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Original Article

Gap junction connexin43 is a key element in mediating phagocytosis activity in human trabecular meshwork cells

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Abstract: Human trabecular meshwork (TM) cells play pivotal roles in maintaining homeostasis of intraocular pressure via regulation of aqueous humor outflow. These cells are capable of phagocytosis, which is considered to be essential for their regulatory function. In addition, there is a strong expression of the gap junction protein connexin43 (Cx43) in the TM. Here, we investigated functional relationships between phagocytosis activity of TM cells and their expression of Cx43. Phagocytosis was measured by showing the ability of TM cells to engulf inert fluorescent particles consisting of pHrodo. We found that internalized pHrodo was partially co-localized with Cx43 and that the phagocytic activity was dramatically reduced after knockdown of Cx43 using lentiviral Cx43 shRNA. These results suggest that Cx43 is involved in the regulation of phagocytosis by TM cells.

Keywords: Trabecular meshwork, gap junction, phagocytosis, connexin43

Introduction

Like other cell types with phagocytic capacity [1], the ability to engulf pathogens, inert particles and tissue debris is an essential element of TM cells [2, 3]. Thus, besides regulation of aqueous humor outflow, aggressive phagocytosis by TM cells is considered obligatory for removing debris and other materials from the aqueous humor [2]. Failure of phagocytosis debilitates the aqueous humor drainage system, and contributes to a variety of pathological conditions including glaucoma [4, 5]. The phagocytosis property of TM has been well studied and verified in cultured TM cells, in trabeculectomy specimens and in anterior segmental organ culture systems [6]. In addition, phagocytosis mediated by TM cells is involved in the turnover of extracellular matrix [7], disturbance of which can lead to increased aqueous humor outflow resistance and increased IOP [8]. Mechanisms underlying phagocytic capacity of various cell types, including that of TM cells, have been extensively studied with respect to regulation by cell signaling pathways, interactions involving cytoskeletal and scaffolding proteins, and actions of drugs [9, 10].

Some of these mechanisms involve direct or indirect interactions with proteins such as integrins, which are known to be associated with Cx43. It is, therefore, not surprising that Cx43 impacts phagocytic activity, such as in Cx43 enhancement of phagocytosis in macrophages after bacterial stimulation [11]. The recent recognition that the gap junction protein Cx43 is strongly expressed in TM cells raises the possibility that this connexin may contribute to the regulation of TM cell phagocytosis [37], as seen in other cell types [11]. We investigated this using pHrodo labeling to monitor phagocytosis activity, and immunofluorescence labeling of Cx43 to define its intercellular localization in relation to that of internalized pHrodo. Further, we determined phagocytic activity of TM cells after administration of highly efficient lentiviral shRNA knockdown of Cx43.

Material and methods

Antibody, HEK 293 cells and reagents

Polyclonal anti-Cx43 (Cat. No. 71-0700) antibody was purchased from Life Technologies (Invitrogen, Carlsbad, CA) and the specificity of this antibody was previously reported [12-16]. HEK293 cells were purchased from ATCC (American Type Culture Collection, Manassas, Virginia, USA).

Human primary trabecular meshwork cell culture

HTM cells were isolated and cultured based on protocols we previously reported [17-19]. These cells were cultured in Dulbecco's Modified Eagle's Medium, containing 50/50% high and low glucose (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and 1% penicillin-streptomycin-fungizone (Sigma-Aldrich, St. Louis, MO). These studies were conducted in accordance to the tenets of the Declaration of Helsinki.

pHrodo phagocytosis assay

HTM cells were grown in 6-well cell culture plates coated with collagen I and incubated with pH sensitive *E. coli*-conjugated pHrodo red particles diluted at 1:2000 (Invitrogen, Carlsbad, CA). When these particles were phagocytosed by HTM cells, the acidic condition inside the cells allows visualization of these particles by monitoring their fluorescence. Red fluorescence was manually quantified using an Olympus confocal microscope after 2 hours of labeling with *E. coli*-conjugated pHrodo red particles in three independent experiments. Quantification of phagocytosis was conducted by comparing the number of punctate phagocytized pHrodo-labeled *E Coli* bioparticles inside lentiviral Cx43 shRNA transduced HTM cells vs. those in cells transduced with scrambled shRNA control. In addition, as shown in **Figure 5**, we only manually count the punctate labeling inside of the cells. The pHrodo labeling outside of the cells is excluded from the count.

