Cell-to-Cell Communication in a Differentiating Ovine Lens Culture System

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Purpose. This study was performed to determine whether the junctions between both the epithelial and the differentiating fiber-like cells of ovine lens cultures, like gap junctions in other tissues, exhibit cell-to-cell communication that is inhibited by n-octanol, and to determine whether lens connexins and the fiber cell membrane proteins MP20 and MP26 are expressed by these ovine lens cell cultures.

Methods. Cells were injected with Lucifer yellow CH to measure cell-to-cell communication. Antibodies to connexin-related lens membrane protein MP70, connexin 43 (Cx43), and connexin 46 (Cx46) and to membrane proteins MP20 and MP26 were used to immunofluorescently label lens cultures and probe Western blots of membranes isolated from lens cultures.

Results. Both epithelial cells and differentiating clear cells exhibited cell-to-cell transfer of Lucifer yellow that was inhibited by n-octanol. Although a Cx43 antibody immunofluorescently labeled small plaques between the epithelial cells, an MP70 antibody labeled large plaques as well as small punctate areas of the differentiating fiber-like cells. It is interesting that Cx43 and MP70 were frequently present in the same plaques at cell interfaces between epithelial cells as well as some of the larger plaques on the differentiating fiber-like cells. Cx46 and MP70 antibodies labeled the same plaques in membranes of differentiating fiber-like cells and late-stage epithelial cells. The electrophoretic mobility of all three connexin proteins was modified after treatment with alkaline phosphatase. Immunohistochemical staining of these differentiating regions and Western immunoblotting of purified membranes derived from differentiated cultures also showed the presence of MP20 and MP26.

Conclusions. The different cell types in the ovine lens culture exhibit gap junction-mediated cell-to-cell communication that is likely effected by one or more of the connexin proteins.

Materials using these antibodies suggest that the Cx43 present are shown to be coupled by functional gap junctions. Fitzgerald and Goodenough, 15 these cells describe by a large number of differentiating lens cells, and, unlike ovine culture system we have established thus contains the junctions joining myocardial cells 12 and is the most frequently identified connexin protein in different tissues. 1 Although antibodies to Cx43 have been shown to stain gap junctions connecting lens epithelial cells, they do not stain fiber–fiber cell junctions. 13 Musil, Beyer, and Goodenough 13 have shown that although chick lens cells synthesize Cx43 as a single 42-kD species, this protein is phosphorylated post-translationally, which converts it to a 45-kD form. In normal rat kidney cells, this phosphorylation appears to be associated with the insertion of Cx43 into junctional plaques. 14 Attempts in our laboratory to obtain a differentiating lens cell system have focused on the culturing of ovine lenses. We have found that trypsin-treated epithelial and outer cortical fiber cells of the ovine lens can be cocultured successfully. Under appropriate conditions, the latter grow on the epithelial layer and appear to further induce some of the contacting epithelial cells to differentiate. In this study, we show that the more differentiated cells of such regions contain the two major fiber cell membrane proteins, 10,11 MP26 and MP20. We also show that most of these differentiating cells, and all of the epithelial cells in the ovine lens cultures, are linked by functional gap junctions. Immunofluorescent antibody staining indicates that the fiber-like cell junctions contain MP70 and Cx46, whereas the junctions between the epithelial-like cells contain Cx43. Furthermore, double-labeling studies using these antibodies suggest that the Cx43 present in the epithelial junctions is replaced gradually by MP70 and Cx46 during differentiation into the fiber-like cells of the culture. Lastly, all three connexin proteins are shown to be phosphorylated in vivo. The ovine culture system we have established thus contains a large number of differentiating lens cells, and, unlike the fiber-like cells of the rat lens culture system described by Fitzgerald and Goodenough, 15 these cells are shown to be coupled by functional gap junctions.

MATERIALS AND METHODS

Materials

Sheep eyes were obtained from Forster's Farm Market (Plymouth, MN) or Farmstead Foods (Albert Lea, MN); Hank's balanced salt solution with calcium and magnesium (HBSS), trypsin, medium 199 (M199), and penicillin-streptomycin were obtained from Gibco (Grand Island, NY); and fetal calf serum was obtained from Hyclone (Salt Lake City, UT). Rhodamine-, fluorescein-, and peroxidase-conjugated secondary antibodies and molecular biology grade calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim (Indianapolis, IN), whereas 125I-labeled secondary antibodies were obtained from Amersham (Arlington Heights, IL). Paraphenylenediamine, poly-DL-ornithine, complete Freund's adjuvant, Limulus polyphemus hemocyanin, Pristane (2,6,10,14-tetramethyl-pentadecane), glutaraldehyde, Tween, Tritton X-100, and all other chemicals were obtained from Sigma Chemical Company (St. Louis, MO). Nitrocellulose (0.2 μm) and membranes for electroelution of proteins from polyacrylamide gels were purchased from Schleicher and Schuell (Keene, NH). All investigations using animal materials adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antibodies

The affinity-purified polyclonal antibody to the eight-amino acid C-terminus of the lens fiber cell membrane protein MP20 was produced in rabbits 11; this antibody immunolabels MP20 and its 28-kD heterodimer on Western immunoblots. 10 Rabbit antisera to the C-terminus of bovine MP26 (residues 256 to 263) was a generous gift from Dr. Larry Takemoto, Kansas State University. The mouse monoclonal antibody to MP26 was a particularly generous gift of Dr. Ross Johnson, University of Minnesota.

