Expression and Function of Muscarinic Receptor Subtypes on Human Cornea and Conjunctiva

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PURPOSE. To investigate the cellular distribution of the muscarinic receptor (MR) subtypes m1–m5 on the ocular surface and to determine their function in cell growth.

METHODS. Human limbal and conjunctival epithelial cells and conjunctival fibroblasts were isolated and cultured. RT-PCR, real-time PCR, immunostaining and Western blot analyses for m1–m5 were performed on cultured cells and tissues and a human conjunctival epithelial cell line (IOBA-NHC). Cell proliferation and p42/44 mitogen-activated protein (MAP) kinase (MAPK) activation in response to MR agonists and antagonists were analyzed by bromodeoxyuridine (BrdU) incorporation and Western blot analysis, respectively.

RESULTS. RT-PCR revealed the presence of m1–m5 transcripts in cultured limbal and conjunctival epithelial cells and conjunctival fibroblasts. Relative quantitative real-time PCR showed that the m1 transcript level in conjunctival cells was higher than that in limbal cells; m2, m3, and m4 expression levels were higher in conjunctival fibroblasts than in epithelial cells. Absolute quantitative real-time PCR showed that the m5 mRNA level in the three cell types was higher than those of m1–m4. Immunohistochemistry and Western blot analysis confirmed the presence of m1–m5 proteins in the cultured cells and in tissues. Carbachol increased the incorporation of BrdU into conjunctival epithelial cells in a dose-dependent manner, which was totally inhibited by atropine, but only partially inhibited by pirenzepine, AF-DX116, and 4-DAMP. Carbachol also activated p42/44 MAPK in a time-dependent manner. Preincubation with U0126 abolished carbachol-induced p42/44 MAPK activation and cell proliferation.

CONCLUSIONS. All five MR subtypes were found on corneal and conjunctival cells. The MRs have a role in epithelial cell proliferation through the phosphorylation of p42/44 MAPK in a time-dependent fashion similar to EGF. (Invest Ophthalmol Vis Sci. 2007;48:2987–2996) DOI:10.1167/iovs.06-0880

Muscarinic cholinergic receptors belong to the superfamily of plasma membrane-bound, G-protein-coupled receptors. Currently, five distinct muscarinic receptor genes have been cloned and sequenced and are referred to as m1 through m5.1,2 Although a large body of evidence has shown that muscarinic receptors have important roles in the nervous system, recent studies have suggested that muscarinic receptors are widely expressed in non-neuronal cells, such as epithelial, endothelial, and immune cells, and muscle fibers.3,4 Non-neuronal acetylcholine appears to be involved in the regulation of diverse cellular activities including but not limited to cell proliferation, differentiation, cytoskeleton arrangement, and locomotion.3,5

In the eye, multiple muscarinic receptor subtypes have been reported in the ocular surface, ciliary body, lens, retina, and sclera.6 Studies have suggested that muscarinic receptors are involved in the regulation of eye development,7 corneal epithelial wound healing,8,9 tear fluid and aqueous humor production,10,11 iris and ciliary muscle contraction,12 lens cell signaling,13 and the regulation of scleral growth.14 The ocular cholinergic system is also a pharmacologic target in the treatment of myopia.15,16 Activation of protein kinase C (PKC) and phospholipase C (PLC), the mobilization of calcium, the transactivation of the EGF receptor, and the subsequent activation of MAPK was observed on stimulation of muscarinic receptor agonists in various ocular cell types.15,17–20

In human conjunctival epithelial cells, the expression of m1, m2, and m3 was identified.21–23 The same subtypes were also reported in the mucin-secreting goblet cells.21,23 It was shown that m3 was the most important MR subtype in goblet cells in regulating mucin secretion. Carbachol-stimulated activation of MAPK via the EGF receptor was observed in cultured goblet cells.19 Certain proinflammatory cytokines such as interferon-γ were found to regulate the expression of m2 in NHC-IJOBA cells.22 Under pathophysiological conditions such as vernal keratoconjunctivitis, m1 expression was reduced and m2 and m3 was irregularly distributed.24 However, the distribution of m4 or m5 on human conjunctival and corneal epithelial cells is not clear. Studies have shown that the activation of MRs increase corneal epithelial cell proliferation.8,9,25 However, this aspect has not been well studied in squamous human conjunctival epithelial cells.

