Significance of Ectodomain Shedding of TNF Receptor 1 in Ocular Surface

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Purpose. We evaluated an anti-inflammatory effect of TNF receptor 1 (TNFR1) ectodomain shedding in ocular surface.

Methods. Human corneal epithelial cell (HCEC) was first pretreated by TNF-α. Ectodomain shedding was stimulated by uridine triphosphate (UTP) or peptidoglycan (PGN), with or without shedding inhibition using TNF-α processing inhibitor (TAPI). The phosphorylation of the NF-kB inhibitory protein, IkB, was assessed by Western blotting and concentrations of soluble TNFR1 (sTNFR1) in culture medium were analyzed by ELISA. Tear fluid from patients with Sjögren syndrome and graft-versus-host disease (GVHD) was collected and analyzed by ELISA for sTNFR1 concentration. Five dry eye patients underwent topical treatment using diquafosol sodium eye drops, a purinergic P2Y2 receptor agonist, and the tears of the patients was sampled before and after the treatment for sTNFR1 ELISA.

Results. Phosphorylation of IkB was diminished by adding UTP or PGN, and this down-regulation of IkB phosphorylation was reversed by adding TAPI. In HCEC medium, sTNFR1 release was increased significantly by adding UTP or PGN, and inhibited significantly by adding TAPI. In the tears of the patients with Sjögren syndrome and GVHD, sTNFR1 expression was upregulated. In the tears of the patients with short breakup time (BUT) dry eye, sTNFR1 concentrations (ng/mL) in the tears were 1.30 ± 0.58 ng/mL for the pretreatment baseline, and 1.64 ± 0.70 after treatment, statistically significantly higher than those for the pretreatment (P < 0.01).

Conclusions. Ectodomain shedding of sTNFR1 blocked TNF-α-induced intracellular signaling in corneal epithelium. The upregulation of sTNFR1 in inflamed ocular surfaces suggests an anti-inflammatory role of sTNFR1 ectodomain shedding at the ocular surface.

Keywords: cytokine receptor, ectodomain shedding, TNF receptor

The signal transduction of TNF-α is transmitted by two functionally distinct receptors, TNF receptors 1 (TNFR1) and 2 (TNFR2).1 The TNF-α and TNF receptors can be processed and released to the extracellular region as soluble form by metalloproteinase TACE (TNF-α converting enzyme) or ADAM17 (a disintegrin and metalloproteinase). This process, termed ectodomain shedding or shedding, generates soluble protein and changes the characteristics of the substrate.2 Ectodomain shedding of cytokines and cytokine receptors has a major role in establishing the balance between inflammation and host defense, as exemplified by TNFRI, which is critical to inflammatory progression.3-5 The TNFR1 ectodomains released in the extracellular space chelate sTNF-α, providing negative feedback to the TNF-α-induced inflammatory loop.6

Recent report elucidate that ligation of TNFRI by TNF-α leads to elevated cytosolic and mitochondrial Ca2+ levels via inositol-1,4,5-triphosphate release, which induce reactive oxygen species (ROS) production by mitochondria to promote ADAM17 activation at the cell surface.3 This process may stimulate ectodomain shedding of TNFR1 and, thus, is considered as one of the possible mechanisms that causes negative feedback of TNF-α signaling. Plus, upregulation of mitochondrial Ca2+ releases ATP to stimulate purinergic receptor P2Y2, which prolongs upregulation of cytosolic and mitochondrial Ca2+, and TNFR1 ectodomain shedding.

Previously, we showed that TACE and TNFR1 were expressed in corneal cells, and showed that soluble TNFR1 (sTNFR1) was generated from corneal epithelium by TACE-dependent ectodomain shedding.7,8 However, because those metalloproteinases also possess the ability to degrade extracellular matrix during tissue turnover, the significance of TNFR1 release by ectodomain shedding in the cornea is not yet established. In this study, we focused on the effect of sTNFR1 shedding from cultured corneal epithelium cells by studying whether TNF-α signal transduction is inhibited by TNFRI shedding on corneal epithelium cells. Furthermore, we evaluated the concentrations of sTNFR1 in tear fluid of patients with inflammatory diseases on ocular surface, and investigated whether sTNFR1 is generated by ectodomain shedding in human ocular surface by applying P2Y2 receptor agonist to the dry eye patients.

