deep cortex.

**Morphological Differentiation**

Lens cultures typically had a 6–8-wk lifespan, during which their appearance changed dramatically. Twenty-four hours after seeding the cultures were washed free of nonadherent cells and debris. Remaining cells, either isolated or in small clumps, were stellate or polygonal in appearance, and covered a small percentage of the culture dish, resembling a typical subconfluent cultured epithelium. The absence of elongated or anucleated cells suggested that only cells derived from the lens epithelium were capable of adherence under the conditions employed.

At 4–6 days, when confluence was being approached, most cells retained an epithelial appearance, but occasional phase-bright mounds of cells could be found (Fig. 3). Over the next 2–5 wk these mounds increased in both size and number, ultimately assuming the very large rounded shape (Fig. 4) of the classically described lentoid.33,34 In parallel with lentoid development, the undifferentiated epithelial cells receded from confluence, ultimately existing in small islands, in the center of which was usually located a lentoid (Fig. 5).

A second class of lentoid was also consistently seen. In contrast to the very tall and rounded lentoid, which has been described in other systems, this class assumed the form of elongated bundles of varying thickness (Fig. 6), which, by Nomarski optics, appeared to be composed of parallel strands of cells (Fig. 7). These bundles existed either as thin, attenuated connections between rounded lentoids (Fig. 4, small arrow), or as larger independent structures (Fig. 6). When found independently they consisted of elongated bundles which were anchored to the substrate at either end, but which for most of their length arched well above the plane of the dish. Thus, they were largely devoid of either cellular or substratum contact, being surrounded only by medium over much of their length (Fig. 8).

In all cases, the onset of morphologic change was accompanied by significant changes in the refractile properties of the cells, so that cells in or entering the differentiation phase were readily identifiable by phase optics in the living state (Figs. 3–6).

One-micron sections of plastic-embedded cultures suggested that the changes involved in the transformation of epithelial cells into lentoid cells mimicked the changes of the differentiating lens epithelial cell in vivo (Fig. 9). Most of the lentoid cells were greatly enlarged, with a homogeneous staining cytoplasm, sug-
Fig. 1. Freeze fracture replica of a rat lens fiber cell in the superficial cortex. The image includes almost the full width of a single cell. An extraordinary density of fiber cell junctions (sunken plaques, an example of which is located between two arrows) is apparent (x14,000).

Fig. 2. Same replica as in Figure 1, except in a different field. Again the field includes almost the entire width of a single fiber cell, however, only a single small fiber cell junction can be seen (arrow). The disparity in junctional density between Figures 1 and 2 underscores the difficulty in estimating the precise percentage of the fiber cell surface involved in intercellular junctions (x28,000).
Fig. 3. Phase contrast micrograph of a live, maturing, pre-confluent lens culture. An emerging lentoid (arrow) is visible, and readily definable by its phase optical properties (×15,000).

Fig. 4. Phase contrast micrograph of a lens culture after confluence has begun to recede. A very well-developed rounded lentoid is apparent (large arrow), which overlies undifferentiated epithelial cells. An attenuated "bridge" (small arrow) connects two nearby lentoids (×1200).

Fig. 5. Phase contrast micrograph of lens culture, at a very mature stage. Phase-bright differentiated structures can be seen (large arrows), as well as some remaining undifferentiated epithelial cells (small arrows) (×1200).

Fig. 6. Phase contrast micrograph of lens culture, displaying a second class of lentoid, the elongated bundle (arrow). The "phase bright" nature of the differentiated cells renders them easily identifiable. Subsequent immunocytochemistry confirmed that these structures also express MIP and crystallins, lens-specific markers of differentiation (×1200).
Ultrastructural examination confirmed many of the cytologic features suggested by light microscopic examination. Cells adherent to the dish were flattened, organelle-rich, epithelioid cells (Fig. 10), joined by occasional gap junctions. A fair amount of overlap occurred between adjacent undifferentiated cells, and, like their in vivo counterparts, obvious zonulae occludentes were not observed.

