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Activation of toll like receptor-3 induces corneal epithelial barrier dysfunction

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ABSTRACT

The epithelial barrier is critical in the maintenance of the homeostasis of the cornea. A number of eye disorders are associated with the corneal epithelial barrier dysfunction. Viral infection is one common eye disease type. This study aims to elucidate the mechanism by which the activation of toll like receptor 3 (TLR3) in the disruption of the corneal epithelial barrier. In this study, HCE cells (a human corneal epithelial cell line) were cultured into epithelial layers using as an in vitro model of the corneal epithelial barrier. PolyI:C was used as a ligand of TLR3. The transepithelial electric resistance (TER) and permeability of the HCE epithelial layer were assessed using as the parameters to evaluate the corneal epithelial barrier integrity. The results showed that exposure to PolyI:C markedly decreased the TER and increased the permeability of the HCE epithelial layers; the levels of cell junction protein, E-cadherin, were repressed by PolyI:C via increasing histone deacetylase-1 (HDAC1), the latter binding to the promoter of E-cadherin and repressed the transcription of E-cadherin. The addition of butyrate (an inhibitor of HDAC1) to the culture blocked the corneal epithelial barrier dysfunction caused by PolyI:C. In conclusion, activation of TLR3 can disrupt the corneal epithelial barrier, which can be blocked by the inhibitor of HDAC1.

1. Introduction

Viral infection is a common eye disease. A report indicates that the prevalence of viral keratitis is 0.065% in central China [1]. Adenoviral infection is one of the causative factors of keratitis nummularis [2]. Herpes stromal keratitis causes multiple destructive pathologies of the eye [3]. Although the research in viral infection has advanced rapidly in recent years, the pathogenesis of the viral keratitis is to be further investigated.

A stratified squamous epithelium consisting of three to five nonkeratinized epithelial cell layers covers the surface of the cornea [4]. These epithelial cells are connected each other by intercellular junctions including tight junctions, adherens junctions and desmosomes [5]. The components of the intercellular junctions consist of several proteins, such as occludin, claudin, zonula occludens-1 (ZO-1). The expression of these proteins plays a critical role in the maintenance of the functional status of the corneal epithelial barrier [5,6]. A number of factors have been recognized inducing epithelial barrier functions, such as hypoxia-reoxigenation [7], psychological stress [8], bacterial infection [9] and viral infection [10]. These factors may affect the expression of the tight junction proteins [11], in which the molecular mechanism is not fully understood yet.

Most viruses synthesize double-stranded RNA (dsRNA) during their replication [12]. The dsRNA virus can activate different epithelial cells, including corneal epithelial cells via activating TLR3 [13–15]. Thus, we hypothesize that activation of TLR3 compromises corneal epithelial barrier. The results of this study showed that activation of TLR3 by PolyI:C (polvriboinsine-polyribocyaidylic acid) markedly increased the corneal epithelial barrier permeability by down regulation of the expression of E-cadherin.

2. Materials and methods

2.1. Reagents

The shRNA kits of TLR3 and HDAC1, antibodies of HDAC1, pHDAC1, E-cadherin were purchased from Santa Cruz Biotech (Shanghai, China). The dextran-FITC, parthenolide, PolyI:C and butyrate were purchased from Sigma Aldrich (Shanghai, China). The reagents for RT-qPCR and Western blotting were purchased from Invitrogen (Shanghai, China).

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2.2. Cell culture

Human corneal epithelial cell line HCE cells were obtained from the RIKEN BioResource Center (Tokyo, Japan). The cells were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% FBS (fetal bovine serum), human epidermal growth factor (10 ng/ml), 0.1 mg/ml streptomycin and 100 U/ml penicillin. HCE cells were seeded at a density of 1×10^6 cells per Transwell insert, or at the same densities in 6-well culture plates. The viability of the cells was greater than 98% when used for further experiments as assessing by the Trypan blue exclusion assay.

