Molecular Cloning of Ovine Connexin44 and Temporal Expression of Gap Junction Proteins in a Lens Cell Culture

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PURPOSE. The lens plasma membranes of several mammalian species have been shown to contain three different connexin proteins. The goal of this study was to clone the sheep homologue of rat connexin46 identified as sheep connexin44 and to determine the temporal changes in the expression of the three sheep connexin proteins in a lens primary cell culture system.

METHODS. A sheep genomic library was screened with a rat lens connexin46 cDNA probe. Lens junctional protein and mRNA levels were determined in a sheep primary cell culture system by Western and Northern blot analyses, respectively.

RESULTS. Sheep connexin44, the homologue of rat lens connexin46, was identified as a single-copy gene with a predicted molecular weight of 43,989 Daltons that is contained within a single exon. Northern blot analysis detected a 2.2kb connexin44 transcript in RNA isolated from lens but not that isolated from heart, kidney, liver, or lung. During the in vitro differentiation of lens epithelial cells from 5 to 20 days in culture, connexin43 mRNA levels declined approximately 75%, whereas connexin49 RNA levels increased approximately 24 fold. The 40% decrease in the level of connexin43 protein and the 21-fold increase in the level of connexin49 protein did not directly correlate with the changes in mRNA levels encoding these proteins during this same period. Although detectable, the amount of connexin44 mRNA and protein remained low throughout the 20-day period during which lens cells were grown in culture. Neither mRNA nor protein encoding MP20 or MP26 transcripts could be detected in even the oldest 20-day lens cultures.

CONCLUSIONS. Steady state mRNA levels of sheep connexin43 and connexin49 do not appear to be the only factor regulating the expression of these genes during in vitro differentiation of lens cells in culture. Although a decreased level of expression of connexin43 was accompanied by an increased level of expression of connexin49 over the 20-day period in culture, connexin44 mRNA and protein levels remained low throughout this 20-day period. Overall, these results suggest that these junctional proteins have a unique temporal pattern of expression during differentiation, and this lens primary cell culture system provides a valuable tool to better understand this process.

Gap junctions are specialized plasma membrane structures that mediate the intercellular transfer of low-molecular-weight (<1 kDa) metabolites and ions.1-3 The structural units of these channels are termed connexons and consist of six integral membrane subunits termed connexins.4,5 The genes encoding the connexin proteins comprise a multigene family that shows significant sequence homology.2,5,6 Different tissues and cell types often show characteristic temporal or spatial patterns of connexin expression,7,8 and this characteristic pattern of connexin gene expression appears to be coordinated with cellular differentiation.9

To date, three connexins have been identified in mammalian and avian lenses. Connexin43 is expressed in lens epithelial cells10 and many other tissues,11 mouse connexin50 is present in lens epithelial12 and fiber cells,13 and rat connexin4614 is essentially lens fiber cell specific. Mouse connexin50 homologues have been detected by immunofluorescence labeling in corneal epithelium15 and heart valves,16 whereas rat connexin46 is expressed in Schwann cells.17 Sheep connexin4918 (initially termed MP7019) is the sheep homologue of mouse connexin50, human connexin50, and chicken connexin45.6. In addition to the connexin proteins, there are two major lens fiber-specific membrane proteins, MP26, a member of the CHIP family of water transporting proteins,20,21 and MP20, a distant member of the epithelial membrane protein MP22/EMP-1 gene family.22-24 Both MP26 and MP20 are present in fiber cell membranes throughout the lens as well as in junctional areas in a restricted region of the lens outer cortex.25,26

Previously, we developed a sheep lens primary cell culture system that undergoes in vitro differentiation from lens epithelial cells into fiberlike lentoids.57 To explore the expression patterns of both mRNA and proteins of lens gap junctions...
in this cell culture system, the relative amounts of these proteins have now been determined by Northern and Western analyses at different times during lens culture growth. Although changes in mRNA levels approximately correlate with changes in protein levels, steady state mRNA levels do not appear to be the only factor regulating the differential expression of connexin43 and connexin49 in this primary ovine lens culture system.