Examination of the uppermost cells of the lentoid confirmed the absence of virtually all traces of cytoplasmic membranous organelles (Fig. 11), a characteristic of the maturing lens fiber in vivo. Thin sections through the arching bundles of cells, parallel to the long axis of the bundle, revealed a highly ordered and parallel alignment of these fiber-like cells, which was virtually indistinguishable from fiber cells in vivo (Fig. 12). Cross-sections through rounded lentoids, while less ordered, were cytologically equally well differentiated (Fig. 11), revealing a uniformly dense cytoplasm and exhibiting a complete or nearly complete absence of cytoplasmic membranous organelles. Adjacent lentoid cells, although closely apposed, seldom exhibited intercellular junctions of any type (Figs. 11-12).

Freeze fracture analysis of mammalian lenses has revealed differences in the intramembrane particle (IMP) packing of the junctions of the epithelium and junctions of the fiber cell. Under conditions routinely employed for electron microscopy, the IMPs of junctions between epithelial cells inevitably exhibit a significant degree of particle condensation into paracrystalline arrays. In contrast, the IMPs of fiber cell junctions are almost always found more loosely packed, lacking any semblance of long-range order. To establish if a comparable dimorphism occurred in vitro, differentiated cultures were examined using the freeze fracture approach. In order to rigorously associate junctional morphology with differentiation status, junctional identification was initially confined to those cells whose adjacent cytoplasm could also be observed. In this manner, plasma membranes of undifferentiated (organelle-rich) and differentiated (organelle-poor) cells could be discriminated from one another. With this as a guideline, it became apparent that differentiated cells had a higher density of IMPs than undifferentiated cells, thus providing a second means of establishing a cell's maturational state.

Using these two criteria (IMP density, or the presence/absence of cytoplasmic organelles) junctional plaques were identified as belonging to either differentiated or undifferentiated cells. Gap junctions joining undifferentiated cells were predominantly of a type that resembled their in vivo counterparts in being comprised of IMPs that were condensed, either in whole or in
part, into ordered arrays (Fig. 13). However, occasional junctions, with disordered arrays of IMPs, reminiscent of those typically found between fiber cells in vivo, were found between undifferentiated cells. These, though, were the exception.

Junctions between clearly differentiated lentoid cells were rare, occupying a small percentage of the cell surface. These junctions displayed the disordered IMP organization characteristic of the lens fiber junction in vivo, and were the only type of junction found between differentiated cells of the lentoid (Fig. 14). Thus, the structural dimorphism between junctions of undifferentiated and differentiated cells in culture appeared to parallel that observed in vivo. Although electrophysiological data demonstrate the presence of low resistance pathways between epithelioid and lentoid cells in culture (see below), we were unable to generate sufficient unequivocal fracture faces of these interfaces to document the morphology of the heterologous cell junctions.
Fig. 11. Low magnification electron micrograph of a section of a classical rounded lentoid, revealing the near absence of organelles in these greatly enlarged cells. Although this type of lentoid lacks the parallel arrangement of cells found in the bundle-shaped lentoid, they exhibit a comparable degree of cytologic differentiation. The free surface of the lentoid is located at the arrow, the epithelium lies to the left, out of the image (×42,000).

Fig. 12. Low magnification electron micrograph of a section passing entirely through a bundle-like lentoid. The highly parallel arrangement of organelle-free cells is apparent. Free surface is located on both sides at the arrows (×45,000).
Biochemical Differentiation

Differentiation in ocular lens cells includes the onset of synthesis of several lens-specific proteins, including the cytoplasmic crystallins, and the integral membrane protein MIP. Thus, these polypeptides are not only lens-specific, but are markers of differentiation as well. To establish whether the observed in vitro morphological differentiation was accompanied by biochemical differentiation, cultures were probed for their ability to incorporate $^{35}$S-methionine into newly synthesized crystallins and MIP.