2.3. Assessment of the corneal epithelial barrier function

The HCE cells were cultured in Transwell system. The trans-epithelial electric resistance (TER) was recorded with an Ohmmeter. When the TER reached or over 1000 Ω cm^2, the HCE layers were regarded confluence and used for assessing the epithelial barrier function. When the confluent HCE layers were stimulated with PolyI:C in the culture, TER was recorded at 0 h and 24 h respectively. Dextran-FITC (4000 kDa) was added to the upper chambers at 1 mg/ml at 0 h; the samples were taken from the basal chambers at 24 h; the fluorescence intensity of the samples was determined with a fluorometer.

2.4. Gene silence of TLR3 and HDAC1

To knock down the genes of TLR3 or HDAC1, HCE cells were treated with shRNA kits of TLR3 or HDAC1 following the manufacturer's instructions. The gene knockdown effect was assessed by Western blotting.

2.5. Western blotting

The total proteins were extracted from the HCE cells, fractioned with SDS-PAGE, and transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk for 30 min, and incubated with the primary antibodies (100 ng/ml) overnight at 4 °C, followed by incubation with the secondary antibodies (conjugated with peroxidase) for 1 h at room temperature. The membranes were washed with TBST (Tris buffered saline Tween 20) after each incubation. The immune blots on the membrane were developed with ECL (enhanced chemilunonescence). The results were photographed with a KOKAK Image Station (4000Pro, KODAK, Shanghai, China). The integrated density of the blots was determined with Photoshop software and presented as a percentage of the internal control β-actin.

2.6. Real time quantitative RT-PCR (RT-qPCR)

The total RNA was extracted from HCE cells with the TRIzol reagent. The cDNA was synthesized with a reverse transcription reagent kit. The qPCR was carried out on a real time PCR device (MiniOpticon, Bio-Rad, Shanghai, China) with the SYBR Green Master Mix following the manufacturer's instructions. The results were presented as the Ct value normalized to a percentage of the internal control β-actin. The primers using in this study are presented in Table 1.

Table 1
Primers using in this study.

Molecules	Forward	Reverse
E-cadherin	cggacgatgatgtgaacacc	ttgctgttgtgcttaacccc
ZO-1	ccagcatcatcaacctctgc	catgcgacgacaatgatggt
Occludin	tttggaccataaccccggaa	atcgtctggggtgtgaaagt
Claudin-8	agagtgtcggccttcattga	agaaggacatcacggaagca
Claudin-10	ggagccgctctgtttattgg	gccagcgagctcttttagac
Claudin-14	caccagctgcctacaaagac	aaactttgtgctggaacccc
HDAC1	attatggacaaggccaccca	gcttgctgtactccgacatg
HDAC2	aggtggctggcttaaggtag	tcattctgcaaactccacgc
HDAC3	acttcgagtactttgcccca	ggcacgtcatgaatctggac
HDAC4	tgggaaacgagcttgatcct	catctggtctcttttcggcg
HDAC5	cagaagttgaacgtgggcaa	gtcctccaccaacctcttca
HDAC6	tgtgctcccaatcctgacat	acgtactcagcactgtgaca

2.7. Chromatin immunoprecipitation

Following the published procedures [16], ChIP was carried out with the HCE cells after treating with PolyI:C. Briefly, the HCE cells were washed with PBS and fixed in 1% formaldehyde (10 ml) at room temperature for 10 min to covalently cross-link any DNA-protein complexes. The cross-link was terminated by adding glycine (570 μl of 2.5 M). After incubating in Mg–NI buffer and Mg–NI-Nonidet P-40 buffer, the cells were centrifuged, the cell pellets were resuspended in lysis buffer and sonicated. Then the samples were centrifuged at 13,000 rpm for 10 min to pellet cell debris, the soluble chromatin was harvested. Immunoprecipitation was performed with IgG or anti-pHDAC1 at 4 °C overnight. Protein A slurry was added to pull down the DNA-protein complexes. Relative binding levels of the E-cadherin promoter were determined by RT-qPCR and normalized to input DNA.

2.8. Statistics

The data are presented as mean ± SD. The difference between two groups was determined by the Student t test, or ANOVA if more than two groups. A $p < 0.05$ was set as a significant criterion.