Lentiviral Cx43 shRNA transduction

High efficiency transfection using lipofectamine 3000 reagents in HEK293 cells as well as generation of high titer lentivirus was conducted as we previously described [20, 37]. Cx43 shRNA was obtained from Santa Cruz Biotechnology (Dallas, TX), the shRNA sequences that target human Cx43 consisted of pools of four different sequences, these sequences were: A: 5'-GATCCCTGCGAACCTACATCATCzATTCAAGAGATGATGATGTAGGTTCGCAGTTTTT-3'; B: 5'-GATCCGAACCTACATCATCAGTATTTCAAGAGAATACTGATGATGTAGGTTCTTTTT-3'; C: 5'-GATCCGTTGGGGATGTCACTTAACATTCAAGAGATGTTAAGTGACATCCCAACTTTTT-3'; D: 5'-GATCCCCTACTTAATACACAGTAATTCAAGAGATTACTGTGTATTAAGTAGGTTTTT-3'. Scrambled lentiviral shRNA was used as a control.

Western immunoblotting

Western blotting procedures were conducted as previously described [21, 22]. Briefly, HTM cells were rinsed in cold PBS buffer (50 mM sodium phosphate buffer, pH 7.4, 0.9% saline) and lysed in an IP buffer (20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of leupeptin, pepstatin A, and aprotinin). Homogenates were centrifuged at 20,000 × g for 20 min at 4°C, and supernatants were subjected to protein determination using the Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Proteins were boiled for 5 minutes and then separated by SDS-PAGE (10 μg of protein per lane) using 12.5% gels, followed by transblotting to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) in standard Tris-glycine transfer buffer, pH 8.3, containing 0.5% SDS. Subsequently, membranes were blocked for 2 h at room temperature in an Odyssey blocking buffer (Li-Cor Biosciences), rinsed for 30 minutes in TBSTw (50 mM Tris-HCl, pH 7.4, containing 1.5% sodium chloride + 0.05% tween 20), and incubated overnight at 4°C with polyclonal anti-Cx43 antibody diluted at 1:1000 in TBSTw. Membranes then were washed four times in TBSTw for 40 min, incubated with specific secondary antibodies (Li-Cor Bio-sciences, Lincoln, NE), and scanned using an imaging system (Odyssey Infrared; Li-Cor) with companion software (Odyssey 2.0; Li-Cor).

Immunofluorescence labeling of Cx43

HTM cells or HTM cells transduced with lentiviral Cx43 shRNA or scrambled control shRNA for

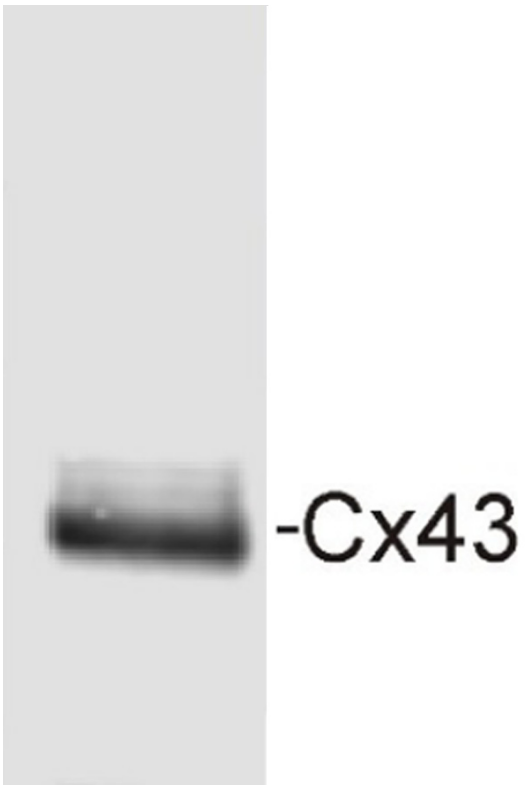

Figure 1. Western blot showing detection of Cx43 in lysates of HTM cells at approximately 43 kDa.