Mouse IgG monoclonal antibody to MP70 (55.35) and rabbit polyclonal antibody directed against residues 370 to 381 of the C-terminus of rat α1 Cx43 were generous gifts from Dr. T. H. Lu of the Department of Genetics and Cell Biology, University of Minnesota, 17 and Dr. Nalin Kumar of The Scripps Institute, San Diego, 18 respectively. Rabbit polyclonal antibody directed against residues 411 to 416 of the C-terminal of rat Cx46 was a generous gift of Dr. Larry Takemoto of Kansas State University, whereas monoclonal antibody 6-4-B2-C6 to MP70 was a generous gift of Dr. Joerg Kistler of the University of Auckland, New Zealand. 5

Lens Cultures

Eyes were removed from freshly slaughtered sheep that were approximately 1 year old and kept on ice for use within 1 to 3 hours. The eyes were rinsed and transferred to a sterile hood, where they were soaked further in a 95% ethanol solution for 30 seconds before dissection of the lens. Adherent ciliary epithelium was removed by gently dabbing the exterior of the dissected lens with a sterile cotton swab. Care was taken not to disrupt the lens capsule, and intact lenses were placed in cold, sterile HBSS. Typically, six sheep lenses in 6 ml 0.4% trypsin diluted in HBSS were digested in
a 50-ml sterile conical polypropylene tube on a single-speed orbital mixer for 30 minutes at 37°C.

After trypsin treatment, medium containing dissociated cells was removed from undigested lens tissue and diluted quickly with seven volumes of cold HBSS. The cell suspension was centrifuged at 250g for 15 minutes, and the cells were resuspended in M199 that included 10% fetal calf serum containing 100 U/ml penicillin and 100 μg/ml streptomycin, to a density of 5 × 10⁶ cells/ml. Equatorial fiber cells, or those epithelial cells beginning to elongate, also were present but were not counted because of clumping and often indiscernible cell boundaries. Two milliliters of an epithelial-rich cell suspension were applied to each 35-mm Petri dish, and cells were left to adhere for 48 hours at 37°C in a humidified atmosphere of 95% air–5% CO₂. Medium was replaced at 24 hours, so the cells not yet adherent (generally a mix of epithelial and outer cortical cells) were pelleted to remove cell debris and reapplied to the culture dish in fresh medium.

Lens cultures were grown on polyornithine-coated 35-mm Falcon dishes for microinjection studies and on polyornithine-coated glass coverslips for immunofluorescence studies. Poly-DL-ornithine coating proceeded as follows: 10 mg polyornithine was dissolved in 100 ml 0.15 M borate buffer (pH 8.6), and 2 ml was added to each 35-mm culture dish for a 2-hour incubation at room temperature. Plates then were rinsed three times with double distilled water and dried under ultraviolet light for 20 to 30 minutes.

Membrane Preparation

Lens membranes were purified according to the procedure of Goodenough through sucrose gradient fractionation after extraction with 4 and 7 M urea. All solutions included a protease inhibitor mixture of 0.5 mM diisopropylfluorophosphate, aprotonin (1 mg/l), pepstatin (1 mg/l), leupeptin (1 mg/l), 0.5 mM benzamidine, 1 mM EGTA, and 1 mM ethylenediaminetetraacetic acid. Lens membrane preparations were frozen in 10 mM HEPES and 10% sucrose (pH 7.0) and kept at −70°C until use.

To prepare a membrane fraction from the lens cultures, cells were sonicated in 5 mM Tris, 1 mM EGTA, and 1 mM ethylenediaminetetraacetic acid containing the above protease inhibitor cocktail, and DNA in the samples was sheared by aspirating the sample several times with a 25-gauge needle and a tuberculin syringe. Membranes then were pelleted at 40,000g for 30 minutes, and the pellet was extracted once with 7 M urea protease inhibitor-containing medium. After extraction, membranes were centrifuged again at 40,000g for 30 minutes, resuspended, and centrifuged twice in 20% sucrose and 20 mM HEPES buffer (pH 7.0), and finally resuspended in 10% sucrose and 10 mM HEPES buffer (pH 7.0). Membranes were used immediately or stored at −70°C until use.

Dephosphorylation of Membranes

Dephosphorylation of membranes derived from lens cell cultures was performed according to a modification of the procedure described by Musil and Goodenough. In this, 12.5 μl 50 mM Tris HCl, 10 mM MgCl₂, 150 mM NaCl, phosphate buffer (pH 8.0) supplemented with 1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride (with 1 mg/l leupeptin, 1 mg/l aprotonin, 1 mg/l pepstatin, and 10 mM EGTA for those experiments involving Cx46 and MP70) was added to 18 μl of urea-washed culture membranes containing approximately 90 μg protein in 10 mM Heps (pH 7.0) and incubated at 60°C for 3 minutes. The mixture then was diluted with 50 μl phosphatase reaction buffer and incubated for 4 hours at 37°C in the presence of 3 units of alkaline phosphatase. The reaction was terminated by the addition of SDS–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Control samples were prepared with or without alkaline phosphatase and in the presence or absence of the alkaline phosphatase inhibitors 10 mM sodium orthovanadate, 10 mM ethylenediaminetetraacetic acid, and 10 mM phosphate (pH 8.0).

