Regulation of the Receptor for TNFα, TNFR1, in Human Conjunctival Epithelial Cells

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Purpose. Previous studies demonstrated that mast cell–derived TNFα stimulation is critical to the upregulation of intercellular adhesion molecule (ICAM)-1 on human conjunctival epithelial cells (HCECs), which is an important feature of ocular allergic inflammation. Shedding of TNFR1 by TNFα-converting enzyme (TACE) is a primary mechanism for the regulation of TNFα-mediated events. This process has not been examined in HCECs. In this study, the authors examined the regulation of TNFR1 expression and shedding by TACE on primary HCECs and the IOBA-NHC conjunctival epithelial cell line.

Methods. Primary human conjunctival mast cells and epithelial cells were obtained from cadaveric conjunctival tissue. HCECs were incubated with and without activators (IgE-activated mast cell supernates, phorbol myristate acetate [PMA; to activate TACE], TNFα, and IFNγ [to upregulate TNFR1]) for 24 hours. Pretreatment with the TACE inhibitor TAPI-2 was used to inhibit shedding of TNFR1. Supernates collected from the incubations were analyzed with ELISA for soluble TNFR1 (sTNFR1). With the use of flow cytometry, cells were harvested from these experiments for analysis of TNFR1 and ICAM-1 receptor expression.

Results. IgE-activated conjunctival mast cell supernates upregulated the expression of TNFR1. TAPI-2 inhibited the PMA-induced release of sTNFR1 receptor and enhanced the surface expression of TNFR1 in HCECs in a dose-dependent manner. Upregulation of TNFR1 expression by priming with TAPI-2 and IFNγ resulted in enhanced ICAM-1 expression in response to TNFα stimulation (significant change in the slope of the dose–response curve).

Conclusions. These results demonstrate that TACE promotes TNFR1 shedding in HCECs and that TNFR1 expression may be a more significant target than TNFα for intervention in ocular inflammation. (*Invest Ophthalmol Vis Sci.* 2008;49:3992–3998) DOI:10.1167/iovs.08-1873

The proinflammatory cytokine TNFα is believed to play an important role in ocular surface inflammation such as conjunctivitis (allergic, bacterial, and viral) and dry eye disease.[1–3] However, the regulation of TNFα on the human conjunctival surface has not been studied. It has been demonstrated in vitro that HCECs respond to recombinant TNFα with proinflammatory responses consistent with those observed in vivo, such as the upregulation of intercellular adhesion molecule (ICAM)-1 expression and increased amounts of the chemokine IL-8. However, regulation of the receptor for TNFα has not been studied in HCECs.

In ocular allergic inflammation, conjunctival mast cells are an important source of TNFα. We have previously demonstrated that IgE-activated human conjunctival mast cell supernates upregulate the expression of ICAM-1 on HCECs by a mechanism specifically dependent on TNFα released from the conjunctival mast cell.[4] Our work has further suggested that conjunctival mast cell supernates render HCECs more sensitive to TNFα-mediated ICAM-1 upregulation. In these experiments, we demonstrated that TNFα in mast cell supernates could promote ICAM-1 upregulation at log (10^{-3}) lower concentrations than recombinant TNFα alone.[4] We hypothesized that IgE-activated conjunctival mast cell supernates upregulate the expression of TNFR1 on HCECs and that the upregulation of TNFR1 expression results in increased sensitivity to TNFα-mediated activation responses (e.g., upregulation of ICAM-1).

Our first experiments confirmed the first part of our hypothesis, demonstrating that supernates from IgE-activated conjunctival mast cells upregulate the expression of TNFR1 on primary HCECs. However, because purified conjunctival mast cell supernates require large amounts of cadaveric conjunctival tissues, we sought an alternative mechanism to upregulate TNFR1 expression and to test the biological significance of changes in expression of this receptor. Although TNFR1 expression has not been studied in HCECs, in other types of cells it has been demonstrated that TNFR1 is constitutively expressed and that it can, in some cases, be induced at the transcriptional level by IFNγ.[5] TNFR1 is primarily regulated posttranscriptionally by the metalloprotease, TNFα-converting enzyme (TACE; also known as a disintegrin and a metalloprotease [ADAM]-17). TACE-mediated proteolytic cleavage of TNFR1 to its soluble form (sTNFR1) is one important mechanism for the downregulation of TNFα-mediated inflammatory responses.[6] TACE is naturally inhibited in vivo by tissue inhibitor of metalloprotease-3 (TIMP-3) and can be inhibited pharmaceutically by a variety of compounds. For the present study, we examined the mechanisms of TNFR1 expression and shedding in HCECs (primary and IOBA-NHC cell line) using the phorbol ester phorbol myristate acetate (PMA), which activates the protein kinase C pathway (a common pathway of HCEC activation) and stimulates reactive oxygen species (known to activate TACE) and the pharmaceutical TACE inhibitor TNFα protease inhibitor-2 (TAPI-2). With these tools we were able to examine the second part of our hypothesis, which demonstrates how changes in surface expression of TNFR1 affect the threshold of responsiveness to TNFα.