Fig. 13. Electron micrograph of a freeze fracture replica of a junction between undifferentiated lens culture cells. This junction is similar to the type of gap junction found between lens epithelial cells in situ, in that it is comprised of IMPs that are clustered in small patches or assembled into larger-order, paracrystalline arrays (X95,000).

Fig. 14. Electron micrograph of freeze fracture replica of lentoid cell junction. The intramembrane particles (IMPs) of the lentoid junction are randomized and uncondensed, rendering them indistinguishable from the fiber cell junctions of rat lens cortical cells (X105,000).

The synthesis of MIP was verified by the immunoprecipitation of detergent-solubilized cultured cell membranes. Antibody was affinity-purified from poly-
Fig. 16. Phase contrast micrograph of formaldehyde-fixed lens cultures. A large lentoid (L) is in the plane of focus, situated over underlying undifferentiated epithelial cells (E). Bare dish (B) occupies part of the image. Figures 16–18 are from cultures which were microelectrode-injected with Lucifer Yellow CH, fixed, and subsequently immunolabeled with affinity-purified anti-MIP antibody and rhodamine-conjugated secondary antibody.

Fig. 17. Same field as in Figure 16, but viewed with filters appropriate for rhodamine. Anti-MIP fluorescence clearly delineates the membranes of the differentiated cells which comprise the lentoid. No sign of fluorescence was detectable in the undifferentiated underlying epithelial cells.

Fig. 18. Same field as in 16 and 17, but viewed with filters appropriate for Lucifer Yellow CH. The site of Lucifer Yellow CH injection is marked with an "x." The dye has transferred rapidly and in an unrestricted fashion to adjacent undifferentiated cells, suggesting extensive gap junction coupling. Although dye has spread rapidly to undifferentiated cells that underlie the lentoid, no dye has spread to the cells of the lentoid. This failure to enter the lentoid is particularly evident at that point where the lentoid is overlying only bare dish (arrow).
Fig. 19. Phase contrast image of large lentoid (L), in the plane of focus, attached to the substratum via underlying undifferentiated epithelium (E). A series of micrographs comparable to 16–18 except that the site of Lucifer Yellow injection is into a lentoid cell.

Fig. 20. Same field as in 19, but viewed with fluorescence and filters appropriate for rhodamine. The MIP-positive membranes of the cells that comprise the lentoid are apparent. MIP labeling is confined to the lentoid.

Fig. 21. Same field as in 19 and 20, but viewed with fluorescence and filters appropriate for Lucifer Yellow. The site of Lucifer injection is marked with an “x.” Clearly the dye has remained confined to a single cell, a result strikingly different from that achieved when an undifferentiated, or MIP-negative cell has been injected. Figures 16–21 ×1800.
specific rabbit antiserum using electrophoretically-purified MIP covalently immobilized on Sepharose 4B. Specificity of the purified antibody was established by probing of nitrocellulose replicas of SDS PAGE-resolved bovine membranes (Fig. 15, lanes a and b). Substitution of nonspecific rabbit IgG, or preadsorption of the antibody with electrophoretically purified MIP eliminated replica labeling. Anti-MIP probing of SDS PAGE-resolved soluble lens proteins produced no labeling, confirming antibody specificity. The purification of the MIP and the purification and characterization of the antibody have been described in a previous report.3

Probing of the membrane extract, which was derived from cultures exhibiting a mix of both differentiated and undifferentiated cells, resulted in the specific immunoprecipitation of MIP from a complex mixture of labeled cultured cell membrane proteins (Fig. 15, lane c). Controls, consisting of the use of nonspecific rabbit IgG, were negative. Immunoprecipitation from cultures that showed no sign of morphological differentiation, did not contain detectable levels of the de novo synthesized MIP. Thus, MIP synthesis accompanies morphological differentiation in vitro.