72 h, which then underwent incubation with *E. coli*-conjugated pHrodo red particles for 2 h, were fixed with 2% cold formaldehyde for 10 min, and washed with PBS. Immunofluorescence labelling was conducted as we previously described [23]. Briefly, cells on slides were incubated in 50 mM Tris-HCl, pH 7.4, containing 1.5% sodium chloride (TBS) and 0.3% Triton X-100 (TBSTr) and 5% normal goat serum, for 2 h at room temperature with anti-Cx43 primary antibody (0.25 μg/ml). Slides were then washed for 30 min in TBSTr and incubated for 1 h at room temperature with Alexa Fluor 488-conjugated goat anti-rabbit IgG diluted at 1:1000 (Molecular Probes, Eugene, Oregon). Slides were then covered with antifade medium and examined under an Olympus confocal microscope.

Statistical analysis

Statistical analyses were carried out using either a Student's *t*-test or one-way ANOVA. *P* values < 0.05 were considered significant. Data are presented as the mean number of fluorescence labeled bioparticles/10 TM cells ± SEM.

Results

TM cells express Cx43

We first investigated gap junction protein Cx43 expression in primary cultured HTM cells using our previously validated Cx43 antibody generated against sequences in rodent Cx43 that have high homology with human Cx43 [12, 14, 20]. As shown by western blotting in **Figure 1**, this antibody recognized a clear band migrating at approximately 43 kDa in lysates of HTM cells, which demonstrated the presence of Cx43 in HTM cells using this well characterized anti-Cx43 antibody.

Partial co-localization of Cx43 labeling with pHrodo bioparticles

Consistent with western blot detection of Cx43 in lysates of HTM cells, primary cultures of these cells showed typically high levels of punctate immunofluorescence labeling for Cx43 (**Figure 2A**). It is well established that TM cells can display phagocytic activity and pHrodo labeling is widely used to detect such activity [39]. Using the pHrodo red *E. coli* bioparticle assay, intracellular pHrodo labeling (**Figure 2B**) was detected in HTM cells. Interestingly, the HTM cells displayed partial co-localization of Cx43 labeling with internalized pHrodo (**Figure 2C**, merge).

Lentiviral shRNA mediated knockdown of Cx43 in TM cells reduces the phagocytic activity using pHrodo labeling

Lentiviral Cx43 shRNA constructs were packaged in HEK293 cells, and used to transduce HTM cells for 3 days, followed by tests of phagocytic activity using the pHrodo red *E. coli* bioparticle assay. As shown in **Figure 3**, western immunoblotting revealed that Cx43 was dramatically knocked down in lysates of Cx43 lentivral shRNA-transduced HTM cells compared with control shRNA-transduced HTM cells. By pHrodo labeling assay, results from Cx43 shRNA-treated HTM cells showed significantly ($P < 0.001$) reduced intracellular pHrodo labeling compared with control Cx43 shRNA-treated cells (**Figure 4**).

Discussion

Our results showed that Cx43 is highly expressed in primary HTM cells, and that following phagocytosis of pHrodo by these cells,