Materials and Methods

The tenets of the Declaration of Helsinki were followed in this work. The study using tears was approved by the Institutional Review Board.
**Stimulation of Cultured Corneal Epithelial Cell**

The SV40-transformed human corneal epithelial cell (HCEC) line was purchased from American Type Culture Collection (Manassas, VA). This cell line was seeded in 24-well plates and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and antibiotics at 37°C, in a 5% CO2 incubator (CPE-1201; Hirasawa Works, Tokyo, Japan). After reaching to 80% subconfluent condition, each well was pretreated by recombinant TNF-α for 30 minutes (10 ng/mL; R&D Systems, Inc., Minneapolis, MN, USA). Subsequently, each well was treated for 1 hour with uridine triphosphate (UTP, 100 μM; Sigma-Aldrich, St. Louis, MO, USA) or 24 hours with peptidoglycan (PGN, 100 μg/mL; Sigma-Aldrich) dissolved in serum-free DMEM. In experiments using TACE inhibitor, TNF-α processing inhibitor-1 (TAPI-1, 250 ng/mL, 30 minutes; EMD Chemicals, Darmstadt, Germany) was dissolved in serum-free DMEM and added 30 minutes before addition of UTP or PGN described above.

**ELISA Test**

After treatment, the culture medium from each well was sampled and analyzed by ELISA for soluble TNFR1 (R&D Systems, Inc.). Those experiments were repeated three times separately and the statistical analysis was performed using Student’s t-test.

**Western Blotting**

After treatment, the cells were lysed in a sample buffer (NuPAGE LDS Sample Buffer; Life Technologies, Grand Island, NY, USA) containing 1% mercaptoethanol, boiled for 3 minutes, and subjected to Western blot analysis. An SDS-PAGE assay was conducted using MULTIGEL II Mini (Cosmo Bio, Tokyo, Japan). After SDS-PAGE, proteins were transferred electrophoretically to a hydrophobic polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA). The membrane was incubated at room temperature (RT) for 1 hour in 1% Tris-buffered saline Tween 20 (TBST) containing 4% skimmed milk (Block Ace; DS Pharma Biomedical, Osaka, Japan), and then incubated at 4°C for overnight in TBST containing primary antibody (rabbit antihuman Phospho-IkBα antibody and total-IkBα antibody; 1:1,000 dilution; Cell Signaling Technology, Beverly, MA, USA). The membrane then was incubated at RT with shaking for 30 minutes in TBST containing the secondary antibody (Phototope-HP Western Blot Detection System, Anti-rabbit IgG, HRP-linked antibody, 1:2000; Cell Signaling Technology). Each step was followed by extensive washing in TBST. Subsequently, the membrane was washed three times for 10 minutes at RT with ×1 TBST. Antigen detection was achieved by incubation of the membrane for 1 minute at RT with a chemiluminescent substrate according to manufacturer’s instruction (Phototope-HP Western Blot Detection System; Cell Signaling Technology). Chemiluminescent image was photographed by LAS-3000 (Fujifilm, Tokyo, Japan), and the signal intensity of individual spots was determined by densitometry using the Gel-Pro analyzer software (Media Cybernetics, Silver Spring, MD, USA). The ratio of total IkBα and phospho-IkBα was calculated and the statistical analysis was performed using Student’s t-test. This experiment was performed three times separately.

**Measurements of sTNFR1 Concentration in the Tear Fluid of Patient With Dry Eye**

Keratoconjunctivitis sicca caused by Sjögren syndrome is now regarded as chronic inflammation of the ocular surface. Various proinflammatory cytokines, including TNF-α and metalloproteinase, are reported to be upregulated in the tear fluid of patients with Sjögren syndrome.

Furthermore, previous reports suggest the strong relationship between graft-versus-host disease (GVHD)-related dry eye and extent of inflammation, evidenced by the upregulation of proinflammatory cytokine in tears and inflammatory cells infiltration in conjunctiva of GVHD patients. Therefore, to investigate expression of sTNFR1 by the inflamed human ocular surface, we collected tear fluid samples from patients with primary Sjögren syndrome (24 eyes, 12 patients) and patients with GVHD-related dry eye (8 eyes, 4 patients). Tear fluid samples from healthy volunteers (20 eyes, 10 individuals) served as normal controls.

Tears were sampled by the Schirmer I method using filter paper (Schirmer Tear Production Measuring Strips; Showa Yakuhin Kako, Tokyo, Japan). After sampling, the Schirmer strips were stored at −20°C until needed. Each Schirmer strip with a sample was thawed and eluted overnight, at room temperature, with 0.5 M NaCl and 0.5% Tween 20 containing 0.05 M PBS (pH 7.2) solution. The amount of tears was calculated by diluting each 1 mm on a wet Schirmer strip to form 1 μL of tear volume with the end concentration of the elution solution containing a 40-fold diluted tear sample. Concentrations of sTNFR1 in tear samples were determined using an ELISA kit (R&D Systems, Inc.). Statistical analysis was performed using a Kruskal-Wallis H test; P values of <0.05 were regarded as significant.

