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Acetylcholine enhances keratocyte proliferation through muscarinic receptor activation

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ABSTRACT

Acetylcholine (ACh), a classical neurotransmitter, has been shown to be present in various non-neuronal cells, including cells of the eye, such as corneal epithelium and endothelium, and to have widespread physiological effects such as cytoskeleton reorganization, cellular proliferation, differentiation, and apoptosis. The aim of this study was to investigate the effect of ACh on corneal keratocyte proliferation, and the underlying mechanisms, in order to explore its possible effect in corneal wound healing. Primary culture of human keratocytes was established from donated corneas. Cell viability and fraction of proliferating cells were detected by MTS assay and BrdU incorporation ELISA, respectively. Expression of proliferative markers, PCNA and Ki-67, was detected by western blot and immunocytochemistry. Activation of the MAPK/Erk signaling pathway and its involvement in ACh-enhanced proliferation was determined by western blot analysis, MTS, and BrdU ELISA. We found that ACh enhanced keratocyte proliferation even at low concentrations. Stimulation of proliferation was mediated through activation of muscarinic ACh receptors (mAChRs). Western blot analysis revealed that ACh stimulation of keratocytes upregulated the expression of PCNA and Ki-67, and Ki-67 immunocytochemistry showed that ACh-treated cells were in an active phase of the cell cycle. ACh activated MAPK signaling, and this step was crucial for the ACh-enhanced proliferation, as inhibition of the MAPK pathway resulted in ACh having no proliferative effect. In conclusion, ACh enhances keratocyte proliferation and might thus play a role in proper corneal wound healing.

1. Introduction

Acetylcholine (ACh) is regarded as a classical neurotransmitter well known for acting in the central and peripheral nervous system, and to be present in both prokaryotes and eukaryotes [1–3]. However, in recent years, there have been extensive evidence demonstrating a non-neuronal cholinergic system, in which ACh and/or its synthesizing enzyme, choline acetyltranferase (ChAT), have been found to be present in non-neuronal human cells, such as keratinocytes [1,4], epithelial [5] and endothelial [6] cells, tendons [7], and various cells of the immune system [3]. ACh has been shown to have widespread physiological effects such as cytoskeleton reorganization, cellular proliferation, differentiation, and apoptosis [1,3,8,9]. Most cell types not innervated by cholinergic neurons still express two types of ACh receptors [10]: nicotinic (nAChRs), which are ion channels, or G protein-coupled muscarinic receptors (mAChRs). Activation of these receptors has been linked to activation of mitogen-activated protein kinase (MAPK)-dependent pathways [9,11,12], in which phosphorylation of Erk1/2 leads to DNA synthesis and cell proliferation [13].

The cornea is the transparent front part of the eye and is composed of three distinct layers: an outermost epithelial layer, a middle, transparent stromal layer, and an innermost endothelial layer. The stroma accounts for up to 90% of the corneal thickness and it is made of collagen fibrils in between which the quiescent cells of the stroma, the keratocytes, reside. The main role of these cells is maintenance and repair of the stroma [14–16]. ACh and ACh receptors have been studied in the cornea; however, most of the studies have been conducted in corneal epithelium and endothelium. Corneal epithelium has been shown to contain high concentrations of ACh and its synthesizing enzymes ChAT, and both corneal epithelium and endothelium express mAChRs [17]. There has been less attention to expression of nAChRs in the cornea. However, a recent study has shown that corneal epithelium expresses several nAChRs, including both α7 and α9 homomeric nAChRs and predominantly the α3β2 ± α5 subtype of heteromeric nAChRs [18]. The role of ACh in the cornea remains unclear, but data suggest that ACh plays an important role in epithelial regrowth after corneal injury and that it promotes chemotaxis [18], therefore facilitating healing of the injured cornea. Recent findings in our lab have shown that *in vitro* cultured human keratocytes secrete ACh and express mAChRs of subtypes M_1, M_3, M_4, and M_5 (unpublished data). Nevertheless, the knowledge on the ACh system in the corneal stroma, and its keratocytes, is still

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scarce, and a deeper understanding of the potential role of ACh in corneal wound healing requires studies on the stromal components. Therefore, in the present study, we evaluate the role ACh in keratocyte proliferation and thus its possible role in corneal wound healing.

2. Materials and methods

2.1. Collection of human corneas

Healthy human corneas from deceased individuals who had chosen, when alive, to donate their corneas post-mortem for transplantation or research, according to Swedish law, were kept in a corneal biobank at the University Hospital of Umeå, Sweden. If these healthy donated corneas were not used for transplantation after their collection, they were delivered to the laboratory for research purpose. If corneas were used for transplantation, some or all of the transplantation graft leftovers were retrieved for study purpose: the healthy donor peripheral part or the healthy donor anterior or posterior central lamella. The study was vetted by the Regional Ethical Review Board in Umeå (2010-373-31 M) without objections. The study was performed according to the principles of the Declaration of Helsinki.

2.2. Chemicals and reagents

Acetylcholine, mecamylamine, atropine, and collagenase were purchased from Sigma–Aldrich (Saint Louis, MO, USA). PD98059 was obtained from Calbiochem (San Diego, CA, USA). Antibodies against β-actin, p-Erk1/2 (Thr202/Tyr204), and PCNA were purchased from Cell Signaling (Danvers, MA, USA). Ki-67 antibody was obtained from Millipore (Billerica, MA, USA). Cell viability assay was purchased from Promega (Fitchburg, WI, USA). BrdU incorporation ELISA was purchased from Roche (Basel, Switzerland). DMEM/F-12 + GlutaMAX™ medium, penicillin-streptomycin, FBS, and Trypsin-EDTA were obtained from Life Technologies (Carlsbad, CA, USA).