We used relative and absolute quantitative real-time PCR to study the distribution of muscarinic receptor subtypes in human conjunctival and limbal epithelial cells and conjunctival fibroblasts. We further determined the localization of muscarinic receptor proteins in human conjunctival and corneal tissue. In addition, we also found that carbachol stimulated the proliferation of conjunctival epithelial cells and that the proliferation was totally inhibited by atropine, but only partially by pirenzepine, AF-DX116, or 4-DAMP. P42/44 MAPK was activated in the presence of carbachol, and the activation was abolished when the cells were preincubated with U0126, indicating that the carbachol-induced cell proliferation was mediated through p42/44 MAPK.

Materials and Methods

Human and Animal Tissues

Human corneal (n = 3), limbal and conjunctival tissues (n = 12) harvested within 24 hours of death were provided by the Singapore
Eye Bank. The protocol was approved by the Institutional Review Board of the Singapore Eye Research Institute and complied with the tenets of the Declaration of Helsinki. Normal monkey brain cortex tissues used as positive controls in this study were obtained from animals euthanatized as part of an approved protocol that conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Isolation and Cultivation of Human Limbal and Conjunctival Epithelial Cells

Human limbal tissues were washed in phosphate-buffered saline (PBS) containing 100 U/mL penicillin, 50 μg/mL gentamicin, and 2 μg/mL amphotericin B. After careful removal of the corneal endothelium, iris, excessive sclera, and conjunctiva, the limbal rings were exposed to Dispase II (1.2 IU/mL in Hanks’ balanced salt solution free of Mg²⁺ and Ca²⁺) at 37°C in humidified air with 5% CO₂ for 3 hours. Loosened epithelial sheets were removed with a cell scraper and separated into single cells by 5 minutes of trypsin digestion. The cells were pelleted at 1000 rpm for 5 minutes and resuspended in supplemented hormonal epithelial medium (SHEM).²⁶ SHEM consists of an equal volume of DMEM containing 100 U/mL penicillin, 50 μg/mL hydrocortisone, 50 μg/mL gentamicin, 2.5 μg/mL epidermal growth factor, 8.4 ng/mL chola toxin A subunit, 0.5% dimethyl sulfoxide, 0.5 μg/mL hydrocortisone, 0.5 μg/mL gentamicin, 1.25 μg/mL amphotericin B, and 5 mM HEPES. The cells were plated at 10⁵ cells/cm² in cell culture dishes containing a mitomycin C (MMC)-treated 3T3 feeder layer and were incubated at 37°C in 5% CO₂, 95% air. The medium was changed every 2 days. On reaching 70% to 80% confluence, the 3T3 feeder layer was removed, and the epithelial cells were subcultured to the next passage.

To prepare the feeder layer, confluent 3T3 fibroblasts were incubated with 4 μg/mL MMC in DMEM with 10% fetal bovine serum (FBS) for 2 hours at 37°C under 5% CO₂, trypsinized, and plated onto new culture dishes at a density of 2.2 × 10⁴ cells/cm². These feeder cells were used 4 to 24 hours after plating.

Human conjunctival epithelial cells were isolated as described earlier. However, the conjunctival epithelial cell culture was initiated in 1:1 medium, which contained equal volumes of DMEM and Ham’s F12 with 10% FBS, 0.5 μg/mL hydrocortisone, 8.4 ng/mL cholera toxin A subunit, 10 ng/mL epidermal growth factor (EGF), and 5 μg/mL insulin for the first day. They were changed to serum-free keratinocyte/H9262/F12 with 10% FBS. After initial cell outgrowth from the explants was observed, the volume of medium was increased. The cultures were passaged on reaching 70% to 80% confluence.

### RNA Isolation and RT-PCR

Total RNA was extracted from limbal and conjunctival epithelial cells and from conjunctival fibroblasts grown to near confluence (TRIzol reagent; Invitrogen-Gibco). Genomic DNA was removed by digestion with DNase I (Amp Grade; Invitrogen-Gibco) for 15 minutes at room temperature. The integrity of the RNA was verified by agarose gel electrophoresis. One microgram of total RNA was reverse-transcribed with random hexamers by using a first-strand cDNA synthesis kit (Invitrogen-Gibco). Three microliters of cDNA was used for PCR amplification. PCR was performed for 35 to 40 cycles at 94°C for 45 seconds, 52°C to 55°C for 45 seconds, and 72°C for 30 seconds. Primer sequences are listed in Table 1.²⁷ Total RNA extracted from monkey brain cortex was used as a positive control. PCR amplification of β-actin was performed in parallel, to detect genomic DNA contamination. The amplified products were analyzed by electrophoresis on 1.2% agarose-TAE gels.