**Materials and Methods**

**Materials**

Sheep eyes, liver, lung, heart, and kidney were obtained from John Morrell (Sioux Falls, SD), Hanks' balanced salt solution (HBSS) with calcium and magnesium, trypsin, medium 199 (M199), and penicillin-streptomycin from Gibco (Grand Island, NY), fetal calf serum from Hyclone (Logan, UT), and poly-DL-ornithine from Sigma (St. Louis, MO). All chemicals used were of reagent grade or the highest grade available.

**Screening of Phage Genomic Libraries**

A sheep genomic library in λ phage EMBL-3 vector obtained from Clontech (Palo Alto, CA) was screened with a 670-bp cDNA fragment of rat connexin46. The coding regions of the restriction enzyme-digested phage inserts were then subcloned into the SalI site of a vector (pBluescript; Stratagene, San Diego, CA) that was transformed into Escherichia coli strain DH5α. Double-stranded DNA cycle sequencing was performed using a kit (AmpliCycle; Perkin-Elmer, Foster City, CA).

**Southern and Northern Blot Analysis**

Total genomic DNA was isolated from sheep blood using a genomic DNA preparation kit (Puregene; Gentra, Minneapolis, MN). Total RNA was isolated from lens primary cultures or intact lenses using the RNeasy kit (Qiagen, Chatsworth, CA). Total RNA was isolated according to the manufacturer's protocol. Northern blot analysis was performed according to a protocol described previously. Quantitative analysis of autoradiographic intensities of individual bands on Northern blot analysis was accomplished using a Phospholmager system (Molecular Dynamics, Sunnyvale, CA).

**Primary Sheep Lens Cultures**

The preparation of primary ovine lens culture has been described previously. In brief, eyes were removed from freshly killed adult sheep and kept in ice until use for approximately 5 to 6 hours during transportation from the abattoir to the laboratory. This differs from our previous study in which eyes were retrieved from less than 1-year-old lambs at a local abattoir and dissected within 1 hour. Eyes were briefly soaked in 95% ethanol before the lenses were manually removed from the eyeballs. The adherent ciliary epithelium was cleaned by gently dabbing the exterior of the dissected lens with a sterile cotton swab without removing the capsule. Approximately 12 to 15 nicks were then cut along the equator of the lens to facilitate dissociation of outer cortical cells in the subsequent trypsinization step. The lenses were then placed in ice-cold sterile HBSS. Typically, six lenses were treated with 0.4% trypsin diluted in 6 ml HBSS in a 50-ml sterile conical polypropylene tube on an orbital shaker at 37°C for 15 minutes. After trypsinization, seven volumes of ice-cold HBSS was added, and the epithelial and outer cortical fiber cells were released into the solution by gently agitating the trypsinized lenses 25 to 30 times with a pipette until the capsules were detached from the lenses. Medium containing dissociated cells was removed, and the cell suspension was centrifuged at 250g for 10 minutes before the cell pellet was resuspended in medium 199 containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Two milliliters of this cell suspension was transferred to a poly-DL-ornithine-coated 35-mm dish with a glass coverslip, as described. The lens cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

**Preparation of Lens Membrane Proteins**

Membranes were isolated by homogenizing either intact sheep lenses or sheep primary lens cultures in 10 mM Tris-HCl (pH 7.5) buffer containing 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin. After centrifugation at 10,000g for 10 minutes, the resultant membrane pellets were extracted with 4 M and 7 M urea, as described previously.

**Immunoblot Analysis of Lens Membranes**

Western transfers of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) fractionated membrane proteins were performed as described by Towbin et al., except that 100 mM NaCl and 0.05% SDS were added to the transfer buffer to enhance protein absorption to the nitrocellulose membranes. A 1:1000 dilution of a mouse anti-connexin43 monoclonal antibody purchased from Chemicon (Temecula, CA) was applied in PBS-Tween 20 buffer containing 5% nonfat dry milk. The anti-MP70 monoclonal antibody (6-B42-C6) kindly provided by Joerg Kistler (University of Auckland, New Zealand) was used at 1:400 dilution in 3% nonfat dry milk. The rabbit polyclonal antibody to residues 411 to 416 of rat connexin46 generously provided by Larry Takemoto (University of Kansas, Lawrence, KS) was applied in 3% nonfat dry milk at 1:5000 dilution. For the detection of sheep connexin43 and sheep connexin49 (MP70), peroxidase-coupled anti-mouse IgG-IgM (1:5000; Kirkegaard & Perry, Gaithersburg, MD) was used. The peroxidase-coupled anti-rabbit IgG (1:400; Pierce, Rockford, IL) was used in 3% nonfat dry milk to detect sheep connexin44 (rat connexin46). The immunoreactive components were then detected with enhanced chemiluminescence Western blot analysis detection reagent (Amersham, Arlington Heights, IL) for 1 minute and immediately exposed to film (XR-P-1; Eastman Kodak, Rochester, NY). After development, films were scanned with a densitometer (Molecular Dynamics) to quantitate the intensity of each immunoreactive band. Each experiment was performed using membrane proteins isolated from at least three batches of sheep primary lens cultures.