3. Results

3.1. TLR3 ligand PolyI:C disrupts corneal epithelial barrier function

TLR3 recognizes virus infection and induces viral inflammation, such as in the viral conjunctivitis. Previous reports indicate that activation of TLR3 causes epithelial barrier disruption [17]. Whether TLR3 is involved in the corneal epithelial barrier dysfunction has not been investigated. To this end, we treated the human corneal epithelial (HCE) cell epithelial layers with PolyI:C at gradient concentrations in Transwells, which markedly decreased the TER and increased the permeability to dextran in the epithelial layers in an PolyI:C dose-dependent manner (Fig. 1A and B). The results suggest that TLR3 activation is one of the causative factors inducing corneal epithelial barrier dysfunction. To confirm the role of TLR3 in the PolyI:C induced corneal barrier dysfunction, in separate experiments, we knocked down the TLR3 gene in HCE cells (Fig. 1C) and repeat the above experiments using the TLR3-null HCE cells. The results showed that the epithelial layers did not respond the stimulation of PolyI:C (Fig. 1A and B).

3.2. Activation of TLR3 represses the expression of cell junction protein E-cadherin in HCE cells

The results of Fig. 1 implicate that the activation of TLR3 by PolyI:C may affect the expression of the cell junction associating proteins. To this end, after exposure to PolyI:C, we assessed the mRNA levels of E-cadherin, ZO-1, occludin and claudin (8, 10 and 14) in HCE cells. The results showed that PolyI:C markedly suppressed the expression of E-cadherin, but did not affect the expression of ZO-1, occludin and claudins (Fig. 2A). To enforce the

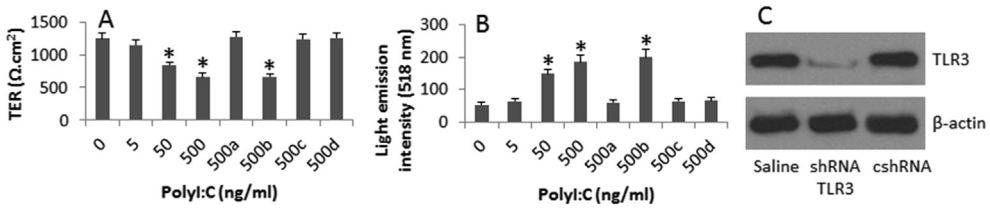

Fig. 1. Assessment of HCE monolayer barrier function. HCE cells were cultured to monolayers in Transwells. PolyI:C and dextran-FITC (4 kDa) was added to the culture at the indicated concentrations. The TER was recorded 24 h later. Samples were taken from the basal chambers at 24 h after the addition of dextran and assessed with a florometer. A, the bars indicate the TER levels. B, the bars indicate the dextran passed through the HCE monolayers. C, results of TLR gene silence. a: TLR3-null HCE cells. b: HCE cells were treated with control shRNA. c: Butyrate was added to the culture at 1 μg/ml d: Parthenolide was added to the culture at 15 μM. The data of bars are presented as mean ± SD. *, $p < 0.01$, compared with the dose "0" group. The data are representative of 3 independent experiments.

results, we observed the effect of PolyI:C on the expression E-cadherin in HCE cells at several time points. The results showed that 12 h after the exposure to PolyI:C, the expression of E-cadherin was not affected much, which was markedly inhibited 24 h after the exposure and was further inhibited 48 h after (Fig. 2B–D); the inhibitory effect was abolished by knocking down the TLR3 gene in HCE cells (Fig. 2D).

3.3. Activation of TLR3 increases HDAC1 phosphorylation in HCE cells

Previous reports indicate that HDAC interferes with the anti-virus activities in the body [18]. HDAC activation is associated with epithelial barrier dysfunction [19]. Thus, we assessed the mRNA levels of HDAC1-6 in HCE cells after exposure to PolyI:C in the culture for 48 h. The results showed the exposure to PolyI:C markedly increased the mRNA levels of HDAC1, but did not alter the mRNA levels of HDAC2-6 (Fig. 3A). We then assessed the protein levels of phosphor (p)HDAC1 in HCE cell extracts. The results showed that the exposure to PolyI:C significantly increased the pHDAC1 protein levels in HCE cells in a PolyI:C time- (B) and dose-dependent manner, which did not occur in TLR3-null HCE cells (Fig. 3C).