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METHODS

Reagents and Solutions

Collagenase (type I), hyaluronidase (type I-S), trypsin-EDTA, 2-(hydroxy ethyl) piperazine-N′-(2-ethane sulfonic acid) (HEPES), Triton X-100, density gradient (Percoll; Sigma Chemical, St. Louis, MO), toluidine blue, trypan blue, gentamicin, penicillin/streptomycin, amphotericin, PMA, dimethyl sulfoxide, and bovine serum albumin (BSA), were obtained from Sigma Chemical. Hanks basic salt solution (HBSS; without Ca^{2+}, Mg^{2+}, or phenol red) and newborn calf serum were obtained from Sigma Chemical or Life Technologies (Rockville, MD). TNFα protease inhibitor-2 (TAPI-2) was obtained from Peptides International (Louisville, KY).

Media (EpiLife) for primary cell culture and defined trypsin inhibitor were obtained from Cascade Biological (Portland, OR). Fibronectin/collagen (FNC Coating Mix) was obtained from AthenaES (Baltimore, MD). Wright staining was performed with a staining kit (Diff-Quik; Baxter Scientific Products, McGaw Park, IL). Recombinant human TNFα and IFNγ were obtained from Genzyme Diagnostics (Cambridge, MA).

The Tyrode physiological salt solution plus gelatin (TG) used in these studies consisted of 137 mM NaCl, 2.6 mM KCl, 0.35 mM NaH_2PO4, 11.9 mM $NaHCO_3$, 5.5 mM glucose, and 1 g/L mM gelatin adjusted to pH 7.4 with HCl. TGCM is TG with added $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM). The density gradient (Percoll; Sigma Chemical) stock solution was prepared by mixing the commercial solution and 10× HEPES buffer plus dH_2O to obtain an osmolality of 285 mOsm/kg H_2O. The desired density of the gradient was prepared by mixing the stock solution with TG.

IOBA-NHC (normal human conjunctiva) cell line was derived from normal human conjunctival epithelium (Instituto de Oftalmobiología Aplicada [IOBA; University of Valladolid, Spain] Spanish Patent and Trade Mark Office register number M 2.537.742).[7]

Culture media for IOBA-NHC cells were prepared by supplementing Dulbecco modified Eagle medium (DMEM) nutrient mixture (F-12 Ham) with mouse EGF (2 ng/mL), bovine insulin (1 μg/mL), cholera toxin (0.1 μg/mL), hydrocortisone (5 μg/mL), and 10% heat-inactivated fetal bovine serum (all obtained from Sigma Chemical).

Mast Cell and Primary Epithelial Cell Isolation, Purification, and Culture

Modifications of previously reported methods for obtaining purified conjunctival mast cells and primary HCECs in monodispersed suspension were used in these studies.[8] Briefly, human conjunctival tissue was obtained with prior consent from organ/tissue donors (8–10 sets of tissue per experiment were obtained through the Lion's Eye Bank of Wisconsin, Madison, WI, and the Kansas Eye Bank and Cornea Research Center, Wichita, KS, and were approved by the University of Wisconsin Human Subjects Committee). Upper and lower bulbar conjunctiva aseptically collected within 8 hours after death (average time, 4.5 hours) were transported in corneal preservation medium (Dexsol; Chiron Ophthalmics, Irvine, CA) and were stored at 4°C for up to 5 days. Eight to 10 sets of tissue weighing 4 to 5 g were used per experiment. Hyaluronidase and collagenase were used to digest tissue. The digestion process (30 minutes at 37°C on a rotating shaker) was first performed at a low concentration of enzymes (2 digests at 200 U/g in 10 mL final volume), followed by tissue digestion at a high concentration of enzymes (3–6 digests at 2000 U/g in 10 mL final volume). Each digest was followed by washing of the enzyme-treated tissue (with TGCM) over a 100-μm nylon mesh filter to collect freed cells. After the digestion procedure, the freed cells were pelleted, pooled, resuspended in TG, and layered over a single-density gradient (Percoll [Sigma Chemical]; 1.041 g/mL) and centrifuged (500*g*, 20 minutes). The resultant top cell layer (epithelial cells) was harvested, washed, and resuspended in media (EpiLife; Cascade Biological) without hydrocortisone, at a concentration of 1×10^6 cells/mL, and was transferred to fibronectin/collagen (FNC Coating Mix; AthenaES)–coated 24-well plates (0.5 mL/well) for culture at 37°C. Media were changed every 48 hours until confluence. Primary HCECs were passaged (10 passages) using trypsin-EDTA and a serum-free defined trypsin inhibitor. The mast cell–enriched pelleted cells were washed in TG, resuspended to a concentration of 1×10^6 cells/mL in mast cell culture medium, and transferred to a 24-well plate (0.5 mL/well) for an equilibration period (up to 72 hours at 37°C). After the equilibration period, the cell suspension was removed from the plate, layered over a double-density gradient (Percoll [Sigma Chemical]; 1.08 g/mL layered over 1.123 g/mL), and centrifuged (500*g*, 20 minutes). Purified conjunctival mast cell suspensions (>90%) harvested from the interface between the densities were washed and allowed to equilibrate in mast cell culture medium for 24 hours at 37°C before experimentation. Differentiation of other cells was obtained using Wright-stained cytospins.