2.3. Isolation and primary culture of human corneal stromal cells

Primary culture of human keratocytes was established by first scraping off any remaining epithelial and endothelial cells from a donated cornea, and then separating and mincing central and peripheral parts with a scalpel. Samples were then digested in 2 mg/ml collagenase diluted in DMEM/F-12 + GlutaMAX™ medium overnight at 37 °C. The samples were then transferred to DMEM / F-12 GlutaMAX™ medium supplemented with 2% FBS and 1% penicillin-streptomycin and cultured at 37 °C with 5% CO_2. Medium was replaced every second to third day until the cells reached confluence. Confluent cells were detached with 0.05% Trypsin-EDTA and split in a 1:2 ratio. Cells from the central cornea in passages 4 to 5 were used for experiments. DMEM/F-12 GlutaMAX™ medium supplemented with 0.1% FBS was used both for seeding cells and performing experiments. In total, 12 corneas were used for experiments. The corneas were assessed individually.

2.4. Cell viability assay

The effect of ACh on the viability and proliferation of cells was measured using MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay) according to manufacturer's instructions. Briefly, keratocytes at a density of 2×10^3/well were seeded in a 96-well plate overnight and treated with various concentrations of ACh for 24 h. To assess type of ACh receptors responsible for ACh effect on proliferation, cells were pretreated with various concentrations of atropine (muscarinic receptors antagonist) and mecamylamine (nicotinic receptors antagonist). Moreover, to assess involvement of MAP kinase cascade in ACh-induced proliferation, cells were pretreated with 25 μM PD98059 (MAP kinase inhibitor). Next, 20 μl of the MTS reagent was added into each well and cells were incubated for 4 h at 37 °C, 5% CO_2. To measure the amount of formazan produced by cellular reduction of MTS, absorbance at 490 nm was measured with a micro-plate reader (BioTek, Winooski, VT, USA).

2.5. BrdU incorporation ELISA

ACh effect on cell proliferation was performed by measurement of BrdU incorporation in newly synthesized cellular DNA according to manufacturer's instructions. Briefly, 3×10^3 keratocytes were seeded into 96-well plate overnight and treated with various concentrations of ACh. Additionally, cells were pretreated with atropine, mecamylamine, or PD98059 as described in a previous section. One hour after treatments, cells were labeled with 10 μM BrdU and incubated for 24 h at 37 °C, 5% CO_2. Next, cells were fixed and the DNA was denatured. Anti-BrdU-POD was added to bind BrdU incorporated in newly synthesized DNA. Immune complexes were detected by measuring the absorbance at 370 nm (with a reference wavelength at 492 nm) with a microplate reader (BioTek).

2.6. Western blot analysis

2.5×10^5 cells/well were plated in 6-well plates. In order to block muscarinic or nicotinic receptors, cells were treated with atropine or mecamylamine for 30 minutes. Afterwards, cells were treated with A-Ch. After indicated time, keratocytes were washed with PBS and freeze/thawed 3 times. Cells were further lysed in RIPA buffer supplemented with proteinase and phosphatase inhibitor cocktail (Fisher Scientific, Waltham, MA, USA). Protein concentration was assessed by Bradford assay (Bio-Rad, Hercules, CA, USA). Aliquots of the lysates (12 μg for p-Erk1/2 (Thr202/Tyr204); 30 μg for PCNA and Ki67 studies) were separated on either 4–20% or 7.5% (Ki67) SDS–polyacrylamide gel and transferred to PVDF membranes. After blocking with either 5% non-fat dry milk or 5% BSA, membranes were incubated with primary antibodies at 4 °C overnight. Then, the membranes were incubated with HRP-conjugated antibodies for 1 h at room temperature. Images were taken by Odyssey® Fc imaging system (LI-COR, Lincoln, NE, USA). Densitometry was performed using Image J analysis software (NIH).

2.7. Immunocytochemistry

10^4 cells per well were seeded in an 8-well chamber slides (Corning, Corning, NY) overnight. Cells were treated with 10^{-7} M ACh. After 24 h, medium was removed and cells were washed twice with PBS. Keratocytes were fixed with 3.7% paraformaldehyde in 1 × PBS for 5 minutes at room temperature, and then blocked for 15 minutes with rabbit normal serum in PBS (1:20). Cells were incubated with Ki-67 antibody (1:200) for 1 h at 37 °C and blocked again for 15 minutes with rabbit normal serum in PBS (1:10). After washing, secondary antibody labeled with TRITC (Dako, Glostrup, Denmark) was incubated with the cells for 30 minutes at 37 °C. Finally, cells were mounted in ProLong® Diamond Antifade Mountant with DAPI (Life Technologies). A control well was also prepared for secondary antibody by replacing the primary antibody with PBS. A Zeiss Axioskop 2 plus microscope equipped with epifluorescence and an Olympus DP70 digital camera were used for analysis.

2.8. Statistical analysis

All experiments were performed in triplicates. Data are presented as mean ± SD. Statistical analysis was carried out using one-way or two-way ANOVA and Bonferroni post hoc test. Differences were considered statistically significant at a p value of < 0.05. The experiments were repeated successfully at least three times.