3.4. Activation of TLR3 interferes with the gene transcription of E-cadherin in HCE cells

We then further observed that the role of activation of TLR3 by PolyI:C in the E-cadherin gene transcription in HCE cells. After exposure to PolyI:C in the culture, the HCE cells were collected, the extracts were analyzed by ChIP. The results showed that the activation of TLR3 by PolyI: C significantly increased the binding rate of the E - cadherin promoter by pHDAC1. The mRNA levels and protein levels of E-cadherin in the HCE extracts were also altered in parallel to the changes of the binding of pHDAC1/E-cadherin promoter, which was abolished by the knockdown of TLR3 gene in the HCE cells (Fig. 4). The results implicate that the HDAC1 phosphorylation mediates the TLR3 activation-induced E-cadherin suppression in HCE cells. To test the inference, we added HDAC1 inhibitors, butyrate sodium or parthenolide, to the HCE culture in the presence of PolyI:C. Indeed, the PolyI:C-induced HCE epithelial barrier dysfunction was abolished (Fig. 1).

4. Discussion

Epithelial barrier integrity is important in maintaining the corneal homeostasis. The disruption of the epithelial barrier is associated with the pathogenesis of a number of eye diseases. Viral

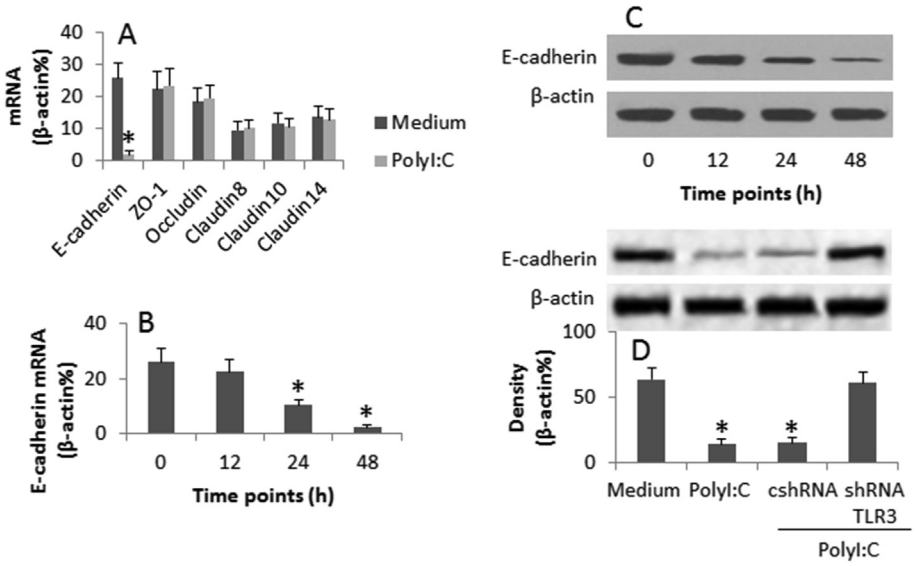

Fig. 2. Activation of TLR3 suppresses E-cadherin in HCE cells. HCE cells were stimulated with PolyI:C (500 ng/ml) in the culture for 48 h. The cell extracts were analyzed by RT-qPCR and Western blotting. A, the bars indicate the mRNA levels of the tight junction proteins denoted on the X axis. B, the bars indicate the mRNA level of E-cadherin in HCE cells at various time points after exposure to PolyI:C in the culture. C, the Western blots indicate the protein level of E-cadherin in HCE cells at various time points after exposure to PolyI:C in the culture. D, the Western blots indicate the protein levels of E-cadherin of HCE cells. The bars below indicate the integrated density of the blots. The data of bars are presented as mean ± SD. *, $p < 0.01$, compared with the medium group. The data are representative of 3 independent experiments.