IOBA-NHC Epithelial Cell Line Culture

IOBA-NHC cells were cultured in the media defined (see Reagents and Solutions) until almost confluent. Media were changed every 2 days. Cells were passaged using incubation with trypsin-EDTA, which was neutralized using DMEM supplemented with 20% newborn calf serum. For storage, the cells were frozen in media containing 20% heat-inactivated fetal bovine serum (FBS) and 10% dimethyl sulfoxide using a cell-freezing container (Mister Frosty; Nalgene Nunc International, Rochester, NY) and stored at −80°C. When needed, cells were quickly thawed in a 37°C water bath and transferred to culture media including antibiotics and extra FBS (20%). After 24 hours, the media were changed to regular culture media.

Challenge of Mast Cells

All mast cell challenges were conducted in TGCM at a concentration of 1×10^4 mast cells/mL (in duplicate). Purified conjunctival mast cells were challenged with anti-IgE antibody at a dose of 10 μg/mL (previously determined to induce mediator release from purified mast cells) or buffer (spontaneous control) for 90 minutes at 37°C.[9,10] The resultant supernates were harvested and stored at −80°C until time of use.

Treatment and Analysis of HCEC Monolayers

HCECs were cultured until almost confluent (24–48 hours after passage) on 24-well plates (3–5 passages for primary HCECs). When mast cell supernates were used, the supernates (in TGCM buffer) were combined 1:1 with media and added to the wells (thus diluted 1:2) and incubated with HCEC monolayers (0.5 mL/well, 2–4 wells/treatment) for 24 hours. Unstimulated HCEC controls were incubated in a 1:1 mixture of TGCM/media for evaluation of constitutive ICAM-1 and TNFR1 expression.

In experiments examining the mechanisms of TNFR1 shedding, similar monolayers were prepared on 24-well plates. To investigate the role of endogenous TACE, TACE inhibitory compounds, TAPI-2 (10 and 100 mM), and EDTA (0.5 and 5 mM) were preincubated with the cells for 15 minutes at room temperature before activation with PMA (10 ng/mL) for 24 hours (0.25 mL/well, 4–6 wells/treatment). Where the effect of TAPI-2 and IFNγ priming on the dose–response curve to TNFα was being examined, the cells were preincubated with TAPI-2 (100 mM) as described, followed by 24-hour incubation with IFNγ (0.5 ng/mL). The cells were then washed and stimulated for 24 hours with TNFα (0.5, 5, and 50 ng/mL).

After 24-hour incubation with the various treatments, supernates were harvested and stored at −80°C for evaluation of sTNFR1 and sICAM-1 by ELISA. HCEC monolayers were harvested with trypsin-EDTA and resuspended in buffer. Cell counts were performed on the harvested cells using a Coulter counter (model ZM; Coulter Corp., Miami, FL) to confirm that cell counts did not vary significantly between cultures. To simultaneously measure ICAM-1 and TNFR1, each tube of 100 μL viable cells (not fixed) was stained with mouse anti–human ICAM-1(CD54)-APC–conjugated antibody and mouse anti–human TNFR1-PE–conjugated antibody or isotype-matched controls using the manufacturer's recommended amounts (10 μL/tube). Stained

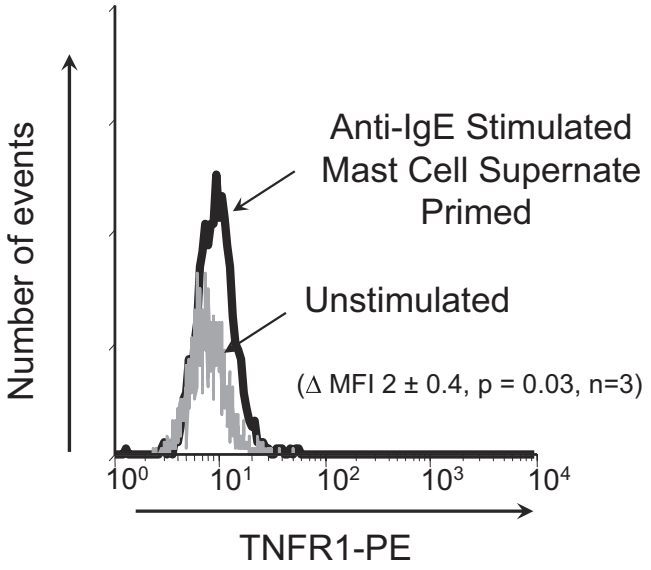

FIGURE 1. Representative overlay histograms ($n = 3$) showing upregulation of staining for TNFR1 (*x*-axis) on primary HCECs after incubation with supernates from anti-IgE–stimulated purified conjunctival mast cells (*black*) compared with unstimulated HCECs (*gray*). MFI was increased by 2 ± 0.4 units ($P < 0.05$).

cells were incubated on ice for 30 minutes, washed, and resuspended in 300 μL/tube of buffer for analysis. Propidium iodide was added to each tube to determine viability. Resultant histograms were analyzed geometrically and based on gating of live cells only.

Statistical Analysis

Data were analyzed using statistical software (Minitab, State College, PA). A general linear model analysis of variance with preplanned comparisons was used to generate two-tailed *P* values. $P < 0.05$ was considered statistically significant. Unless otherwise stated, all data are presented as the mean ± SEM of three to seven separate experiments.