**Statistical Analysis**

Where appropriate, the data were analyzed by analysis of variance (ANOVA) on computer (SuperANOVA ver. 1.11; Abacus Concepts, Berkeley, CA). If the ANOVA revealed a significant difference (P < 0.05), means were separated by Fisher's least-significant difference.
RESULTS

Cloning of Sheep Connexin44

The sheep homologue of rat connexin46 (Cx46) was isolated from a sheep genomic λ phage library using a cDNA probe encoding the first 670 bp of the coding region of connexin46. A single positive clone designated SHX46 was isolated that contained a 15.0-kb insert. Subcloning of this insert identified the complete 1242-bp coding sequence for the sheep homologue of connexin46 encoding a 413–amino acid protein with a predicted molecular weight of 43,989 Daltons, and therefore, by current convention, it was termed sheep lens connexin44 (GenBank accession number AF177912). The data presented in Table 1 indicate significant homology among sheep connexin44, bovine connexin44, and rat connexin46. In contrast, the amino acid sequences of sheep connexin44 and sheep lens connexin49 have only 45% identity, supporting the conclusion that sheep connexin44 belongs to the rat lens connexin46 subfamily. The interspecies comparison between nucleotide and peptide sequences of sheep lens connexin49, mouse connexin50, chicken connexin45.6, and human connexin50 are reported in Table 1.

As with all the other connexins so far identified, Southern blot analysis indicated connexin44 is a single-copy gene. Only single bands were detected on Southern blot analysis in which sheep genomic DNA was separately digested with five restriction enzymes (SalI, BamHI, Xbol, HindIII, and EcoRI) and subsequently hybridized with a genomic DNA fragment covering base pairs 99 to 1035 of connexin44 (data not shown). One unique feature of the sheep connexin44 nucleotide sequence is its high guanine-cytosine (GC) content (80%) in the 540-bp segment encoding the C-terminal cytoplasmic domain. Within this connexin subfamily such a high GC content has only been identified in bovine connexin44. Northern blot analysis of total RNA isolated from a variety of sheep tissues including heart, kidney, liver, lung, and lens using this connexin44 probe indicated that expression of this connexin is restricted to the lens (data not shown).

### Table 1. Percentage Identity of Sheep Connexin44 to Lens Connexins in Other Species

<table>
<thead>
<tr>
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<th>Nucleotide Sequence Identity with Sheep Connexin44 (%)</th>
<th>Protein Sequence Identity with Sheep Connexin44 (%)</th>
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<tbody>
<tr>
<td>Bovine connexin44</td>
<td>96</td>
<td>88</td>
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<td>Rat connexin46</td>
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<td>71</td>
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<td>Human connexin50</td>
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![Figure 1.](image)

**Figure 1.** Northern and Western blot analyses of connexin43 in lens cultures. Total RNA containing equal amounts of poly(A)-mRNA isolated from cell cultures at each time point (lanes 1 through 4, respective to time points in D) was electrophoretically fractionated and stained with ethidium bromide (A), transferred to a nylon membrane, and hybridized with 32P-labeled rat connexin43 (Cx43) cDNA probe to determine the relative amount of connexin43 transcript expressed (B). The positions of 28S and 18S rRNA are indicated by *lines*. Equal amounts of total membrane protein isolated from cell cultures at each time point were electrophoretically fractionated and immunoblotted with connexin43 antibody (C). The expression of connexin43 mRNA (open bars) and protein (hatched bars) in different aged lens cultures are compared with that of the 5-day-old cultures arbitrarily set to 100. Intensities from both the major, faster migrating bands and the barely detectable, slower migrating bands of connexin43 were combined to obtain the results. Data (means ± SD) are derived from three batches of cell cultures with each P representing the significance of this data point compared with the 5-day value (either mRNA or protein level of connexin43); *P < 0.05.
Amounts of Connexin mRNA and Protein in a Differentiating Lens Culture System