### Relative and Absolute Quantitative Real-Time PCR

Real-time PCR primers for transcripts of m1–m5 and β-actin were purchased from Applied Biosystems Inc. (Taqman Gene Expression System; ABI; Foster City, CA). Real-time PCR reactions were performed on a sequence-detection system (Prism 7700; ABI) with 500 ng total cDNA per reaction in a final volume of 25 μL.

Two types of real-time PCR analysis were used in the study. The first type determined the relative level of gene expression quantitatively, which compared the transcript abundance of a single gene among different cell types. The Ct of each reaction was obtained by using a constant threshold. β-actin was used as an internal control. ΔCt was calculated by subtracting the average Ct of β-actin from the average Ct of target gene. The expression level of each gene in cultured limbal epithelial cells was used for calibration. ΔΔCt of other samples was calculated by subtracting ΔCt of limbal epithelial cells from the ΔCt of each sample. The relative change of other samples compared with limbal epithelial cells was determined as 2⁻ΔΔCt. Statistical analysis was performed by ANOVA. Significance levels of P < 0.05 were considered significant. The levels of m1–m5 in different sample types were compared by the Fisher least-significant difference (LSD) test.

To compare the abundance of m1–m5 gene transcription in the same cell type, we performed absolute quantitative real-time PCR. DNA templates for transcripts of m1–m5 were obtained by using the primers listed in Table 2, which include the regions of real-time PCR amplicons, and the sequences were confirmed. The sizes of all templates were between 694 and 721 bp. DNA templates were purified and the

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### Table 1. Sequences for PCR Primers for Receptor Subtypes m1–m5

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<td>X15264</td>
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<td>m5</td>
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<tr>
<td>β-Actin</td>
<td>X00351</td>
<td>5′-CACTCTTTCCAGGCCTCTCTC-3′/5′-CTGCTCTACTCTCGTCTGTC-3′</td>
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<td>820–1133</td>
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</table>

β-Actin PCR primer pair was selected to span a 206-bp intron."
concentrations determined. Serial dilutions of the templates were used in real-time PCR amplifications to generate a linear standard curve of the logarithm of DNA quantity versus Ct for each muscarinic receptor subtype.

The absolute copy numbers of transcripts for m1–m5 in samples were calculated by using standard curves, which were run in parallel with each analysis. Data were expressed as the mean ± SE and analyzed by ANOVA. The expression levels of the genes for m1–m5 in the same sample type were compared by the Fisher LSD test. A probability of P < 0.05 was considered statistically significant.

**Immunocytochemistry and Immunohistochemistry**

Human limbal and conjunctival epithelial cells and conjunctival fibroblasts were grown on four-well chamber slides and fixed in 4% paraformaldehyde for 10 minutes. After the reaction was blocked with 3% bovine serum albumin (BSA), 0.3% Triton X-100, and PBS for 30 minutes at room temperature, the cells were incubated for 2 hours at room temperature with anti-muscarinic receptor subtype–specific antibodies (Research & Diagnostic Antibodies, Berkeley, CA) at a concentration of 1:500 diluted in 1% BSA/PBS. After they were stained with FITC-conjugated secondary antibody (Chemicon International) at a dilution of 1:2500 for 1 hour at room temperature, the slides were mounted on coverslips (Fluosave; Calbiochem, San Diego, CA).

Human cornea and conjunctival tissues embedded in optimal cutting temperature (OCT) compound were cut at 6 μm thickness and placed on poly-L-lysine–coated slides. The slides were then processed and stained similarly, as just described. The tissues were mounted on the slides with antibiotic medium containing DAPI (4,6-diamidino-2-phenylindole; Vectashield; Vector Laboratories, Burlingame, CA). DAPI was used as a counterstain to visualize the cell nuclei. For negative controls, primary antibodies were omitted.

A fluorescence microscope (Axioplan2; Carl Zeiss Meditec, GmbH, Oberkochen, Germany) was used to examine the slides and take photographs.

**Electrophoresis and Immunoblotting**

Nearly confluent limbal and conjunctival epithelial cells and fibroblasts were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer plus protease inhibitors (10 mM Tris-HCl [pH 7.4], containing 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 10 mg/mL phenylmethylsulfonyl fluoride, 5 μL/mL aprotnin, and 100 mM sodium orthovanadate). After homogenization, the samples were centrifuged at 14,000 g for 10 minutes at 4°C. Proteins in the supernatant were separated by SDS-PAGE, transferred to nitrocellulose membranes, blocked in 5% BSA in TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.1% Tween-20) for 2 hours at room temperature, and incubated with the same anti-muscarinic receptor antibodies as mentioned earlier at a dilution of 1:1000 for 1 hour at room temperature. The membranes were washed three times in TBST and incubated with HRP-conjugated secondary antibody (Chemicon International) at a dilution of 1:2500 for 1 hour at room temperature. Immunoreactive bands were visualized using the enhanced chemiluminescence method (GE Healthcare, Buckinghamshire, UK).