Immunoblotting

Samples of lens culture or lens membranes were analyzed by SDS-PAGE on 12% polyacrylamide gels, and the proteins were transferred to 0.2 μm nitrocellulose membranes according to the method of Towbin, Staehelin, and Gordon, except that 150 mM NaCl and 0.05% SDS were added to the transfer buffer to enhance protein adsorption. Membranes were rinsed in double-distilled water, dried, and treated for 1 to 2 hours with phosphate-buffered saline (PBS) containing 0.05% Tween (PBS-Tween) and 3% nonfat dry milk to block nonspecific binding sites. Primary antibody was applied in fresh blocking solution and incubated at room temperature for 2 hours. Transfers then were washed three times for 10 minutes in PBS-Tween, followed by the addition of either peroxidase-conjugated second antibody diluted in blocking buffer or the 125I-labeled second antibody for 1 hour at room temperature. Transfers were washed finally in three changes of PBS and the peroxidase label detected with diaminobenzidine as a substrate.

Immunofluorescence

Lens cell cultures were fixed in freshly prepared 3.5% formaldehyde in HBSS for 20 minutes at room temperature. Fixation leads to the loss of the phase bright boundaries demarcating the region of differentiating cells overlying the confluent epithelial layer but illust
nates individual cell boundaries within a highly differentiated region ("lenioid"). Cells were rinsed with HBSS and permeabilized with 0.1% Triton X-100 in PBS for 45 minutes at 37°C. The cultures then were washed and blocked with M199 plus 20% goat or calf serum for 1 hour before the addition of primary antibody at specified dilutions in the same medium. The cells were incubated with antibody for 1 hour at 37°C, washed three times for 5 minutes with HBSS, and then incubated with a 1:250 dilution of secondary antibody conjugated to rhodamine in blocking solution for 1 hour at 37°C. The culture dishes finally were washed several times with PBS-Tween and covered with a glass coverslip applied over a drop of 100 mM Tris buffer (pH 9.0) and 90% glycerol containing 0.1% para phen ylenediamine to prevent fading of fluorescence. Cultures were viewed on a Zeiss (Chicago, IL) inverted microscope under epifluorescence, with a mercury lamp.

Dye Injection and Octanol Treatment

The cultures were placed in M199 containing 10% fetal calf serum and 5 mM Hepes (pH 7.4), and cells were microinjected iontophoretically with a 3.4% solution (wt/vol) of Lucifer yellow CH either by repeated 0.1-second pulses of 1.1 nA current or by overcompensation of the negative capacitance circuit in the amplifier until the impaled cell was brightly fluorescent. A Zeiss IM fluorescence microscope with a halogen lamp was used to monitor the movement of dye to neighboring cells, and results were recorded on videotape as described by Atkinson and Sheridan. For analysis of the dye transfer between differentiating cells, the rate of dye escape from the injected cell was determined by digital analysis. The data are expressed as percentages of the rate obtained for the control injections.

The same culture was used for each of the different treatments in studies involving octanol. Cultures first were microinjected with fluorescent dye while bathed in M199 containing 5 mM Hepes (pH 7.4) and Lucifer yellow vehicle (0.1% dimethyl sulfoxide), then washed and treated for 10 minutes with either octanol or amyl alcohol (both solubilized in 0.1% dimethyl sulfoxide) before the final set of microinjections. Recovery of dye transfer was examined immediately after the alcohols were removed and the culture was washed once with fresh medium. During the microinjections, cultures were maintained at room temperature (approximately 22°C) and exposed to room air.

RESULTS

A variety of conditions have been tested in the culturing of lens epithelial cells. We have observed that cells in the early stages of fiber cell differentiation (ie, outer cortical fiber cells) can be maintained in simple medium on polyornithine-coated culture plastic when co-cultured with undifferentiated epithelial cells. After trypsinization of the lens, the differentiating outermost cortical fiber cells appear transparent and slightly elongated and adhere as aggregates to the trypsinized epithelial cells. These dissociated cells attach to the culture dish, typically through the epithelial cells, then flatten to form a clear layer of cells demarcated from the epithelium by its phase-bright boundaries. These differentiating fiber cells appear to induce additional differentiation of adjacent epithelial cells that become enlarged and transparent. Such areas of differentiation are very similar to lentoid structures described previously. These associations of lens cells are referred to as differentiating fiber-like cells.