RESULTS

Mast Cell Upregulation of TNFR1

The representative overlay histograms ($n = 3$) in Figure 1 demonstrate that stimulation of primary HCECs with supernates from IgE-activated conjunctival mast cells significantly upregulated the expression of TNFR1 (increase of 2 ± 0.4 mean fluorescence intensity [MFI] units; $P = 0.03$).

TAPI-2 Inhibition of sTNFR1 Release

The goal of these experiments was to demonstrate that TAPI-2 inhibition of TACE resulted in the inhibition of shedding of sTNFR1. If positively demonstrated, we could then use TAPI-2 to increase the expression of TNFR1. Because our hypothesis was to demonstrate that increased expression of TNFR1 would result in increased sensitivity to TNFα-stimulated ICAM-1 upregulation, it was also important to examine whether TAPI-2 had any effect on the release of soluble ICAM-1 (sICAM-1). Stimulation of HCECs with PMA (which activates TACE) resulted in the significant release of sTNFR1 in the IOBA-NHC line (Fig. 2A; $n = 4$, $P < 0.05$). This was significantly inhibited by the TACE inhibitor TAPI-2, suggesting that shedding of sTNFR1 is TACE mediated ($P < 0.05$). Results were similar in primary cells, though unstimulated primary HCECs appeared to release higher constitutive amounts of sTNFR1 ($n = 2$; Fig. 2B). This inhibition was dose dependent, as shown in the representative curve (showing percentage inhibition) in Figure 3A (IOBA-NHC) and 3B (primary HCECs). Figure 3 also demonstrates inhibition with EDTA (positive control) that nonspecifically inhibited metalloprotease-mediated shedding by chelation of divalent metal ions (in this case, zinc). Given that ICAM-1 can also be shed in a soluble form, it was important to examine whether TAPI-2 has an effect on sICAM-1 shedding. Figure 4 (representative bar graphs showing percentage inhibition; $n = 2$) compares the effects of TAPI-2 and EDTA (5 mM [EDTA concentrations 5 mM promote the detachment of IOBA-NHC cells and primary HCECs]) on PMA-stimulated sTNFR1 concentrations to sICAM-1 concentrations in the same supernates. In IOBA-NHC (Fig. 4A), TAPI-2 inhibited sTNFR1 production but had a negligible effect on sICAM-1, whereas EDTA inhibited sTNFR1 and, to a lesser extent, sICAM-1. In primary HCECs (Fig. 4B), results were similar for the inhibitory effect of TAPI2 and EDTA on sTNFR1 production. However, primary HCECs appeared to be more sensitive to the EDTA inhibition of sICAM-1 release. These data were important in ruling out a direct effect of TAPI-2 on ICAM-1 in subsequent experiments.

Enhanced Response to TNFα through Increased TNFR1 Expression

To further support our data suggesting that increased expression of TNFR1 results in increased responsiveness to TNFα-mediated ICAM-1 expression, we had to find a way to increase TNFR1 expression on HCECs to an extent similar to that observed with IgE-activated mast cell supernates. In these experiments, we preincubated HCECs with TAPI-2, followed that by

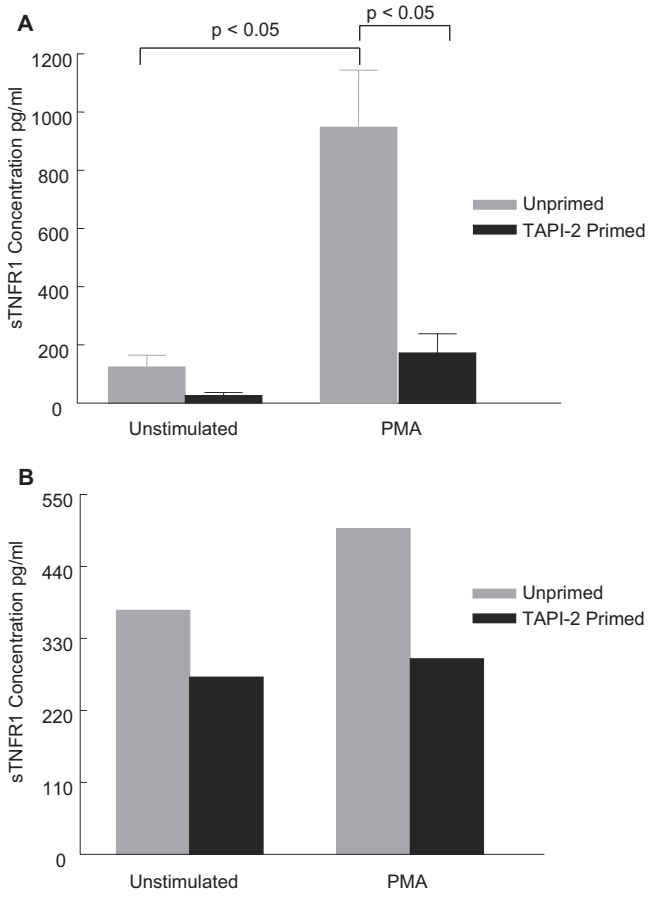

FIGURE 2. Effect of PMA stimulation on sTNFR1 release from unprimed (*gray bars*) versus TAPI-2–primed (*black bars*) IOBA-NHC cells (**A**; $n = 4$, $P < 0.05$) and primary HCECs (**B**; $n = 2$).