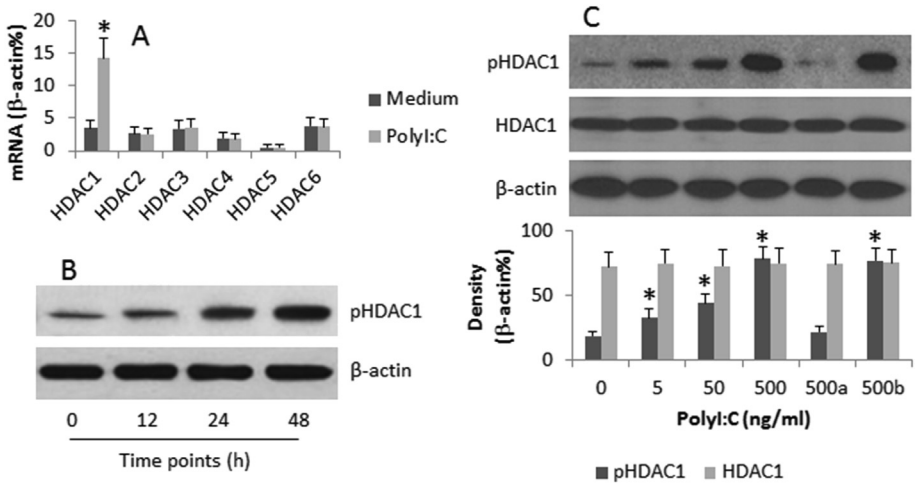

Fig. 3. Activation of TLR3 increases HDAC1 phosphorylation. HCE cells were cultured in the presence or absence of PolyI:C for 48 h. The cell extracts were analyzed by RT-qPCR and Western blotting. A, the bars indicate the mRNA levels of HDAC1-6 when the PolyI:C concentration was 500 ng/ml. B, HCE cells were cultured in the presence of PolyI:C (500 ng/ml) for 0–48 h. Cell extracts were prepared at indicated time points and analyzed by Western blotting. The blots indicate the pHDAC1 levels. C, HCE cells were cultured in the presence of PolyI:C at gradient concentrations as denoted on the X axis for 48 h. The Western blots indicate the protein levels of pHDAC1 and HDAC1. The bars below show the integrated density of the blots. a: TLR3-null HCE cells. b: HCE cells were treated with control shRNA. The data of bars are presented as mean ± SD. *, $p < 0.01$, compared with the medium group (A), or the dose "0" group. The data are representative of 3 independent experiments.

infection is one of the causative factors compromising the epithelial barrier function. Therefore, to elucidate the mechanism by which viral infection compromises the corneal epithelial barrier function is of significance. The present data show that activation of TLR3 markedly compromises the corneal epithelial barrier function by showing the elevation of permeability to a macromolecular tracer, dextran, and down regulation the TER of the HCE layer. The exposure to PolyI:C uniquely suppresses the expression of E-cadherin, one of the tight junction proteins.

The tight junction associated proteins are well studied, including OZ-1, occludin, claudins, E-cadherin, etc. The abnormality of the quantity or quality in any of the proteins may induce the epithelial barrier dysfunction. In modern life, psychological stress is a common causative factor to compromise epithelial barrier function. Yang et al. reported a rat model treated by water-avoided-stress, from which the stress induced significantly intestinal epithelial barrier dysfunction [20]. Liu et al. found that allergic reaction markedly suppressed the expression of claudin-2, one of the tight junction proteins, leading to the hyperpermeability of the epithelial barrier [21]. Hirao et al. observed that infection by HIV induced the tight junction protein ZO-1 mislocation and repressed its expression [22]. Our results are in line with those pioneer studies by showing that exposure to PolyI: C induced significant HCE layer barrier dysfunction via a mechanism of repressing the