Intercellular Communication in Ovine Lens Cell Cultures

The ability of cells to exchange small-molecular weight compounds, such as fluorescent dyes, has been used to show the presence of gap junction-mediated communication between cells. In this study of cultured ovine lens cells, we have focused on the gap junctions of both the confluent epithelial cells and the enlarged differentiating transparent cells growing on the epithelial cells. Trypan blue (0.4%) was used to identify nonviable cells that were avoided for microinjection studies.

Immediately after adherence to the culture dish, the lens epithelial cells displayed little or no dye transfer (data not shown). The epithelial cells were well coupled by 5 to 7 days in culture (1 to 2 days postconfluence), however, with the Lucifer yellow dye diffusing outward from the injected cell through at least four tiers of cells (Figs. 1A, B). Although quantification of the number of cells receiving dye in this system was complicated by the finding that the cells had numerous contacts with multiple layers of neighboring cells, estimates were facilitated because the dye preferentially stained cell nuclei. The epithelial cells cultured for 10 to 15 days appeared to be better coupled than did the cells at 5 days; however, epithelial cells from cultures older than 3 weeks did not consistently transfer dye as well as those cultured for 10 to 15 days (data not shown).

The more differentiated fiber-like lens cells in culture were also well coupled (Figs. 1C, D), with transferred dye often showing well-demarcated cell boundaries. These cells included both differentiating outer cortical fiber cells and any adjacent epithelial cells that had been induced to differentiate. The boundaries of the differentiating fiber-like cells were less obvious, thus hindering the determination of the number of such cells receiving Lucifer yellow dye. To circumvent
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FIGURE 1. Lucifer yellow dye transfer between cells in 12-day ovine lens cultures. In this case, Lucifer yellow dye injected into epithelial cells passed to approximately five tiers of cells: phase contrast (A) and fluorescence (B). Dye transfer is shown in a cluster of clear, differentiating cells: phase contrast (C) and fluorescence (D). Bar = 25 μm.

this difficulty, dye transfer between these differentiating cells was quantitated by digitizing the fluorescence intensity of the injected cell so the rate at which dye was transferred out of this cell could be followed. At the same time, the fluorescence intensities of two adjacent clear cells that appeared to be receiving dye were monitored to ensure that the Lucifer yellow was not escaping to either the surrounding medium or to the underlying epithelial cells. These differentiating fiber-like cells infrequently were observed to transfer dye to one to two neighboring or underlying epithelial cells (data not shown), which is consistent with findings of a previously reported study of differentiating rat lens cultures.15

Octanol Regulation of Intercellular Communication in Ovine Lens Cell Cultures

As in other cell types,26,27 n-octanol significantly inhibited Lucifer yellow dye transfer between the 15-day epithelial cells of the ovine lens cultures; 1 mM n-octanol was required for complete inhibition compared with dye transfer between cells of the same cultures bathed in control medium or medium containing 0.1% dimethylsulfoxide (Fig. 2A). This inhibition was reversible, and communication was restored by washing the octanol-treated cultures with control medium (data not shown). Amyl alcohol (1 mM), which is not effective in blocking junctional conductance in other cell types,26 did not inhibit dye transfer between the epithelial cells in the lens cultures (data not shown). These results provide strong evidence that the dye transfer between the epithelial cells in the lens cultures is mediated by gap junctions.

Although 1 mM octanol was required to completely inhibit dye transfer between the undifferentiated epithelial cells, 2 mM octanol was required to completely inhibit transfer between the differentiating fiber-like cells in 10- to 11-day cultures (Fig. 2B). Only a partial recovery of dye transfer was achieved within 10 minutes after removal of the octanol, although these same cells appeared viable when stained with trypan blue (data not shown).

Cx43 in Ovine Lens Cell Cultures

Immunofluorescence labeling of cell cultures with a polyclonal antibody to rat Cx43 showed that by the time the lens epithelial cells reached confluence at 5 days, this protein was located in numerous small plaques at interfaces between the epithelial cells (Figs. 3A, B); the number of such plaques increased by 15 days in culture (data not shown).

Western immunoblotting of purified membranes with this antibody identified two forms of Cx43 with Mr, s of 42 and 45 kD, both of which accumulated during the culture period (Fig. 4A). As shown previously with chicken Cx43,14 alkaline phosphatase treatment
FIGURE 2. (A) The effect of different concentrations of octanol on Lucifer yellow dye transfer between cells in 10-day cultures of ovine lens epithelial cells. Data are expressed as the mean number of cells observed to have received dye 2 minutes after microinjection; n = 5 for each condition. (B) The effect of different concentrations of octanol on Lucifer yellow dye transfer between differentiating fiber-like cells in 10- to 11-day lens cultures. Data are the mean of two experiments; rates of dye transfer out of the injected cell are expressed as percentages of the control values.

of ovine lens culture membranes led to the disappearance of a component with an M_ of 45 kD recognized by the Cx43 antibody on Western immunoblots and resulted in a concomitant increase in a component with an M_ of 42 kD (Fig. 4B, lane 3). These changes were prevented by phosphatase inhibitors (Fig. 4B, lane 2) and probably did not result from protease contamination because the serine protease inhibitor phenylmethylsulfanyl fluoride was included in the reaction mixture. Furthermore, the Coomassie blue SDS-PAGE protein patterns appeared identical before and after treatment of membranes with phosphatase in the presence of inhibitors (data not shown).