Application of antisera against beta and gamma crystallins, though less clean due to the use of unfractionated antisera, confirmed that these markers of differentiation were being synthesized as well (not shown).

**Immunofluorescence Characterization**

To establish whether MIP (and crystallin) expression coincided with the morphological differentiation, differentiated lens cultures were characterized by immunofluorescence, using affinity-purified anti-MIP, followed by rhodamine-conjugated secondary antibody. This type of analysis revealed that MIP immunoreactivity was strictly limited to structures defined morphologically as lentoids (Figs. 16–17, and 19–20). That is, MIP (and crystallins) were detected only in aggregates of cells that had begun morphological differentiation. The demarcation was usually particularly sharp, with the cell membranes of the lentoids exhibiting strong fluorescence, and the adjacent, or underlying epithelial cells being negative. MIP presence was confirmed in both the classical rounded lentoids, as well as in the bundle-shaped lentoids and in the attenuated processes that joined nearby lentoids.

**Dye Transfer Experiments**

The MIP is a putative gap junction protein, and its presence would be expected to be accompanied by extensive intercellular coupling. To correlate the coincidence of MIP expression and intercellular coupling, the fluorescent dye Lucifer Yellow, which is capable of gap junction-mediated diffusion between coupled cells, was iontophoresed via glass microelectrodes into cells at several sites in the culture. The cells were subsequently fixed and processed for specific immunofluorescence using anti-MIP.

The degree of dye transfer between lentoid cells and between epithelioid cells was qualitatively different. Injection of Lucifer Yellow into a region of epithelial cells that exhibited no detectable morphological differentiation resulted in the rapid spread of fluorescence to all adjacent cells, a result expected of gap junction-coupled cells (Figs. 16–18). However, dye injected into the epithelial monolayer was never observed to transfer into an overlying lentoid, as defined by phase optics in the living state, and by subsequent immunofluorescence using anti-MIP.

In marked contrast, dye injected into the differentiated cells of the lentoid failed to spread to adjacent cells, and remained strictly confined to the injected cell (Figs. 19–21, 22–24). Only in two instances was any sign of dye transfer suspected, and this was at the limits of detection, and after extended periods of time (Fig. 24). Thus, by dye transfer criteria the cells of the lentoid appeared not to be coupled, or coupled to a degree that was radically less than nonlentoid cells.

**Electrical Coupling**

Our ultrastructural examinations of the cultured lens cells only rarely revealed gap junctions between the more mature, differentiated cells of the lentoid. The scarcity of junctions between differentiated cells is consistent with the apparent absence of dye transfer between these cells but contrasts sharply with the fiber cell in vivo, where morphologically similar junctions are abundant, and dye transfer is rapid and extensive. To establish if the lack of dye transfer between lentoid cells represented a true lack of intercellular communication, a rare phenomenon, the cultures were tested for electrical coupling using paired electrodes. The electrodes also contained Lucifer Yellow so that dye transfer and electrical coupling could be monitored simultaneously (Figs. 22–25).

In all cases tested, selected cell pairs were electrically coupled, as shown in Figure 25. In each cell pair studied, a high-resistance (80–120 Mohm) micropipette was inserted into each cell. Cells were separated by distances of 20–400 μm. Resting membrane potentials were −50 to −65 mV in all successful cases. The potentials recorded were usually within 10 mV of each other.

Injection of 10 nA current pulses, 500 ms in duration through one micropipette evoked electrotonic potential changes at the site of the second micropipette (Fig. 25).
Fig. 22. Following measurement of electrotonic coupling with Lucifer Yellow-filled micropipettes, this fluorescence photomicrograph was taken that recorded the positions of the two injected cells. The injected cells are labeled 1 and 2. Phase and fluorescence photomicrographs of rat lens cells in tissue culture.

Fig. 23. This phase micrograph shows the lentoid in which cells 1 and 2 were positioned. A second, separate lentoid contained cell 3 (arrow). This second lentoid rests on the same epithelial bed but shows no detectable direct connection with the first lentoid.