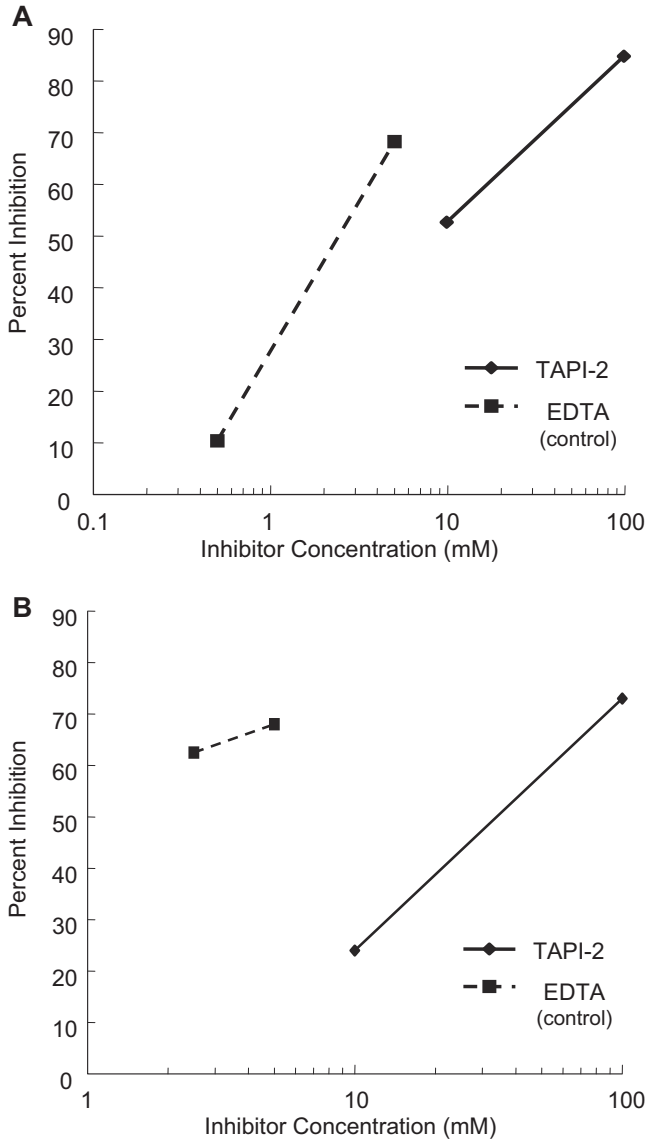

FIGURE 3. Percentage inhibition of PMA-stimulated release of sTNFR1 by EDTA (*dashed line* [■]) and TAPI-2 (*solid line* [◆]) from IOBA-NHC cells (**A**; $n = 2$) and primary HCECs (**B**; $n = 2$).

challenge with IFNγ, and examined TNFR1 expression using flow cytometry. The overlay histogram in Figure 5 demonstrates that this treatment significantly upregulated TNFR1 expression by a magnitude similar to that observed with IgE-activated mast cell supernates (increase in MFI of 2.7 ± 0.9; $P = 0.04$; $n = 4$). TAPI-2 alone slightly, though not significantly, enhanced TNFR1 expression, and IFNγ alone did not upregulate TNFR1 (data not shown). Responses were similar using the IOBA-NHC line (data not shown).

Upregulation of ICAM-1 expression in response to TNFα in unprimed versus TAPI-2/IFNγ-primed HCECs is shown in Figure 6. ICAM-1 expression in unstimulated cells was significantly greater in the IOBA-NHC cell line (82.4% ± 8.2% positive cells) than in primary HCECs (22% ± 3.2%); therefore, the data in Figure 6 are expressed as percentage positive cells greater than unstimulated cells for the purpose of comparison of the responses between the cell line and primary HCECs. The dose–response curves to TNFα in Figure 6A demonstrate that increasing TNFR1 expression using priming with IFNγ and TAPI-2 correlated with significantly enhanced sensitivity to TNFα-mediated upregulation of ICAM-1 expression on IOBA-NHC (left *y*-axis; percentage ICAM-1 expression greater than unstimulated cells; $P < 0.05$ for all comparisons of TNFα-challenged cells between unprimed and primed treatments, including slopes; $n = 4$). To reinforce our findings that TAPI-2/IFNγ priming does not affect the release of sICAM-1, we measured sICAM-1 released into supernates from the same experiments. These results, also shown in Figure 6A, demonstrated that though TNFα appeared to promote the release of sICAM-1, there was no effect of TAPI-2/IFNγ priming on this process (right *y*-axis; sICAM-1 concentration [minus unstimulated] in pg/mL). In primary HCECs, TAPI-2/IFNγ priming resulted in a similarly enhanced response to TNFα-mediated ICAM-1 upregulation (Fig. 6B; 5 ng/mL concentration of TNFα shown; $n = 2$). In IOBA-NHC cells and primary HCECs, priming with TAPI-2/IFNγ alone (no TNFα) resulted in a slight but nonsignificant upregulation of ICAM-1 expression.