MP70 in Ovine Lens Cell Cultures

The 70-kD connexin-related protein, MP70, has been immunolocalized to ovine lens fiber cell gap junctions. In this study, differentiating ovine lens cell cultures were labeled with a mouse monoclonal antibody (55.35) that binds to the 16- to 17-nm gap junctions of purified calf lens fiber cell membranes and labels a 70-kD component in Western immunoblots of both sheep and calf membranes. Both large plaque-like and smaller punctate MP70 immunofluorescence labeling was observed on the membranes of the differentiating fiber-like cells in 10-day cultures (Figs. 5C, D). Labeling also was observed on exposed surfaces of most of the differentiating fiber-like cells when cultures were examined by confocal microscopy (data not shown). This indicates that MP70 was in regions of the cell membrane not forming junctions with other cells. In addition to the immunofluorescent labeling of differentiating fiber-like cells in the 10-day culture, most of the interfaces of the morphologically epithelial-like cells also were labeled immunofluorescently in 10-day culture with this MP70 antibody (Figs. 5E, F); such labeling was observed as early as 5 days. Identical MP70 immunofluorescence labeling also was seen with another monoclonal antibody (6-4-B2-C6) to MP70 (data not shown). There was little or no labeling of cell membranes when nonspecific ascites was substituted for MP70 antibody (Figs. 5A, B).

To confirm the developmental appearance of MP70 protein in the ovine cultures, urea-washed membranes were purified from 5-, 10-, and 15-day lens cell cultures, fractionated electrophoretically, and immunoblotted with antibody to MP70. Immunoreactivity in the 60- to 64-kD region was present at all time points (Fig. 6), supporting the conclusion that the immunofluorescence detected in Figure 5 resulted from MP70-related protein in the lens cultures. The amount of MP70 detected by immunoblotting at these three time points differed somewhat from experiment to experiment; however, at preconfluence, the amount of MP70 detected on immunoblots was always
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FIGURE 3. Immunolocalization of MP70 and Cx43 in the epithelial and differentiating fiber-like cells of 10-day ovine lens cultures. Phase contrast (A) of morphologically epithelial cells colabeled with both anti-Cx43 (1:100) detected with rhodamine-conjugated secondary antibody (1:250) (B) and anti-MP70 (1:100) detected with fluorescein-conjugated secondary antibody (1:100) (C). Phase contrast (D) of a differentiating region colabeled immunofluorescently with anti-Cx43 (E) and anti-MP70 (F). Bar = 10 μm.

less than that after confluence of the cultured cells occurred (data not shown). The variability from 5 to 15 days likely results from variability in the extent of epithelial to fiber cell differentiation in a given culture dish. Lower percentage acrylamide gels resolved the

FIGURE 4. Western immunoblot of Cx43 in membranes derived from ovine lens cells cultured on polyornithine-coated glass coverslips. (A) Immunoblot of membranes prepared from 5-(lane 1), 10-(lane 2), and 15-day (lane 3) ovine lens cultures probed with anti-Cx43 (1:500). (B) Control membranes (lane 1), membranes treated with alkaline phosphatase and alkaline phosphatase inhibitors (lane 2), and membranes treated with alkaline phosphatase (lane 3). MP70 immunoreactive band into two components at 60 and 64 kD (Fig. 6, lane 5). Alkaline phosphatase treatment of lens culture membranes before electrophoresis appeared to slightly increase the content of the 60-kD component and concomitantly decrease the content of the 64-kD component (Fig. 7C). A similar effect of alkaline phosphatase on the relative distribution of the components between 64 and 70 kD was observed in Western immunoblots of urea-washed whole ovine lens membranes (Fig. 7A).

Cx46 in Ovine Lens Cell Cultures

The presence of lens-specific Cx46 also was examined in these differentiating cultures with a Cx46 C-terminal polyclonal antipeptide antibody. Cx46 immunoreactivity was detected between the very enlarged epithelial cells and differentiating fiber-like cells (Fig. 8C). It also was detected in nonadjacent membranes of the transparent differentiating fiber-like cells. In all cases, Cx46 immunoreactivity completely coincided with that of MP70 (Fig. 8B).

Cx46 protein also was identified in lens cultures by Western immunoblotting. The Cx46 antibody labeled three major components of 57, 60, and 74 kD in membranes derived from differentiating cultures (Fig.
7D). Protein phosphatase treatment of these membranes before electrophoresis and immunoblotting resulted in a loss of immunoreactivity at 60 kD and an increase in immunoreactivity at 57 kD (Fig. 7D, lane 4). A Cx46 mobility shift also was detected after treatment of urea-washed whole ovine lens membranes with alkaline phosphatase (Fig. 7B). The Cx46 immunoreactivity at 74 kD on Western immunoblots of membranes derived from lens cultures was not affected by alkaline phosphatase treatment (Fig. 7D, lane 4). This suggests that the antibody detects a Cx46-related protein (or possibly an additional connexin) in the ovine lens cultures that is either absent or not present in a significant quantity in the intact ovine lens.