Fig. 24. The pipette in cell 2 was removed and used to impale cell 3 within the adjacent lentoid, but the pipette in cell 1 was untouched. Following measurement of electrotonic coupling between cells 1 and 3, this second fluorescence photomicrograph was taken, marking the relative position of all three cells. Note that during the 15-min period between the taking of photomicrographs 22 and 23, only weak dye transfer could be detected between cells in the first lentoid (arrow); magnification 1800×.
Both depolarizing and hyperpolarizing pulses were passed, and the role of the microelectrodes was reversed, indicating the absence of rectification at the level of the current passed. Moving one of the micropipettes to an extracellular location, as judged by a loss of the resting potential, resulted in the complete loss of any measurable electrotonic potential changes, indicating that the current was not passing via an extracellular pathway.

We impaled cell pairs consisting of both epithelioid cells (n = 3), both lentoid cells (n = 6), and mixed pairs of epithelial and lentoid cells (n = 3). In all cases, cells were found to be electrotonically coupled.

These data correlate well with the morphological data obtained from the freeze-fracture replicas. The undifferentiated cells, which are joined by gap junctions at a density typical of epithelial cells, exhibited rapid and extensive dye transfer to other epithelial cells, and were electrotonically coupled. The lentoid cells, on the other hand, exhibited far fewer junctions per unit area of plasma membrane, were not detectably dye coupled, but were electronically coupled.

Due to the ambiguity in defining epithelium-to-lentoid junctions in freeze fracture, the heterologous electrotonic coupling observed in vitro does not yet have a morphological counterpart.

Discussion

The study of lens and developmental biology has historically made impressive use of in vitro systems, whether in propagating cell lines of lens epithelium, or in organ culture.\textsuperscript{29,31,37,39,44} Most of the emphasis, however, has been on the occurrence or induction of either crystallin synthesis or morphological changes. The relatively recent escalation of interest in the lens as a model system in which to study developmental membrane and communication biology has underscored the absence of a differentiating culture system to augment such work. Menko et al\textsuperscript{26} have approached the problem using embryonic chick lenses as a source and have documented the expression of MIP in that system. However, significant differences exist between chicken MIP and mammalian MIP, particularly by immunologic criteria (refs. 14, 45, and unpublished observations). Because of the potential value of antibodies in the probing of the molecular dynamics of gap junction synthesis, expression, and control, and because a great deal of work has already been performed on mammalian lens and MIP, we elected to pursue the development and characterization of a mammalian culture system that differentiates in vitro and expresses the MIP.
The system described here requires no exogenous growth factors, and offers an impressive mimicry of most of the documented developmental changes that occur in the lens bow region, the site in the lens at which the differentiation of epithelium into fiber cells occurs. Co-existing in this culture system are both undifferentiated epithelium-like cells, and cells that have differentiated in a morphologically lens-specific fashion. Cytologic differentiation includes enlargement and thickening of the cell and a loss of most intracellular membranous organelles, features that are characteristic of cells in the developing bow region of the lens.

Even at the tissue level, a significant degree of lens-specific multicellular organization is expressed. Microdomains of lentoid cells assume the highly ordered, parallel alignment characteristic of their counterparts in vivo. Undifferentiated epithelial cells in vitro were always found in contact with the collagenous substratum, whereas differentiated lentoid cells were only rarely found in contact with the collagen. The latter were usually anchored to the culture dish via an intervening layer of epithelium. This relationship of cultured cells to the substratum mimics that of the epithelium and fiber cell to the lens capsule, the natural substrate for lens cells.

The expression of lens-specific cytology in this culture system is matched by a comparable degree of biochemical differentiation. The onset of expression of at least three lens-specific markers of differentiation, particularly the putative junctional integral membrane protein MIP, underscores the potential of this culture system as a workable model for in vitro studies of mammalian lens biology and lens membranes in particular.