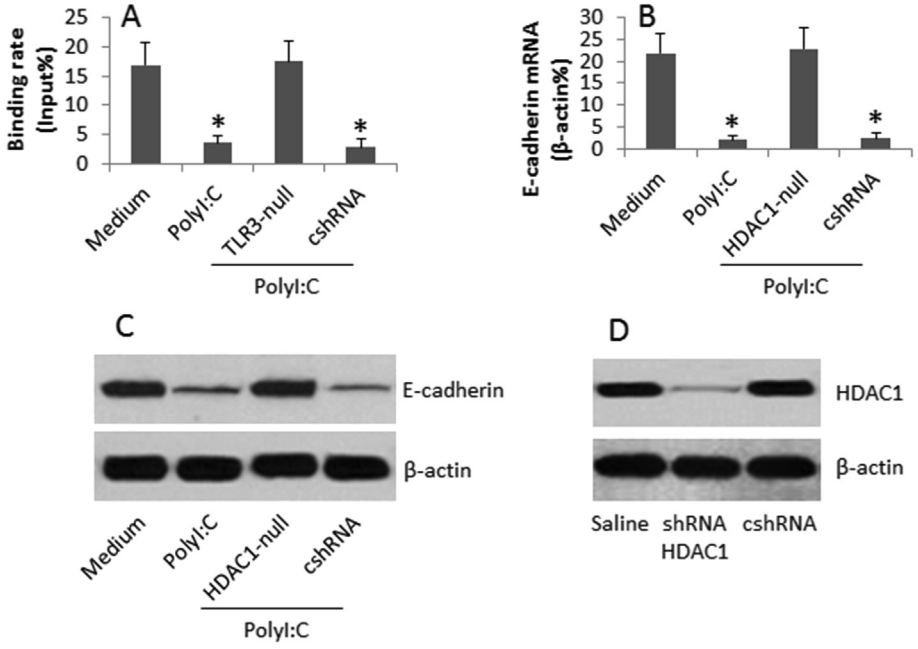

Fig. 4. HDAC1 mediates TLR3 activation-repressed E-cadherin transcription. HCE cells were cultured in the presence of PolyI:C (500 ng/ml) for 48 h. A, the cell extracts were analyzed by ChIP. The bars indicate the rate of pHDAC1 binding to the E-cadherin promoter. B, the bars indicate the mRNA levels of E-cadherin (by RT-qPCR). C, the Western blots indicate the protein levels of E-cadherin. D, the Western blots show the HDAC1 gene silence results. The data of bars are presented as mean ± SD. *, $p < 0.01$, compared with the medium group. The data are representative of 3 independent experiments.

expression of the tight junction protein E-cadherin. Others also suggest that E-cadherin expression is a critical in the maintenance of airway epithelial barrier function, which is markedly suppressed in the airway allergy [23]. Contreras-Ruiz et al. indicate that infection can compromise the corneal barrier integrity by repressing the expression of most tight junction proteins, including E-cadherin [24].

The results show that activation of TLR3 by PolyI:C induces HDAC1 phosphorylation. HDAC1 is one of the deacetylases of histone; its activation usually decreases gene transcription. Our data show that the TLR3 activation-induced pHDAC1 bound the promoter of E-cadherin and repressed the gene transcription of E-cadherin in HCE cells. Supporting data have been reported, Jin et al. indicate that oncogenic STRAP reppresses E-cadherin via promoting HDAC1 phosphorylation [25]. The HDAC1 phosphorylation is also involved in repressing other gene transcription, such as Datta et al. indicate that HDAC1 phosphorylation is involved in the transcription of gastrin [26].

Since the data show that HDAC1 plays a critical role in the suppression of E-cadherin in Corneal epithelial cells, which may be one of the key factors in the compromise of corneal epithelial barrier function during the TLR3 activation. Thus, to block the activity of HDAC1 during viral infection may be beneficial. The subsequent data support this inference. The addition of HDAC1 inhibitor butyrate abolished the PolyI:C-induced HCE barrier dysfunction. Whether this can benefit patients with eye viral infection is worth being further investigated.

In summary, the present data show that activation of TLR3 by PolyI: C induces corneal epithelial barrier dysfunction via increasing HDAC1 phosphrylation, and repressing E-cadherin expression, which can be blocked by the inhibitor of HDAC1.

Conflict of interest

None to declare.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.080.

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