**Colocalization of MP70 and Cx43 in Differentiating Ovine Lens Cell Cultures**

Cx43 antibody labeled a small number of differentiating fiber-like cells and all of the epithelial cells in ovine lens cultures that were 10 days or older (Fig. 3); however, MP70 antibody labeled most epithelial interfaces by 10 days and all of the differentiating clear cell mem-

**FIGURE 5. Immunolocalization of MP70 in 10-day ovine lens cell cultures.** Phase contrast of a region of differentiating fiber-like cells (A, C) and epithelial-like cells (E). Labeling with 1:20 nonspecific mouse ascites (B) or anti-MP70 (1:100 dilution) (D, F) detected with fluorescein-conjugated secondary antibody (1:250). Bar = 20 μm.
they provide excellent markers of lens differentiation.\textsuperscript{11,53}

To determine whether MP20 or MP26 was present in the ovine lens cultures, polyclonal antibodies to MP20 and MP26 were used to label the cultures immunofluorescently. Only the transparent fiber-like cells appeared to possess MP20 or MP26, with the presumably least differentiated cells (the clear cells with nonpyknotic nuclei located at the outer perimeter of the differentiating regions) staining weakly for MP26 and not at all for MP20. Membranes in regions of more aggregated fiber-like cells showed considerable immunofluorescence with either antibody (Fig. 9), although immunofluorescence labeling of MP20 was much weaker than that of MP26, correlating with the relative abundance of these two proteins in lens membranes. The labeling with antisera to MP26 and MP20 was not

Fiber Cell Membrane Proteins MP20 and MP26 in Differentiating Lens Cultures

The two major proteins of differentiated lens fiber cells have Mr of 18 and 26 kD on SDS-PAGE and have been termed MP20 and MP26.\textsuperscript{28,29} The localization of these proteins to junctional regions of the fiber cell membrane in some studies\textsuperscript{20-22} indicates that they may play a role in cell-to-cell communication. Neither MP20 nor MP26 is found in lens epithelial cells, so

\begin{figure}[h]
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\caption{Western immunoblot of MP70 in membranes derived from whole ovine lens and lens cultures. (A) Coomassie-stained 12% SDS-PAGE gel of an ovine lens membrane preparation (50 $\mu$g) (lane 1) and membranes derived from 5-lane 2), 10-(lane 3), and 15-day (lane 4) ovine lens cultures. (B) Lanes 1 to 4: immunoblot corresponding to (A) probed with anti-MP70. Lane 5: sample in lane 4 fractionated on a 10% SDS-PAGE gel.}
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\begin{figure}[h]
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\caption{Western immunoblots of MP70 and Cx46 in alkaline phosphatase-treated lens membranes. Immunoblots of intact lens membranes (A, B) or ovine lens culture membranes (C, D) probed with anti-MP70 (1:900) (A, C) or anti-Cx46 (1:4000) (B, D). Lane 1: control membranes; lane 2: membranes treated as in lane 1 in the absence of alkaline phosphatase inhibitors; lane 3: membranes treated with alkaline phosphatase and phosphatase inhibitors; lane 4: membranes treated with alkaline phosphatase.}
\end{figure}
FIGURE 8. Immunolocalization of both MP70 and Cx46 in 10-day ovine lens cell cultures. Phase contrast of differentiating clear cells (A) colabeled with anti-MP70 (1:100) detected with fluorescein-conjugated secondary antibody (B) and anti-Cx46 detected with rhodamine-conjugated secondary antibody (C). Bar = 20 μm.

restricted to cell interfaces of the differentiating fiber-like cells. The epithelial-like cells did not stain positively for either MP20 or MP26. Substitution of preimmune sera for the MP20 or MP26 antibodies resulted in no immunofluorescence labeling of the lens cultures, indicating the specificity of these two antibodies.

To confirm the immunofluorescence results, urea-washed membranes were prepared from 5-, 10-, and 15-day cultures (Fig. 10A) and analyzed by Western immunoblotting. The amount of MP20 (Fig. 10B) and MP26 immunoreactivity (Fig. 10C) per milligram of membrane protein increased significantly from 5 to 15 days in culture, which corresponds with the increase in the area of regions of differentiating fiber-like cells.

DISCUSSION

The regulation of gap junction permeability in the newly differentiating and differentiated compartments of the lens remains poorly defined, in part because of the difficulty of manipulating conditions in the intact tissue. For this reason, we have established a mammalian primary lens culture system in which these junctions are readily accessible for study. Both differentiating and nondifferentiated ovine lens cells in this culture system exhibit cell-to-cell communication that is inhibited by n-octanol, indicating that the junctions between these cells are similar to gap junctions of other tissues. Furthermore, immunolocalization of connexin-related MP70, Cx46, and Cx43 to the regions of contact between the cells in culture indicates that the gap junctions in culture probably are formed by these previously described lens gap junction proteins.