**Cell Proliferation Assay**

Cell proliferation was assessed by measuring 5-bromo-2′-deoxyuridine (BrDU) incorporation during DNA synthesis in proliferating cells (Cell Proliferation Biotrak ELISA System; GE Healthcare). Primary cultured conjunctival epithelial cells were plated at the density of 10³ cells/well in 96-well dishes and allowed to settle in SFM. On the following day, the cells were starved for 24 hours with MCDB153 (Sigma-Aldrich, St. Louis, MO) supplemented with 0.09 mM Ca²⁺. The cells were then treated with the muscarinic receptor agonist carbachol or the pan-receptor antagonist atropine at different concentrations in MCDB153 for 24 hours. BrDU at 10 μM was added 4 hours before the termination of the incubation. To study the combined effect of atropine and carbachol, we added atropine at 150 μM 2 hours before carbachol stimulation. The detection of BrDU was performed according to the manufacturer’s instruction.

IOBA-NHC cells were plated in complete medium overnight, which contained DMEM/F-12 supplemented with 2 ng/mL EGF, 1 μg/mL insulin, 0.1 μg/mL cholera toxin, 0.5 μg/mL hydrocortisone, and 10% FBS and starved in DMEM for 24 hours. The muscarinic receptor subtype–selective antagonist pirenzepine (M1 antagonist; Sigma-Aldrich), AF-DX116 (M2 antagonist; Sigma-Aldrich), or 4-DAMP (M3 antagonist, Tocris Cookson Inc., Ellisville, MO) was added 2 hours before carbachol stimulation. Other procedures were the same as have been described.

Data are expressed as the mean ± SE, and n is the number of independent experiments. Statistical analysis was performed with ANOVA and the Fisher LSD test. P < 0.05 was considered significant.

**p42/44 MAPK Activation**

Cultured IOBA-NHC cells were serum starved for 24 hours in DMEM and then incubated with 1 mM carbachol or 1 ng/mL EGF for 1, 5, 10, 30, or 60 minutes. After stimulation, the cells were washed once with ice-cold PBS and lysed in PBS containing 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and a cocktail of proteinase inhibitors. The cell lysates were collected and centrifuged at 10,000g for 5 minutes at 4°C, and the supernatants were used as total cell lysates. Aliquots of 20 μL of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The detection of phosphorylated p42/44 MAPK and total p42/44 MAPK proteins was performed according to the instructions provided by the company (Cell Signaling Technology, Danvers, MA). The MEK inhibitor U0126 (Promega, Madison, WI) was added to the medium at 10 μM in 0.1% dimethyl sulfoxide (DMSO) 1 hour before carbachol stimulation. The assays to determine levels of phosphorylated p42/44 MAPK and total p42/44 MAPK proteins and the effect of U0126 on MAPK activation were performed as has been described.

**RESULTS**

**Human Limbal and Conjunctival Cells in Culture**

In primary limbal epithelial cell culture, colonies of 4 to 16 cells were visualized by microscopic examination on the third day after seeding onto the 3T3 feeder layer. The cells in the colonies were small and tightly arranged, whereas the 3T3 cells surrounding these colonies showed a distinct colony margin (Figs. 1A, 1B). In the 10 limbal cultures, the cell-doubling time was approximately 20 hours. However, passage-1 cultures appeared to grow more quickly, and colonies of 4 to 16 cells were observed on the second day after plating.

**TABLE 2. PCR Primer Sequences Used for Preparing Absolute Real-Time PCR Standard Curve**

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<th>Primer Pair</th>
<th>Sequence Sense/Antisense</th>
<th>Product Size (bp)</th>
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<td>m2</td>
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<tr>
<td></td>
<td>5′-GGCAGAGAAAGGAGACCATTTA-3′</td>
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Human conjunctival epithelial cells were cultivated in serum-free medium. Single attached cells divided to form four-cell colonies by days 2 and 3 and continued to proliferate to form 50- to 60-cell colonies by day 6. Each colony consisted of a collection of small, round or ovoid cells (Fig. 1C). The cells were seen mainly as a monolayer, with focal areas showing two or more layers after 8 days. Confluent colonies had cobblestone morphology. Conjunctival fibroblasts migrated from the explants 6 to 7 days after seeding. They assumed a spindle-shaped morphology at confluence (Fig. 1D).