**Measurements of TNFR1 Concentration in the Tear Fluid After Topical Application of P2Y2 Receptor Agonist**

The P2Y2 receptor agonist eye drops, diquafosol sodium (Diquas; Santen Pharmaceutical Co., Osaka, Japan), promotes aqueous and mucin secretion from conjunctival epithelium and goblet cells, respectively. It has been shown to be effective for the treatment of dry eye diseases by extending tear break-up time (BUT) and increasing tear volumes. On the other hand, it has been shown that P2Y2 receptor may have the possible involvement in TNFR1 ectodomain shedding, as described above. Therefore, we investigated the effect of diquafosol sodium in disposition of sTNFR1 concentration in the tear fluid. The subjects who had dry eye symptoms, BUT ≤ 5 seconds, and minimal corneal and/or conjunctival epithelial damage were recruited. Because diquafosol sodium eye drops is reported to be effective in those with short BUT type of dry eye. Nine eyes of five dry eye patients who met those criteria underwent topical treatment using diquafosol sodium eye drops 4 times per day, and the tear fluid of the patients was sampled before and 4 weeks after the treatment.

**Results**

**sTNFR1 Release From Cultured Corneal Epithelial Cells Is Upregulated by Uridine Triphosphate and Peptidoglycan**

In the culture medium of HCEC, the concentration of sTNFR1 was increased significantly by adding UTP (P < 0.05), and this increased concentration of sTNFR1 also was inhibited by TAPI-
Because UTP is one of the P2Y agonists, this result indicates possible involvement of P2Y agonist in TNFR1 ectodomain shedding. Additionally, the concentration of sTNFR1 was increased significantly by adding PGN (P < 0.05), and this increased concentration of sTNFR1 was inhibited significantly by adding TAPI-1 (P < 0.05), which is consistent with our previous report (Fig. 2).

![Figure 1](image1.png) Figure 1. Relationship between TNFR1 ectodomain shedding and phosphorylation of the endogenous NF-kB inhibitor IkBz using UTP. Ectodomain shedding of TNFR1 is stimulated by UTP, with or without ectodomain shedding inhibitor, TAPI. The UTP stimulation significantly upregulates sTNFR1 release in HCEC culture medium, whereas addition of UTP downregulates phosphorylation of the endogenous NF-kB inhibitor IkBz induced by TNF-α. The upregulated release of sTNFR1 is significantly inhibited by TAPI, and inhibition of ectodomain shedding by TAPI reverses the TNF-α-induced phosphorylation (activation) of IkBz (phospho-IkBz). Statistical analysis is performed using unpaired Student’s t-test. * P < 0.05.

![Figure 2](image2.png) Figure 2. The PGN induces ectodomain shedding of TNFR1 and downregulates IkBz phosphorylation. Ectodomain shedding of TNFR1 is stimulated by PGN in the presence or absence of TAPI. The PGN stimulation significantly upregulates sTNFR1 release in HCEC culture medium. On the other hand, IkBz phosphorylation is diminished by PGN. The sTNFR1 release in culture medium and downregulation of IkBz phosphorylation (phospho-IkBz) are reversed by addition of ectodomain shedding inhibitor, TAPI. * P < 0.05.

![Figure 3](image3.png) Figure 3. The sTNFR1 concentration is upregulated in tears of inflammatory disease on ocular surface. In the tear fluid of the patients with Sjögren syndrome and GVHD, sTNFR1 expression is significantly upregulated (Sjögren syndrome, 1.92 ± 0.90 ng/mL; GVHD, 8.24 ± 3.81 ng/mL; normal control, 0.34 ± 0.25 ng/mL; average ± SD) compared with normal control. * P < 0.05, ** P < 0.01.

Intracellular Signaling of TNF-α Is Blocked by Addition of Uridine Triphosphate and Peptidoglycan

We next examined the effect of TNFR1 ectodomain shedding on TNF-α-induced signaling by NF-kB pathways. Western blotting analysis revealed that addition of UTP or PGN inhibited the phosphorylation of the endogenous NF-kB inhibitor IkBz induced by TNF-α (P < 0.05), whereas inhibition of ectodomain shedding by TAPI reversed the TNF-α-induced phosphorylation of IkBz (P < 0.05). Therefore, it can be said that release of TNFR1 by ectodomain shedding is able to block TNF-α signaling.

sTNFR1 Concentration Is Upregulated in Tears of Inflammatory Disease on Ocular Surface

In the tear fluid of the patients with Sjögren syndrome and GVHD, sTNFR1 expression was significantly upregulated (Sjögren syndrome, 1.92 ± 0.90 ng/mL; GVHD, 8.24 ± 3.81 ng/mL; normal control, 0.34 ± 0.25 ng/mL; average ± SD) compared with normal control (Fig. 3). Therefore, it can be said that TNFR1 ectodomain shedding is upregulated to inhibit excessive proinflammatory signal in inflammatory disease on ocular surface.