DISCUSSION

Our data demonstrate that IgE-activated conjunctival mast cell supernates upregulate TNFR1 on HCECs. Manipulation of TNFR1 has led us to a better understanding of the mechanisms and consequences of TNFR1 regulation on HCECs. Using PMA to activate TACE and the TACE inhibitor TAPI-2, we confirmed

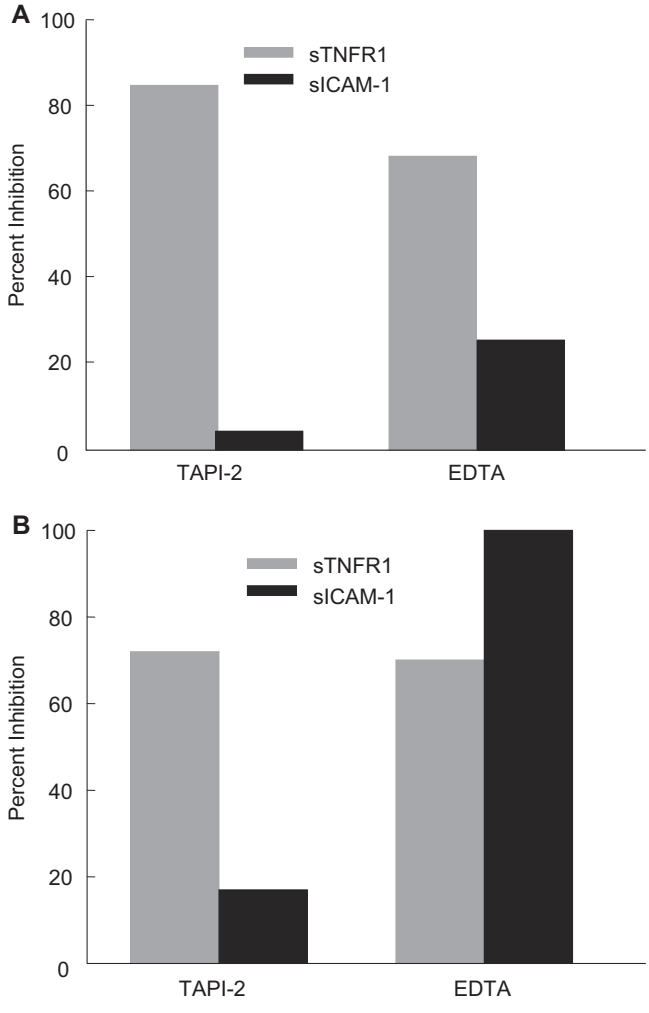

FIGURE 4. Comparison of effect of TAPI-2 and EDTA on release of sTNFR1 (*gray bars*) versus sICAM-1 (*black bars*) from IOBA-NHC cells (**A**; $n = 2$) and primary HCECs (**B**; $n = 2$).

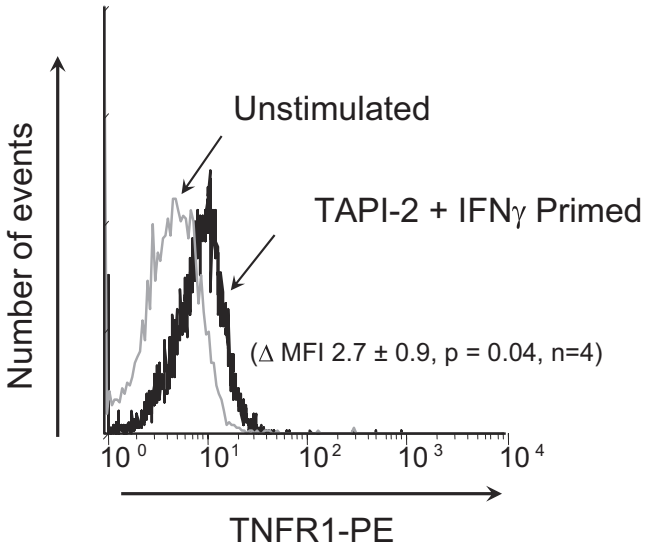

FIGURE 5. Representative overlay histograms ($n = 4$) showing upregulation of staining for TNFR1 (*x*-axis) on primary HCECs after priming with TAPI-2 and IFNγ (*black*) compared with unstimulated HCECs (*gray*). MFI was increased by of 2.7 ± 0.9 units ($P < 0.05$).

that the regulation of TNFR1 on the cell surface involves TACE-mediated cleavage of the receptor to its soluble form. We were further able to demonstrate (using IFNγ and TAPI-2 priming to increase TNFR1 expression) that even small changes in TNFR1 can result in significantly enhanced responses to TNFα, such as ICAM-1 upregulation. This is important because, in ocular allergic inflammation, ICAM-1 expression is upregulated on the conjunctival epithelium and is believed to play an important role in the maintenance of inflammation on the ocular surface by participation in the migration and adhesion of proinflammatory leukocytes.[11] In murine allergic conjunctivitis, antibodies targeting ICAM-1 have been successful at reducing symptoms and infiltration of inflammatory cells.[12] Therefore, therapies targeted toward inhibiting TNFα-mediated upregulation of ICAM-1 expression could be beneficial. Our data suggest that TNFR1 expression is more important than TNFα levels in regulating these inflammatory processes on the ocular surface.