Muscarinic Receptor Gene Expression

By conventional PCR, expression of all five types of muscarinic receptor was detected in cultured conjunctival epithelial cells, conjunctival fibroblasts, and limbal epithelial cells (Fig. 2). The identity of each PCR product was further confirmed by sequencing. Because none of the five MR genes has introns, the PCR product from genomic DNA would yield the same-sized amplicons. To rule out possible contamination by genomic DNA, we chose a pair of β-actin primers at two adjacent exons that spanned a 206-bp intron on cDNA derived from β-actin mRNA. A single 314-bp PCR product band for β-actin mRNA (without intron) was identified in all cDNA samples used.

Quantitative real-time PCR was used to compare the relative abundance of each MR subtype transcript in the different cell types. Compared to m1–m5 gene expression in monkey brain, the muscarinic receptor transcript levels in cultured limbal, conjunctival epithelial cells and conjunctival fibroblasts were very low (P < 0.001; data not shown). As shown in Table 3, the normalized m1 expression level in cultured conjunctival epithelial cells and conjunctival fibroblasts was similar (P > 0.05, n = 3 for each cell type), but both were higher than that in cultured limbal epithelial cells (P < 0.001). Similar levels of m2, m3, and m4 were observed in both epithelial cell types (P > 0.05, n = 3 for each cell type). Fibroblasts had higher levels of m2, m3, and m4 when compared to both epithelial cell types (P < 0.01). RNA levels for m5 in limbal epithelial cells and in conjunctival fibroblasts were not significantly different (P > 0.05, n = 3 for each cell type); however, they were higher than the expression levels in conjunctival epithelial cells (P < 0.001; Table 3).

Although relative quantitative real-time PCR analysis is sufficient for the comparison of the expression of one gene among different samples, it is not suitable to assess the expression of multiple genes within one cell. To compare the relative abundance of m1–m5 transcripts in each of the above cell types, we designed another set of PCR primers (Table 2) to amplify a segment of DNA, which contained the region that was amplified by real-time PCR for each of the muscarinic receptor subtypes. These PCR amplicons were purified, quantified, and used as templates to generate standard curves of cDNA input and Ct values. Using these individual standard curves, we further compared the number of copies of five muscarinic receptor subtypes within the same cell type. For the purpose of analysis, m1 expression in each cell type was set at 1. As shown in Table 4, in the conjunctival epithelial cells, the amount of mRNA of m5 was 24 times higher than that of m1 (P < 0.001, n = 9), whereas that of the other four subtypes was not significantly different from one another (P > 0.05). In limbal epithelial cells, the amount of m5 and m4 was significantly higher than m1 (P < 0.01, n = 9) with no significant difference among m1, m2, and m3 (P > 0.05). In conjunctival fibroblasts, the amount of m5 and m2 was higher than that of m1 (P < 0.001, n = 9), with no significant differences among the levels of m1, m3, and m4 (P > 0.05; Table 4).

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933444/)

**Figure 1.** Cultured human limbal and conjunctival epithelial cells and fibroblasts. Clonal growth of P0 limbal epithelial cells in the presence of a 3T3 feeder layer on the (A) third and (B) sixth days after seeding. (C) Cultured P1 human conjunctival epithelial cells in serum free medium. (D) Cultured P1 human conjunctival fibroblasts. Magnification: (A, B) ×1000; (C, D) ×100.

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933444/)

**Figure 2.** RT-PCR identified cDNA for m1–m5 in cultured human limbal and conjunctival epithelial cells and conjunctival fibroblasts. Lane 1: monkey brain as a positive control; lane 2: limbal epithelial cells; lane 3: conjunctival epithelial cells; lane 4: corneal fibroblasts; lane 5: conjunctival fibroblasts; lane 6: H2O control. PCR products of the expected sizes were as follows: m1 538 bp; m2, 654 bp; m3, 560 bp; m4, 505 bp; m5, 752 bp; and β-actin, 314 bp. The identity of the products was confirmed by sequencing.
We examined the proliferative response of human conjunctival epithelial cells to carbachol stimulation using BrdU as an indicator. After 24 hours of starvation, the cells were treated for 24 hours with 50, 100, or 150 μM or 1 mM carbachol in medium free of serum and growth factors. A dose-dependent increase in BrdU incorporation was observed. A significant increase of 1.84-fold in BrdU incorporation was achieved with 1 mM carbachol when compared with cells in basal medium (P < 0.05, n = 9). The fully supplemented conjunctival epithelial cell culture medium evoked a 3.59-fold increase for the same time (P < 0.01, n = 9; Fig. 7).