Examination of the lentoids by both freeze fracture and thin section electron microscopy revealed that the structural dimorphism of epithelial cell gap junctions and fiber junctions demonstrated in vivo, appears to be manifested in vitro as well. Such dimorphism is not absolute in vivo, since semi-crystalline junctions have been reported in fiber cells, and domains of noncrystalline connexons have been found between epithelial cells. This dimorphism is not found to be absolute in vitro as well. The meaning of this junctional dimorphism has not been rigorously defined. However, immunocytochemical and physiological data, which demonstrate differences in immunoreactivity and coupling sensitivity between epithelial cell gap junctions and fiber junctions, suggest the coexistence of two discrete junctional types in the lens.

In contrast to the fiber cell, however, the differentiated cells of the lentoid assemble relatively few intercellular junctions. Whether this represents an incompleteness in the in vitro differentiation, or is a manifestation of a very plastic response of the maturing fiber cell to specific conditions is unknown. The fiber cell in vivo is quite isolated from a nutrient source, and is apparently reliant upon metabolic coupling with the surface epithelium for the acquisition of nutrients and dissemination of wastes. In contrast, the lentoid cell in vitro is directly bathed in its nutrient source. Thus the low number of fiber cell junctions in vitro may reflect a reduced reliance upon metabolic coupling, manifested by a reduced number of intercellular junctions that mediate this function.

Interestingly, the lentoids which evolve in vitro from embryonic chick lens cells are quite rich in intercellular junctions. However, this report did not include freeze fracture data, and whether the numerous junctions that were found in the avian lentoid were of the epithelial cell type or the fiber cell type was not stated. The marked difference in junctional density between the avian and mammalian systems remains unexplained. However, the presence of two systems, both of which express the MIP, but only one of which assembles junctions, could well provide interesting data on conditions necessary for junction formation, or, indeed, whether the MIP has any junctional role at all.

The presence in undifferentiated cells of numerous gap junctions and strong dye transfer can be well correlated, but the absence of extensive dye transfer between differentiated cells of the lentoid is less well understood. Although it is possible that the differences in dye transfer properties between epithelial cells and between lentoid cells may reflect the presence of two distinct populations of junctions with dissimilar conductance properties, as has been demonstrated in larval insects, it may well be that this observation simply reflects the presence of fewer channels between lentoid cells compared to epithelioid cells. The latter explanation is supported by both our ultrastructural observation that fewer and smaller junctions occur between lentoid cells than epithelioid cells, and by in vivo demonstrations of rapid dye transfer between the junction-rich fiber cells. The two interpretations, however, are not mutually exclusive. Indeed, physiological, structural, and immunocytochemical evidence exists which suggests the possible presence of two unique populations of gap junctions in the lens, which appear at developmentally different stages.

Other examples of electrically coupled cells that do not show dye transfer have been reported. Warner and Lawrence report the presence of cells in larval insects that are generally electrically coupled to surrounding cells, but which show selective dye transfer. The mechanism underlying this apparently selective dye transfer remains to be defined.

Of particular interest is our observation in vitro that cells which are MIP-rich are junction-poor, a finding which contrasts sharply with studies on chick lentoids...
where MIP and lentoid junctions are both present in abundance. MIP and fiber cell junctions are both present in abundance in fiber cells membranes in vivo, an observation suggesting that the MIP is a structural protein in these junctions. Efforts have been made to unequivocally demonstrate a junctional structural role for the MIP, but there remains controversy on this point. Results presented here would suggest that the presence of abundant MIP is neither sufficient for the assembly of large numbers of junctions, nor sufficient for the conferral of extensive dye transfer capability typical of the native lens fiber.

Thus, while the precise function of the MIP remains controversial at present, its extraordinary abundance in the lenses of all vertebrates thus far examined suggests an indispensable role in lens biology. The cultured lens cells described here will be a valuable system for the study of the MIP, intercellular communication, and lens biology.

Key words: MIP, lens culture, intercellular coupling

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