To determine the temporal expression levels of connexin43, connexin49, and connexin44 mRNA and protein during differentiation of lens cells in culture, Northern and Western analyses were conducted to determine the relative amounts of the connexin mRNA and protein in the differentiating sheep lens culture system developed in this laboratory. During the first 24 hours in culture, cells released from trypsinized sheep lenses adhered to the poly-ornithine-coated coverslips. These adherent cells kept dividing until they covered the surface of the coverslips, which took approximately 5 days; these cell cultures are referred to as 5-day-old cultures in this report. Typically, 4 of a total of 16 dishes of these 5-day-old cultures were frozen in liquid nitrogen for subsequent RNA or membrane protein isolation; the remaining 12 dishes were continued in culture with four dishes, each being frozen for subsequent RNA and membrane protein preparation at 5, 10, and 15 days after confluence and are referred to as 10-, 15-, and 20-day-old cultures, respectively. The 5-day-old cultures represented cells that had just reached confluence and primarily comprised epithelial cells with nuclei. Foci of differentiation usually appear in the cultures between 5 and 10 days, whereas the 20-day-old cultures represent the more differentiated epithelial cells.

To ensure equal loading of mRNA in Northern blot analysis, duplicate aliquots of total RNA isolated from 5- to 20-day-old cultures were first bound to nylon membranes and hybridized to 32P-labeled oligo-dT to quantitatively determine the mRNA content in each total RNA sample (data not shown). A representative example of connexin43 mRNA and protein amounts in the differentiating lens culture is shown in Figures 1B and 1C. The averaged data from three similar experiments are shown in Figure 1D. The results indicate that the amount of connexin43 mRNA declined to 46.5% ± 8.2% by 10 days in culture and 75.3% ± 5.2% by 20 days in culture. The decline in the total amount of connexin43 protein was slower than the decline in connexin43 mRNA, so that this was only significantly different from the 5-day value by day 15 in culture (protein decreased to 45.5% ± 6.8%; Fig. 1D). Note that results are from Western blot analysis using a mouse anti-connexin43 monoclonal antibody that apparently recognized one major band of higher mobility and one very minor, barely detectable band of lower mobility. The autoradiographic intensities of both bands were combined for the results shown in Figure 1. Although it is not clear whether the affinity of this antibody was identical for both the phosphorylated and dephosphorylated forms of connexin43, the same antibody has been shown to recognize both the phosphorylated and dephosphorylated forms of connexin43 in several mammalian species including bovine, canine, mouse, and rat.

In contrast to the decreased amount of connexin43 mRNA and protein, the relative concentrations of connexin49 mRNA and protein both increased from 5 to 20 days (Fig. 2). The amount of connexin49 mRNA increased 21-fold between 5 and 10 days in culture, whereas the amount of connexin49 protein increased only 3.4-fold during the same time period. The amount of connexin49 protein increased 24.4-fold over the 20-day period in culture, but as with connexin43, this increase in connexin49 protein lagged the increase in mRNA.

![Figure 2](image_url)
The pattern of connexin44 mRNA and protein expression is shown in Figure 3. The total RNA and membrane protein isolated from intact lenses was included for comparison. The results indicate that the amounts of connexin44 mRNA and protein remained very low throughout the 20-day period (Fig. 3B, 3C, lanes 1 to 4). The summarized data derived from the analyses of four different batches of cell cultures are depicted in Figure 3D. Because of the variability in absolute connexin44 mRNA amounts, the increase in connexin44 mRNA, which never exceeded 2.5-fold when the amounts of connexin44 mRNA in 5- and 20-day-old cultures were compared, was not statistically significant \((P = 0.37)\). Similarly, no significant changes in the amount of connexin44 protein (as determined by Western blot analysis) were observed over the 20 days that the lens cells were grown in culture (Fig. 3D, \(P = 0.48\)).