Similar effects were observed in IOBA-NHC cells. A significant increase in BrdU incorporation was observed with 50, 100, and 150 μM and 1 mM carbachol (P < 0.05, n = 6). The effect of carbachol at 1 mM was not significantly different from 150 μM carbachol (P > 0.05, n = 6; Fig. 7).

To determine whether the effect of carbachol was through the activation of muscarinic receptors, IOBA-NHC cells were preincubated with the muscarinic receptor antagonist atropine. Atropine (150 μM) completely inhibited carbachol (150 μM)-induced cell proliferation (P > 0.05, n = 6). Atropine alone did not have a significant effect on conjunctival epithelial cell proliferation (P > 0.05, n = 7). This result indicated that muscarinic receptors play a role in mediating conjunctival epithelial cell growth.

To determine further the contribution of m1, m2, and m3 to carbachol-induced proliferation in IOBA-NHC cells, we preincubated cells with pirenzepine, the M1-selective antagonist; AF-DX116, the M2-selective antagonist; and 4-DAMP, the M3-selective antagonist. Cytotoxicity analysis (CellTiter-Blue; Promega, Madison, WI) showed that pirenzepine and 4-DAMP had little effect on cell viability at concentrations up to 100 μM. However, significant cell death was observed with 100 μM AF-DX116 (data not shown). We therefore studied the carbachol-induced IOBA-NHC cell proliferation in the presence of pirenzepine and 4-DAMP at concentrations of 1, 10, and 100 μM, and AF-DX116 at concentrations of 1 and 10 μM (Fig. 8). All three antagonists at 10 μM inhibited carbachol-induced proliferation by approximately 42% (P < 0.05, n = 8). However, these did not completely inhibit carbachol stimulation of cell proliferation, as observed with atropine.

**Effect of Carbachol on MAPK Activity**

We examined the activation of p42/44 MAPK in the muscarinic receptor-mediated responses in conjunctival epithelial cells. On stimulation with 1 mM carbachol, p42/44 MAPK was activated in a time-dependent manner (Figs. 9A, 9C). Phospho-p42/44 MAPK was observed at 1 minute of 1 mM carbachol stimulation and peaked at 10 minutes (P < 0.05, n = 3).

As a comparison to the activation of p42/44 kinase elicited by carbachol, we tested epidermal growth factor (EGF), known for eliciting proliferation in epithelial cells. EGF at 1 ng/mL was also found to activate p42/44 MAPK in a time-dependent manner (Figs. 9B, 9C). EGF significantly increased p42/44 phosphorylation a maximum of 10 times in 10 minutes (P < 0.05, n = 3).
By 1 hour, the level of phospho-p42/44 MAPK returned to basal levels ($P > 0.05$, $n = 3$). The p42/44 MAPK activation patterns between carbachol and EGF were similar (Figs. 9B, 9C).

The MEK inhibitor U0126 at 10 $\mu$M completely inhibited the activation of p42/44 MAPK at 1, 5, 10, 30, and 60 minutes (Fig. 9D). U0126 at 10 $\mu$M also completely inhibited carbachol (150 $\mu$M)-induced cell proliferation ($P > 0.05$, $n = 8$, data not shown).

**DISCUSSION**

Previous studies of MR distribution have revealed the expression of m1–m5 on rabbit corneal epithelial and endothelial cells; m1 and m2 in bovine corneal epithelial cells; m1–m3 on mouse, rat, and human conjunctival goblet cells and nongoblet cells. In this study, we detected all five MR subtypes in corneal, limbal, and conjunctival epithelial cells, including conjunctival goblet cells and corneal endothelial...
cells, when analyzed by immunofluorescent staining. In conjunctival tissue, m3 immunostaining was found predominately in the basal cell layer. RT-PCR and Western blot analysis on the cultured cells further confirmed the existence of MR subtypes in limbal and conjunctival epithelial cells as well as in conjunctival fibroblasts.