Members of the TNF family of receptors have been identified on the conjunctival epithelium in a rabbit model of ocular inflammation but have not been previously studied in HCECs.[13] However, TNFα-mediated upregulation of ICAM-1 through TNFR1 has been demonstrated in human retinal pigment epithelial cells.[14] There are two distinct membrane receptors for TNF (TNFRs), the p55-kDa TNFR1 (or CD120a) and the p75-kDa TNFR2 (or CD120b). Although most cells coexpress both receptor types, cellular responses for soluble TNFα seem to be dominated by the interaction with TNFR1, whereas cell surface TNFα is the prime physiological activator of TNFR2.[15] TNFR1 activation appears to induce inflammation and defense against intracellular pathogens.[16,17] It has further been demonstrated that the level of surface expression of TNFR1 can be an important factor in modulating the magnitude of TNFα-mediated signal transduction events.[18]

Although TNFα plays a pivotal role in the immune response to infection, left unchecked, TNFα-mediated inflammation can turn from beneficial to harmful. Regulation of the availability of TNFα receptors provides a protective mechanism against excessive TNFα activity. Proteolytic cleavage (facilitated by TACE and inhibited by tissue inhibitor of metallopreases-3 [TIMP-3]) results in receptor downregulation that may serve to decrease cellular sensitivity to TNFα (Fig. 7). Therapies targeting TNFα (e.g., antibody, soluble receptor) have been developed to manage chronic diseases, such as rheumatoid arthritis and inflammatory bowel disease, in which the dysregulation of TNFα appears to play a critical role in driving inflammation, but TNFα has not specifically been targeted in chronic allergic conjunctivitis. Recently, pharmaceutical TACE inhibitors have been developed to inhibit TNFα because TACE also cleaves TNFα from its inactive, proform on the cell surface to its active form.[19] The compound we used in our studies, TAPI-2, is one such compound. A derivative of hydroxamic acid, TAPI-2 is known to inhibit TACE by binding to the active site of this metalloprotease, thus interfering with its zinc-dependent activation.[20]

Interestingly, however, our study suggests that TACE inhibitors may have some unintended consequences that may conflict with their ability to neutralize TNFα-mediated inflammation (e.g., inhibition shedding of TNFR1 and enhanced responsiveness to TNFα-mediated ICAM-1 upregulation). Furthermore, TACE has multiple additional targets that affect inflammation, including IL-6 and its receptor (IL-6R), transforming growth factor-α (TGFα), membrane-bound epidermal growth factor (EGF) family ligand, L-selectin, and macrophage

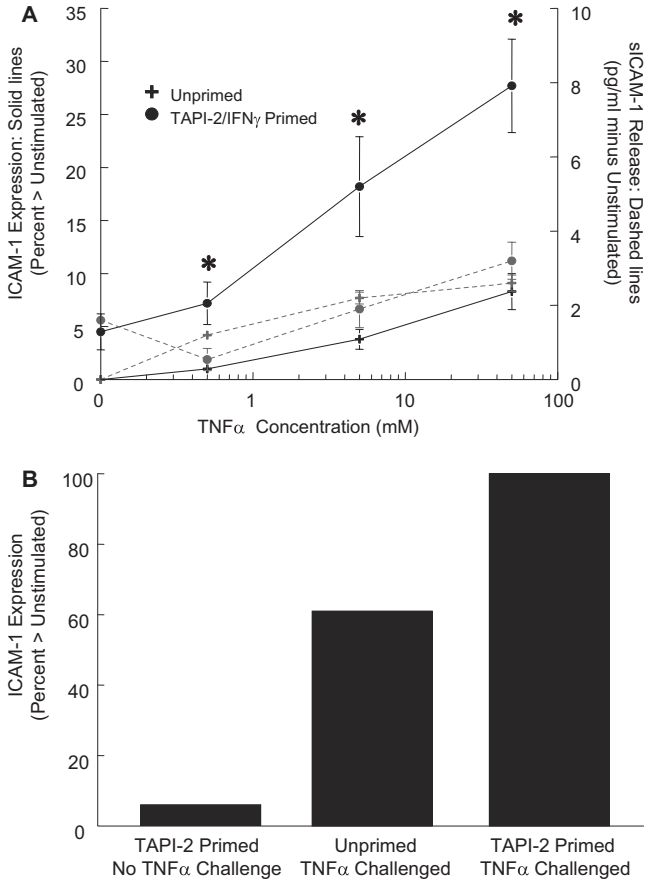

FIGURE 6. Effect of TAPI-2/IFNγ priming on TNFα-mediated upregulation of ICAM-1 expression on IOBA-NHC cells (**A**) and primary HCECs (**B**). (**A**) Dose–response curves to TNFα with and without TAPI-2/IFNγ priming (*left y*-axis; percentage ICAM-1 expression greater than unstimulated cells; $^*P < 0.05$ for comparisons between unprimed and primed treatments, including slopes; $n = 4$). Also shown is the lack of effect of TAPI-2/IFNγ priming on TNFα-stimulated release of sICAM-1 into supernates from the same experiments (*right y*-axis; sICAM-1 concentration [minus unstimulated] in pg/mL). (**B**) In primary HCECs, TAPI-2/IFNγ priming resulted in a similar enhanced response to TNFα-mediated ICAM-1 upregulation (5 ng/mL concentration of TNFα shown; $n = 2$).