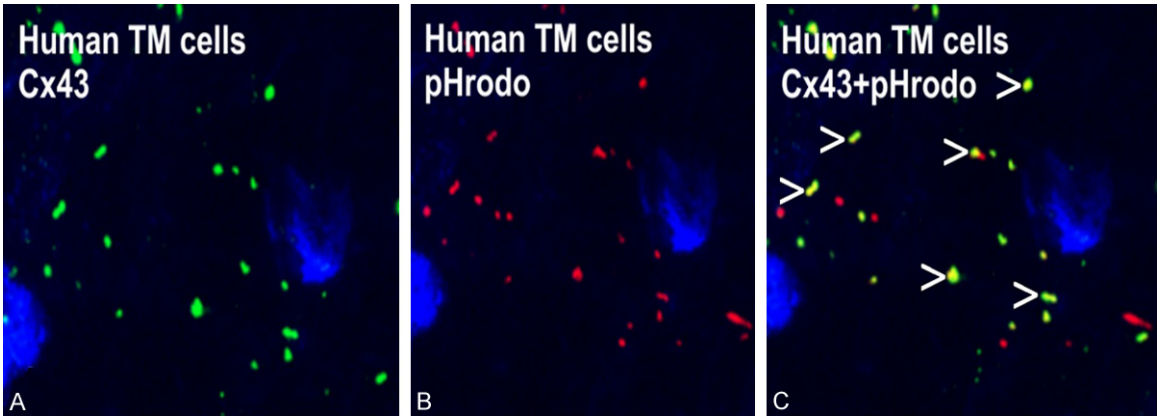

Figure 2. Partial co-localization of Cx43 (A) and pHrodo (B) labeling in human TM cells, with overlay shown in (C) (arrowheads); cell nuclei were labeled with DAPI (blue).

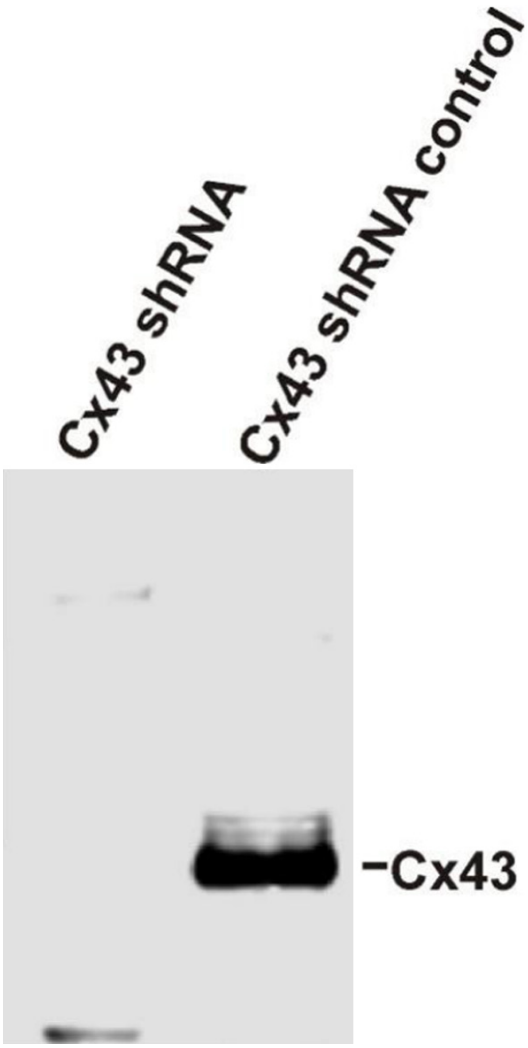

Figure 3. Western blot showing knockdown of Cx43 in HTM cells using lentiviral Cx43 shRNA (lane 1), with scrambled lentiviral Cx43 shRNA used as a control (lane 2).

labeling for pHrodo is partially co-localized with Cx43. Further, HTM cells with Cx43 knockdown using lentiviral Cx43 shRNA showed significantly reduced phagocytic activity, indicating possible involvement of Cx43 in regulation of HTM cell phagocytosis.

A key feature of TM cells is their phagocytic capacity, which allows them to clear cellular debris from aqueous humor. Failure of this activity is a major risk factor for development of glaucoma [3, 24]. Our results provide evidence suggesting that normal expression of Cx43 is required to support the phagocytic activity of TM cells, similar to the reported promotion of phagocytosis by Cx43 in macrophages where it was reported that Cx43 was localized in phagosome of macrophages, demonstrating the involvement of Cx43 in the phagocytosis activity of these cells [11]. In contrast, another study reported that Cx43 was dispensable for phagocytosis by macrophages [38]. This discrepancy could be due to the use of different methods for preparation and isolation of macrophages and detection of phagocytosis activity.