The antibody used in this study readily detected both phosphorylated and dephosphorylated connexin44 protein in plasma membranes isolated from intact lenses (Fig. 3C, lanes 6 and 7). Treatment of cell membranes isolated from lens cultures with alkaline phosphatase before Western blot analysis did not significantly enhance connexin44 immunoreactivity to this anti-rat connexin46 antibody or another connexin46 monoclonal antibody (5H1.3), which is also specific for sheep lens connexin44 \(^{26}\) (data not shown). These results appear to exclude the possibility that the low amount of connexin44 immunoreactivity in Western blot analysis of membranes derived from lens cultures is due to changes in the phosphorylation state of this connexin protein in the lens cultures.

**mRNA Levels of MP26 and MP20 in Culture**

The two most abundant lens fiber cell membrane proteins, MP26 and MP20, have been proposed to play important roles in the formation and/or regulation of lens fiber junctions.\(^{25}\) However, we were unable to detect either MP26 or MP20 mRNA in lens cultures at any time point by Northern blot analysis or RNase protection assay, a procedure approximately 10 times more sensitive than Northern blot analysis (data not shown). In contrast, mRNA encoding both MP26 and MP20 were readily detected in the total RNA isolated from the intact lens (data not shown).

**DISCUSSION**

This study demonstrates that as the epithelial cells start to differentiate in this lens culture system, there is a differential expression of the connexin mRNAs and proteins. Specifically, the amount of connexin43 mRNA and protein declines, whereas the amount of connexin49 mRNA and protein increases. Our results indicate, however, that change in mRNA concentration is not the only factor regulating this differential expression of these two gap junction proteins during lens fiber cell differentiation, because changes in protein level lag changes in mRNA level. Thus, the fourfold increase in connexin49 protein between 5 and 10 days in culture did not directly correlate with the 21-fold increase in connexin49 mRNA level during this same period (Fig. 2). Despite the “delay” in the increased expression of connexin49 between day 5 and day 10 in culture, the 24.4-fold increase in connexin49 mRNA between day 5 and day 20 is comparable with the 21.4-fold increase in the connexin49 protein during this same period. Similarly, the decrease in the amount of con-
nexin43 mRNA between 5 and 10 days in culture did not result in immediate downregulation of connexin43 protein expression; yet by 20 days in culture both mRNA and protein levels of connexin43 had decreased by similar amounts.

The detectable yet low levels of connexin44 mRNA present throughout the 20-day culture period was initially surprising, given the significant amount of connexin44 mRNA that can be detected in total RNA isolated from intact lenses (Fig. 5). The detectable yet low level of connexin44 protein determined by Western blot analysis, also contrasts with our previous report characterizing this primary lens culture system in which connexin44 was immunofluorescently colocalized to some of the same junctional plaques with connexin49 in the cell cultures. Although at present we are unable to resolve this discrepancy, one possible explanation is that different abattoirs in different states were used in the two studies, and the age or breeding seasons of the lambs from which the eyes were removed may therefore have differed. Clearly, the cultures described here appeared to differentiate less than those described in our previous report.27 This is supported by our observation that in contrast to our earlier studies, we failed to detect mRNA encoding the fiber cell–specific proteins MP26 and MP20 in the culture system reported here. Indeed, the discrepancy in the extent of differentiation between the primary cultures described here and those described in our previous report is not without precedent. For example, it has been reported that the age of rats can affect the response of lens epithelial explant cultures to fibroblast growth factor.

The physiological importance of the differential expression of these lens junctional proteins remains unclear. Of note, results from the recently available knockout mice for the three connexins indicate that only in connexin50 knockout mice did the lenses exhibit a reduced rate of growth, suggesting a potentially important role of connexin50 in the early stages of lens development.36 This is further supported by the recent immunolocalization of connexin50 to lens epithelial cells in mice.12 In addition, in the developing embryonic chicken lens, both lens fiber–specific connexins, (connexin45.6 and connexin56) can be immunolocalized to interepithelial gap junctions.

In conclusion, the expression of both mRNA and protein encoding the lens connexin genes appears to be differentially regulated in the sheep lens culture system reported here. However, changes in mRNA concentration do not appear to be the only factor regulating the expression of these genes. This sheep lens cell culture system provides a valuable tool for identifying the other factors responsible for regulating the differential expression of these connexin proteins, which appears to be critical for the maintenance of the specialized functions of this transparent tissue.

Acknowledgments

The authors thank David Paul, Harvard University, for generously providing rat connexin46 cDNA and Nalin Kumar, Scripps Institute, for generously providing the rat MP20 and MP26 cDNA clones.

References