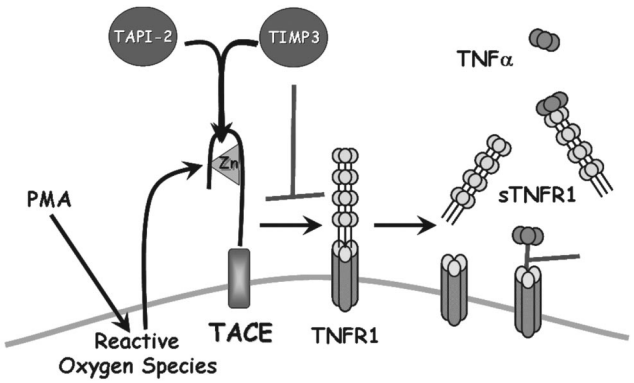

FIGURE 7. Schematic representation of regulation of TNFR1 shedding and its role in TNFα regulation. Regulation of the availability of TNFα receptors provides a protective mechanism against excessive TNFα activity. Proteolytic cleavage of TNFR1 is facilitated by TACE, which can be activated in vivo by the release of reactive oxygen species (ROS) and inhibited by TIMP-3. Shedding of TNFR1 results in the downregulation of receptor numbers on the cell surface, which decreases cellular sensitivity to TNFα. Soluble forms of TNFR1 can also bind to circulating TNFα, resulting in neutralization of TNFα-mediated responses. In our in vitro studies, PMA was used to stimulate ROS, and TAPI-2 was used to inhibit TACE. TAPI-2, a derivative of hydroxamic acid, inhibits TACE by interfering with zinc-dependent activation in the catalytic site of the metalloprotease.

colony-stimulating factor receptor.[21–24] A recent study has also suggested that TACE promotes the shedding of soluble ICAM-1 receptor in endothelial cells.[25] Therefore, TACE inhibitors might be expected to upregulate ICAM-1 expression as well as TNFR1 and IL-6R through the inhibition of shedding. Our studies, however, suggest that ICAM-1 shedding in HCECs is facilitated by a metalloprotease distinct from TACE (not inhibited by TAPI-2 but inhibited by EDTA). One explanation for this discrepancy could be that mechanisms of shedding of certain receptors are cell specific and vary depending on cell type. For example, another study has suggested that the matrix metalloprotease MMP-9 can regulate ICAM-1 shedding in osteoblasts[26]; we will examine these mechanisms further in HCECs.

Another important observation resulting from our studies is that there are some constitutive differences between primary HCECs (derived from cadaveric conjunctival tissues) compared with the spontaneously immortalized IOBA-NHC cell line. For example, constitutive expression of ICAM-1 is higher in unstimulated IOBA-NHC cells than in primary HCECs. Conversely, constitutive release of sTNFR1 is greater in primary HCECs than in the IOBA-NHC cell line. These baseline differences are important when interpreting the magnitude of responses to various stimuli. Another interesting difference observed in our studies was that IOBA-NHC cells appeared to be less sensitive to EDTA inhibition of the metalloprotease involved in the shedding of sICAM-1. Although further examination of this process is required, one interpretation could be that IOBA-NHC cells produce larger concentrations of certain metalloproteases (or divalent metal ions) than primary HCECs. These differences illustrate that though the IOBA-NHC cell line is useful for the execution of large experiments, it is essential to confirm any research using cell lines with parallel studies in primary cells. This is supported by gene expression profiles deposited in GenBank (accession number GSE8633), which compare conjunctival tissue with primary HCECs and the IOBA-NHC and ChWK cell lines (Gene Expression Omnibus Web site). These data suggested that, globally, primary HCECs clustered more closely to conjunctival tissue than either of the two cell lines and that, in general, the IOBA-NHC cell line was better correlated with primary HCECs than the ChWK cell line.

In conclusion, TNFα is increased on the ocular surface in various types of ocular surface inflammation such as chronic ocular allergic inflammation and dry eye disease.[27] In these diseases, HCEC activation by TNFα, including the upregulation of HCEC receptors such as ICAM-1, is believed to play a significant role. Our in vitro studies have demonstrated that mediators from IgE-activated human conjunctival mast cells upregulate the expression of TNFR1 on HCECs. Although the mechanism through which mast cells upregulate TNFR1 needs further investigation, we have demonstrated that the magnitude of this upregulation correlates with an increased sensitivity of the HCECs to TNFα-mediated upregulation of ICAM-1. The potential use of therapies specifically targeting TNFα in ocular surface inflammation, such as dry eye disease and chronic allergic conjunctivitis, has been suggested. Although TACE inhibitors such as the one used in our study were initially developed for pharmaceutical inhibition of TACE-mediated TNFα activation, it is likely that we still do not have a comprehensive understanding of the targets of TACE and the consequences of TACE inhibition. Our study suggests that upsetting the balances of these processes may have conflicting consequences and that downregulation or blocking of TNFR1 may be a more beneficial avenue of inhibiting TNFα-mediated responses on the ocular surface than targeting TNFα itself.

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