Mechanisms underlying phagocytic capacity of various cell types, including that of TM cells, have been extensively studied with respect to regulation by cell signaling pathways, interactions involving cytoskeletal and scaffolding proteins, such as integrins, transmembrane proteins, actins and actions of drugs such as dexamethasone [9, 10, 25]. Some of these mechanisms involve direct or indirect interactions with integrins or other proteins, which are known to be directly or indirectly associated with Cx43

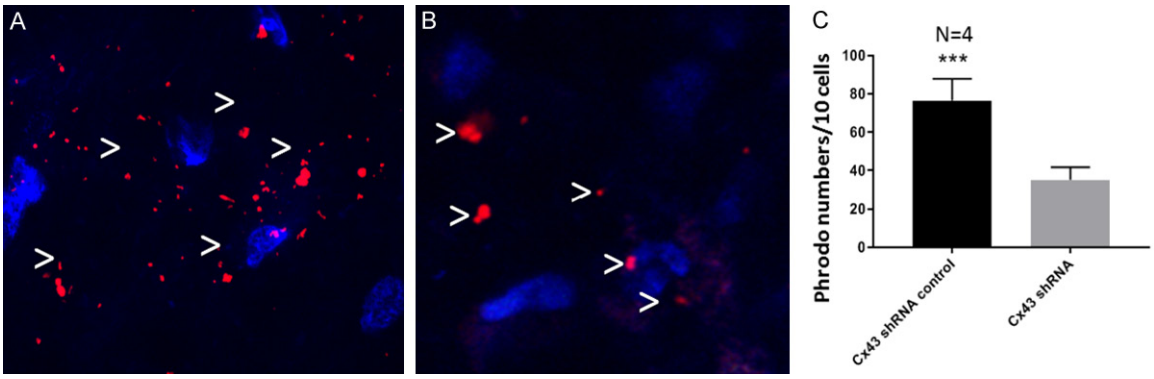

Figure 4. HTM cells treated with control shRNAs show phagocytosis of pHrodo (A, red), while significantly reduced pHrodo phagocytosis is seen in HTM cells treated with Cx43 shRNA (B), as shown quantitatively (C, ***P < 0.001); arrowheads showing representative pHrodo labeling; nuclei were labeled with DAPI (blue).

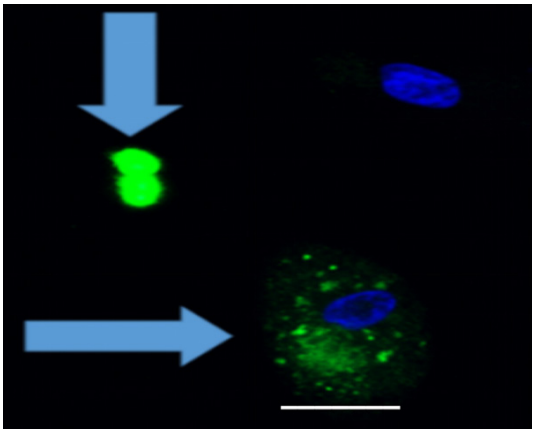

Figure 5. HTM cell showing phagocytosis of pHrodo (green) with horizontal arrow, while vertical arrow shows false positive pHrodo *E Coli* bioparticle (green) outside of HTM cell. DAPI stained nuclei are labeled blue. Scale bar: 20 μm.

[26-30]. Such interactions may underlie events whereby Cx43 has an impact on phagocytic activity, as in Cx43 enhancement of phagocytosis in macrophages after bacterial stimulation [11]. However, it appears that there are discrepancies in dexamethasone actions on phagocytosis in different cellular populations. In human monocytes, dexamethasone promotes phagocytosis [31], while it inhibits phagocytic activity in TM cells [25], which may therefore reflect a cell-type dependent mechanism.

Although it is not clear how Cx43 in HTM cells promotes phagocytic activity, it is well established that pH changes can regulate Cx43 function [32-34]. Since the pHrodo phagocytosis assay is related to pH changes, it is possible that Cx43 may be involved in the uptake of the pHrodo *E Coli* bioparticles. Our results showing that elimination of Cx43 was accompanied by reduced HTM cells phagocytic activity suggests that development of drugs that stimulate expression of Cx43 or its binding partners may provide a means for ameliorating progression of primary open angle glaucoma.

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Disclosure of conflict of interest

None.

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