However, the abundance of each muscarinic receptor subtype in the three cell types was found to vary, as revealed by quantitative real-time PCR. We found that conjunctival fibroblasts expressed higher levels of m2, m5, and m4 transcripts than either limbal or conjunctival epithelial cells, and this difference was greatest for m2 transcripts. This finding is consistent with a previous report that suggested that the m2 transcript in skin fibroblasts is significantly higher than that in keratinocytes. The transcript level for m1 was the lowest in limbal epithelial cells, and the level for m5 was the lowest in conjunctival epithelial cells. When the abundance of muscarinic receptor subtypes was compared within the same cell type, we found that the m5 transcript was the most abundant, followed by m4 in conjunctival and limbal epithelial cells. In
conjunctival fibroblasts, m2 was the second most abundant muscarinic receptor subtype. Previous studies have shown that m5 plays an important role in the regulation of conjunctival goblet cell functions.\textsuperscript{21,23} Our results suggest that m4 and m5 may also be important in regulating muscarinic receptor-mediated functions in the ocular surface.

However, it has been reported that MR gene expression changes in cultured lens epithelial cells when compared with native lens cells.\textsuperscript{32} The expression of m1, which was the most abundant subtype found in native lens cells, decreased in cultured lens cells. In contrast, the expression of m3 increased and became the most abundant subtype in cultured lens cells.\textsuperscript{32} Changes in MR expression in cultured conjunctival epithelial cells in the presence of IFN-γ and in patients with vernal keratoconjunctivitis has also been reported.\textsuperscript{32,24} It remains to be determined whether cell culture systems change the expression of MR genes in limbal and conjunctival epithelial cells compared with cells in vivo.

Studies of the MR in non-neural cells show that they are involved in the regulation of cellular proliferation. For example, the MRs were shown to be involved in the modulation of keratinocyte proliferation, as well as migration, cell-differentiation, and cell-to-cell contact in the skin.\textsuperscript{3,33} Carbachol-stimulated goblet cell proliferation has been reported.\textsuperscript{24} In the present study, carbachol stimulated conjunctival epithelial cell proliferation in a time-dependent manner, and a significant increase in BrdU incorporation was found with 1 mM carbachol. The effect of carbachol was inhibited by preincubating the cells with atropine, which is consistent with previous study performed in corneal cells.\textsuperscript{8,9} We further demonstrated that m1, m2, and m3 subtype-selective antagonists alone blocked only approximately 40% of carbachol-induced cell proliferation. Thus, m1-m3 may contribute equally to the mediation of the carbachol-induced cell proliferation effect. Because m4 and m5 were more abundant in both the limbal and conjunctival epithelial cells, m4 and m5 may also participate in carbachol-induced cell proliferation. Little is known about the function of m5. In the brain, it has been reported that m5 constitutes less than 2% of the total MR population with most of it concentrated in the hippocampus, hypothalamus, and distinct mid-brain dopamine cell body.\textsuperscript{35,36} However, m5 knockout mice (m5\textsuperscript{−/−}) did not show significant changes in locomotor activity or dopamine agonist-induced activities.\textsuperscript{37} More specific subtype-selective antagonists are needed to study the function of each subtype further.

Carbachol-stimulated conjunctival epithelial cell proliferation correlates with its activation of p42/44 MAPK. Our study showed that 1 mM carbachol activated p42/44 MAPK with kinetics similar to EGF. Preincubation with U0126, a p42/44 MAPK inhibitor, completely blocked carbachol-induced p42/44 MAPK activation, as well as cell proliferation. Carbachol-stimulated activation of p42/44 MAPK has been observed in goblet cells through increased intracellular Ca\textsuperscript{2+} concentration or transactivation of EGF receptor via phosphorylation of Pyk2 and Src.\textsuperscript{19,20,38} Collectively, these results suggest that p42/44 MAPK is the major protein kinase responsible for muscarinic receptor-mediated cell proliferation.

**FIGURE 5.** Immunofluorescence detection of muscarinic receptor subtypes in human conjunctival tissue. Receptor subtypes m1–m5 were expressed in epithelial cells as well as goblet cells. m3 was predominantly found on basal epithelial cells, whereas other subtypes were expressed on all epithelial cells. Arrows: goblet cells.

**FIGURE 6.** Western blot analysis of muscarinic receptors m1–m5 in cultured human limbal and conjunctival epithelial cells and fibroblasts. Major bands for m1–m5 were obtained at 55, 50, 55.9, and 55.9 kDa, respectively. Depicted are representative data from at least three independent experiments. Lane 1: P0 limbal epithelial cells; lane 2: P1 conjunctival epithelial cells; lane 3: P1 conjunctival fibroblasts.