Examination of the most differentiated transparent cells of the ovine cultures has shown that they possess gap junctions and other phenotypic characteristics of differentiating fiber cells in the outer cortex of the adult ovine lens. The differentiating regions of chick embryo cultures resemble the most differentiated regions seen in the ovine cultures (see Fig. 2A of Menko, Klukas, and Johnson), although there is a tendency for the differentiating ovine cells to be larger and flatter than the differentiating chicken cells and to less frequently form the discrete clumps of cells referred to as lentoids. The differentiating lens cells in cultures of both species possess fiber-like characteristics, such as increased cell size, loss of organelles, and pyknosis of nuclei, as viewed by transmission electron microscopy.

The enlarged, irregularly shaped clear cells in the cultures do not resemble the thin, elongated, and tightly packed differentiating fiber cells of lens tissue, which implies that one or more of the differentiation signals present in vivo are absent in the cultures. The differentiating cells of the culture are bathed directly in medium so it is likely that they have less need for nutrient transport and less need to align and communicate through extensive gap junctional complexes such as those formed between the fiber cells in vivo. It is interesting that the more differentiated cells (as indicated by immunofluorescence labeling with antibodies to MP20 and MP26 and their very pyknotic nuclei) at
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FIGURE 9. Immunolocalization of MP20 and MP26 in differentiating ovine lens cultures. Phase contrast (A) and immunofluorescence (B) of a region of differentiating fiber-like cells in a 10-day culture labeled with MP26 polyclonal antibody and detected with rhodamine-conjugated goat antirabbit IgG, as described in Materials and Methods (bar = 50 μm). Similar differentiating structures in a 10-day culture viewed in phase contrast (C) and labeled with anti-MP20 (D) (bar = 21 μm).

FIGURE 10. Western immunoblot of MP20 and MP26 in ovine lens culture membrane preparations. Coomassie blue-stained gel of membranes isolated from ovine cultures (150 μg/lane) at 15 (lane 2), 10 (lane 3), and 5 days (lane 4) and membranes from sheep lens (50 μg/lane) (lane 1) (A). Western blot of a duplicate gel probed with anti-MP20 detected with 125I-labeled secondary antibody (B). Western blot of a duplicate gel probed with MP26 monoclonal antibody and detected with peroxidase-conjugated secondary antibody (C).

the surface of the differentiating clear cell regions of the cell cultures eventually dissociate from the underlying cells, suggesting that direct exposure to the culture environment is not conducive to the maintenance of the formed fiber-like gap junctions.

The developmental expression of lens-specific membrane proteins in the ovine lens cultures appears to mirror that in the intact lens. On adherence of the trypsin-removed lens epithelial cells to the culture dish, connexins rarely are observed at cell interfaces by immunofluorescent labeling (data not shown); however, by 5 to 7 days the epithelial cells have flattened and divided to reach confluence, and cell interfaces now label with antibodies to Cx43. Similarly, at 10 days these epithelial-like cells are well labeled with Cx43 antibody but additionally exhibit some MP70 immunoreactivity at cell-to-cell interfaces. The cells then begin to enlarge, remain morphologically epithelial-like, but stain immunofluorescently for Cx43, MP70, and, less frequently, Cx46. We also have found that the cells at this stage have begun to accumulate γ-crystallin, a conventional marker of lens fiber cell differentiation (data not shown). A fraction of these cells (especially those neighboring the plated-down outer cortical cells) continue to enlarge from 10 to 15 days to form more differentiated regions consisting of large, trans-
junction precursors are present in single-membrane
with Cx43 antibody. Antibodies to rat Cx43 reacted in
cell communication in situ. The significant labeling of
differentiating fiber-like cell membranes of the lens
immunoblots with both a 42-kD and a 45-kD compo-
layer in the ovine lens cultures were immunolabeled
nonjunctional hemichannels must be closed.

MP20 and MP26 are found only in the differenti-
tiated fiber cells of the intact ovine lens,\textsuperscript{11,33} which
indicates that the enlarged transparent cells of the cul-
tures are representative of developing lens fiber cells
in vivo. The finding that the transparent cells in the
outer portions of the differentiated regions often con-
tained nonpyknotic nuclei and stained only weakly for
MP26 but not at all for MP20 indicates that these cells
were the least differentiated of the clear fiber-like
cells. The cell membranes in the center of regions of
aggregated clear cells stained intensely for both of
these proteins and contained pyknotic nuclei, suggest-
ing they were more differentiated than the cells in the
outer perimeter of the clear cell foci. It is interesting
that the MP70 antibody-labeled epithelial-like cells in
the lens culture were not labeled by antibodies to ei-
ther MP20 or MP26. Thus, it appears that MP70 ex-
pression in the differentiating cells of the ovine lens
culture system precedes that of MP20 and MP26. Al-
though MP70 has not been localized to epithelial cell
interfaces in the intact lens,\textsuperscript{11} it is possible that equato-
rial epithelial cells begin to synthesize MP70 immedi-
aply before elongation and cryosections have not cap-
tured this small zone of differentiating epithelial cells.