![Figure 4](image4.png) Figure 4. The sTNFR1 concentration is upregulated in tears of patients with short BUT dry eye. In the tear fluid of the patients with short BUT dry eye, sTNFR1 concentrations (ng/mL) in the tears were 1.30 ± 0.58 ng/mL for the pretreatment baseline, and 1.64 ± 0.70 after diquafosol sodium treatment, statistically significantly higher than those for the pretreatment in five patients (P < 0.01, Fig. 4). This result indicates the possible involvement of P2Y2 receptor agonist in the ectodomain shedding process on the ocular surface.

**DISCUSSION**

In this study, we revealed that the stimulation of sTNFR1 ectodomain shedding blocked TNF-α-induced intracellular signaling in the corneal epithelium. The release of sTNFR1 from ocular surfaces suggests an anti-inflammatory role of sTNFR1 ectodomain shedding at the ocular surface.
These results suggested the physiological significance of sTNFR1 ectodomain shedding in the ocular surface. Whereas TNF-β expression is limited to hematocytes, TNFR1 is expressed ubiquitously in almost all type of cells. The TNFR1 and TNFR2 receptors share only approximately 30% homology in their extracellular domain and no homology in their intracellular domain. In contrast to TNFR1, TNFR2 has no death domain on its intracellular region, suggesting activation of different downstream transduction pathways. The TNFR1 activation leads to activation of the transcriptional factor AP-1 and NF-kB, subsequently leading to the release of proinflammatory cytokines, chemokines, adhesion molecules, and matrix metalloproteinases. Furthermore, TNFR1 signal also leads to the activation of caspase 8 to trigger the apoptotic process. Therefore, TNFR1 is considered to have an important role in the regulation of various inflammatory conditions. Indeed, the level of sTNFR1 in biological fluids had been suggested as a useful biomarker in various inflammatory diseases, such as sepsis, acute lung injury, myocardial infarction, diabetic nephropathy, and GVHD. Impaired TNFR1 ectodomain shedding from the cell surface has been proposed to be responsible for an autoimmune disease, termed TNFR1-associated periodic syndromes (TRAPS). Therefore, these evidences suggest the pivotal role of ectodomain shedding of TNFR1 in inflammatory conditions.

Our in vitro results indicated that TNFR1 shedding may act as a negative feedback mechanism to regulate the TNF-α-TNFRI signaling pathway on the ocular surface, evidenced by the downregulation of NF-kB inhibitor IkBα phosphorylation by stimulating ectodomain shedding in corneal epithelial cells. Although we only used TAPI for the inhibition of ectodomain shedding in this in vitro study, we previously showed that TIP-3, an inhibitor of the ADAM family, also downregulated TNFR1 ectodomain shedding. Furthermore, TNF-α is also processed by ADAM-mediated ectodomain shedding. However, we previously indicated that the target protein of ectodomain shedding in the corneal epithelium is the TNF receptor, rather than TNF-α. Therefore, it can be said that the existence of sTNFR1 in tears of patients with inflamed ocular surface diseases is consistent with these in vitro results.

The ADAM-mediated ectodomain shedding is constitutive and inducible, dependent on G-protein coupled receptors, intracellular Ca²⁺ levels, protein kinase C, membrane lipid composition, and other experimental and natural stimuli. However, it is challenging to induce or stimulate ectodomain shedding or metalloproteinase function in the practical clinic. The P2Y2 receptor is one of the G-protein coupled receptors and P2Y2 receptor agonist eye drops are shown to increase intracellular Ca²⁺ levels in corneal and conjunctival epithelial cells. Therefore, we applied P2Y2 receptor agonist eye drops, which had been commercially available for the treatment of dry eye patients in Japan, to patients with short BUT dry eye. The concentration of sTNFR1 was significantly upregulated by applying P2Y2 receptor agonist eye drops for 4 weeks. This result, showing the possible relationship between P2Y2 receptor stimulation and TNFR1 shedding, was consistent with the investigation by Rowlands et al., and may indicate future application of P2Y2 receptor agonist eye drops to inflammatory diseases on ocular surface.

During the past decade biologic therapies, such as monoclonal antibodies and fusion proteins, have applied to various autoimmune diseases and autoinflammatory syndrome. By targeting key cytokines, cytokine receptors, and immune cells biologics have provided more specific therapeutic interventions. Several cytokine receptors have attracted attention to their application in biologic therapies, such as etanercept (humanized soluble TNFR2), anakinra (anti-IL-1R antibody) and tocilizumab (anti-IL-6R antibody). However, in ocular surfaces, the clinical applications of those biologic therapies, or rather, the pathophysioligic significances of cytokine receptors are not yet established. We believe that our present study shed light on physiologic significance of TNFR1 shedding in ocular surface.

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References