**FIGURE 7.** The effect of carbachol on conjunctival epithelial cell proliferation. After treatment with carbachol, BrdU incorporation into conjunctival epithelial cells showed a dose-dependent effect and was increased by 1.84 times in the presence of 1 mM carbachol, compared with the cells in the basal medium MCDB153 (P < 0.05, n = 9). The fully complemented keratinocytes growth medium increased cell proliferation by 3.59 times (P < 0.01, n = 9). In IOBA-NHC, carbachol at 50 µM began to stimulate proliferation and a maximum effect was observed at 150 µM (P < 0.05, n = 6). The effect of carbachol at 1 mM was not significantly different from that at 150 µM (P > 0.05, n = 6). (○) IOBA-NHC cells; (●) cultured primary human conjunctival epithelial cells; (□) IOBA-NHC cells in complete medium; (■) cultured primary human conjunctival epithelial cells in complete medium.
Muscarinic receptors have been found in the cornea. However, the distribution of MRs in limbal epithelial cells has not been reported. The limbus is a specialized narrow band between the cornea and conjunctiva and is innervated by sensory, sympathetic, and parasympathetic nerves. The basal layer of limbal epithelium contains corneal stem cells responsible for the renewal of corneal epithelium. The role of acetylcholine in the regulation of neural stem cell differentiation has been reported by different groups. The finding of all five muscarinic receptor transcripts and proteins in cultured limbal epithelial cells suggests that these receptors may play a role in the regulation of limbal stem cell function.

Not only are the multiple muscarinic receptor subtypes widely distributed in the conjunctival and corneal tissues, their natural ligand acetylcholine is also abundant in the ocular surface. Acetylcholine is secreted by both parasympathetic neurons and ocular surface epithelial cells. In fact, corneal epithelium contains the highest concentrations of acetylcholine among the various mammalian tissues that have been studied. The existence of both ligand and receptors make muscarinic receptors physiologically relevant targets for the control of ocular surface epithelial cell functions. Because both the secretion of acetylcholine and the expression of muscarinic receptors are subjected to changes by cytokines and in disease conditions, it is possible that muscarinic receptors participate in the pathophysiological changes of ocular surface epithelial cells.

In summary, our study showed for the first time the quantitative distribution of the five muscarinic receptor subtypes in human limbal and conjunctival epithelial cells and conjunctival fibroblasts. Activation of muscarinic receptors by carbachol led to increased conjunctival epithelial cell proliferation. The mitogenic effect of carbachol correlated with the activation of p42/44 MAPK. Both atropine, a general muscarinic receptor antagonist, and U0126, an inhibitor of p42/44 MAPK, blocked carbachol-induced cell proliferation, indicating that p42/44 MAPK is the major protein kinase responsible for muscarinic

**Figure 8.** Effect of muscarinic receptor subtype-selective antagonists on carbachol-induced IOBANHC cell proliferation. Pirenzepine at 1, 10, and 100 μM; AF-DX116 at 1 and 10 μM; or 4-DAMP at 1, 10, and 100 μM was added 2 hours before carbachol stimulation. The antagonists showed a dose-dependent inhibition of carbachol-induced cell proliferation. The antagonists at 10 μM inhibited carbachol-induced proliferation approximately 42% (P < 0.05, n = 8); however, there was no significant difference among subtype-selective antagonists (P > 0.05). Data are presented as percentage of inhibition of carbachol response over baseline levels.

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**Figure 9.** Activation of p42/44 MAPK by 1 mM carbachol and 1 ng/mL EGF in cultured conjunctival epithelial cells. (A) Time-dependent change of phospho-p42/44 MAPK in the presence of 1 mM carbachol. (B) Time-dependent change of phospho-p42/44 MAPK in the presence of 1 ng/mL recombinant EGF. (C) Quantitative results of the p42/44 MAPK activation by carbachol and EGF. The values for the two bands for phosphorylated p42/44 MAPK were analyzed together to determine the amount of phosphorylated MAPK. These data were normalized to the amount of total MAPK. Data were expressed as the percentage of increase above basal values, which was set to 1. By 1 hour, phospho-p42/44 MAPK activation had returned to basal levels (P > 0.05, n = 3). EGF (1 ng/mL) activated p42/44 MAPK in a time-dependent manner, increasing it significantly with a maximum of 10 times at 10 minutes (P < 0.05, n = 3). (D) Absence of phosphorylated p42/44 MAPK when the cells were incubated with 10 μM U0126 before 1 mM carbachol.
receptor-mediated cell proliferation. Preincubation with pirenzepine, AF-DX116, and 4-DAMP equally blocked approximately 40% of carbacoh-induced cell proliferation, indicating the equal contributions of m1, m2, and m3. The fact that the m5 gene is abundantly expressed in ocular surface cells should be considered in the future design and selection of muscarinic receptor agonists or antagonists for use in topical eye drops.

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