Immunofluorescence labeling of the clear, fiber-
like cells in culture with antibodies to either MP26 or
MP20 was diffuse and present on all membrane sur-
faces. Like MP26 and MP20, MP70 and Cx46 labeling
in such regions also was not restricted to only the junc-
tional contacts; however, in contrast to MP26 and
MP20, MP70 and Cx46 labeling was found consist-
tently to be restricted to well-demarcated plaque-like
regions either on nonadjacent cell plasma membranes
or at junctional contacts between the newly differen-
tiating cells. The finding that gap junction proteins
MP70 and Cx46 could be immunocolocalized to the
same plaques at interfaces of enlarged epithelial and
differentiating fiber-like cell membranes of the lens
cultures indicates that both connexins may mediate
cell communication in situ. The significant labeling of
nonadjacent cell surfaces by these antibodies further
indicates that lens connexin hemichannels or gap
junction precursors are present in single-membrane
regions of the more differentiated cultured cells. For
the lens cells to remain viable in the cultures, these
nonjunctional hemichannels must be closed.

As expected, gap junctions of the epithelial cell
layer in the ovine lens cultures were immunolabeled
with Cx43 antibody. Antibodies to rat Cx43 reacted in
immunoblots with both a 42-kD and a 45-kD compo-
nent of ovine culture membranes (Fig. 4). The 45-kD
component could be converted qualitatively to a 42-
kD component by alkaline phosphatase (Fig. 4B), as
was described previously with the Cx43-related dou-
blet of embryonic chick lens cultures.\textsuperscript{14} This indicates
that, as in chick lens cultures, a significant fraction of
Cx43 is phosphorylated in the ovine lens cultures.

Cx43 also was identified in larger plaques located
in junctional and nonjunctional membranes of a small
number of the differentiating fiber-like cells of the
cultures (Fig. 3). This is similar to findings reported by
Musil, Beyer, and Goodenough,\textsuperscript{13} who showed that
junctions containing Cx43 were initially present dur-
ing lens fiber differentiation in the bow region of the
chick lens and that these plaques were larger than
those in the nondifferentiating epithelium. It also was
shown that the Cx43 immunoreactivity diminished as
one moved deeper into the outer cortex. This might
explain why only a fraction of the differentiating cul-
tured ovine cells were labeled with Cx43 antisera and
why these labeled cells included only the presumably
least differentiated clear cells that had nonpyknotic
nuclei. These observations suggest that Cx43 is re-
placed by Cx46 and MP70 during the differentiation
of epithelial to fiber cell in vivo.

We have shown recently that alkaline phosphatase
treatment increases the electrophoretic mobility of
bovine lens membrane Cx46.\textsuperscript{11} Such a mobility shift of
Cx46 now is shown to occur in purified ovine lens
membranes (Fig. 7B) and urea-washed membranes pu-
rified from ovine lens cell cultures (Fig. 7D). It is inter-
esting that the increase in the amount of the lower
molecular weight 57-kD component in ovine lens cell
culture membrane preparations treated with alkaline
phosphatase is similar to the mobility shift of Cx43 in
alkaline phosphatase-treated ovine lens culture mem-
branes (Fig. 4).

Alkaline phosphatase treatment of whole lens
membranes resulted in an increase in the immunoreac-
tivity of the 64-kD region of MP70 in Western blots
(Fig. 7A). Although the 64-kD component immunola-
babeled by MP70 antibody in whole lens membranes not
 treated with alkaline phosphatase may reflect the previ-
ously described proteolytic product of MP70,\textsuperscript{7} the in-
creased MP70 antibody immunoreactivity at 64 kD
after treatment of these lens membranes with alkaline
phosphatase probably does not result from additional
proteolytic digestion of MP70 because protease inhibi-
tors were included with this highly purified phospha-
tase enzyme. A similar increase in the lower molecular
weight MP70 antibody immunoreactivity was observed
after alkaline phosphatase treatment of membranes
purified from differentiating fiber-like lens cell cul-
tures (Fig. 7C). This may reflect a phosphorylation-de-
pendent shift in mobility of MP70 analogous to that
exhibited by Cx43 and Cx46 in the ovine culture.
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Thus, a significant fraction of all three connexins appears to be phosphorylated in the ovine lens cultures. It is interesting that the MP70-immunoreactive components detected in Western blots of culture membrane preparations were of slightly lower molecular weight compared with those detected in whole lens preparations (compare Figs. 7A and C).

Together, these data indicate that differentiation of ovine lens cells in culture resembles the process of differentiation occurring in the outermost cortical regions of the lens. The differentiating fiber-like cells not only display conventional markers of differentiation, but also possess connexin proteins in junctional regions between cells. Dye transfer between these differentiating fiber-like cells is inhibited by n-octanol, indicating that cell communication between these cells is mediated by the same type of gap junction proteins that effect this communication in other tissues. Thus, the ovine lens culture system provides easy access to cells that are analogous to those in the intact lens. This should permit a closer examination of the factors regulating communication between the cells of this tissue. Additional definition of the junctional differences between the subpopulations of the differentiating ovine lens cells in culture also should aid in understanding the developmental changes that occur as lens gap junctions are formed in vivo.

Key Words
lens, communication, cultures, connexins, phosphorylation

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References


