Interaction between conjunctival epithelial cells and mast cells induces CCL2 expression and piecemeal degranulation in mast cells.

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**Abstract**

**Purpose:** Intraepithelial mast cells are observed in giant papillae tissue samples obtained
from patients with atopic keratoconjunctivits (AVC)/vernal keratoconunctivitis (VKC) patients. In this study, we examined the roles of interaction between the conjunctival epithelial cells and mast cells.

Methods: The interaction between human mast cells and conjunctival epithelial cells (HCjE) was investigated using a coculture model. Protein array analysis, ELISA and realtime PCR were carried out to test the interaction. Tissue samples (n=6) from Giant papillae were resected for therapeutic purposes, and subjected to immunohistological analysis of CCL2 expression. Recombinant CCL2 (10ng/ml) was reacted with the cultured human mast cells, and ultrastructural analysis was carried out. A ragweed (RW)-induced mouse experimental allergic conjunctivitis model was used to examine ccl2 mRNA expression and mast cell morphology.

Results: Protein array and real-time PCR analyses showed that CCL2 protein/mRNA expression was induced by mast cell-HCjE coculture. Upregulation of CCL2 mRNA was observed in mast cells, whereas in situ CCL2 expression was observed at the conjunctival epithelium of the giant papillae by immunohistochemistry. Ultrastructural analysis showed that recombinant CCL2 treatment induced piecemeal degranulation (PMD) in the mast cells. Ultrastructural analysis of tissues from the giant papillae showed PMD of mast cells within the conjunctival epithelial cells. The RW-induced experimental allergic
conjunctivitis model showed increased ccl2 mRNA expression and PMD morphology in the conjunctivae.

**Conclusions:** Mast cell-conjunctival epithelial cell interaction induces CCL2 expression and subsequent PMD.

**Introduction**

Mast cell activation and migration within and around the conjunctival epithelium is one of the histopathological features of severe chronic allergic conjunctivitis, atopic keratoconjunctivitis (AKC)\(^1\) and vernal keratoconjunctivitis (VKC).\(^2\) In this study we investigated possible interactions of mast cells and conjunctival epithelial cells using *in vitro* coculture models, and we found that CCL2 expression in mast cells was upregulated by coculture. A previous report showed that CCL2 could induce piecemeal degranulation (PMD) in basophils.\(^3\) PMD and anaphylactic degranulation are known as two distinct types of mast cell degranulation.\(^4\) Anaphylactic degranulation is a degranulation style with antecedent granule-to-granule and/or granule-to-plasma membrane fusions. On the other hand, gradual emptying of cytoplasmic secretory granules in the absence of granule-to-granule or granule-to-plasma membrane fusion events is observed in PMD. Previously, we reported that 34 of 168 mast cells observed in the giant papillae of eight eyes obtained from VKC patients showed PMD, whereas only 28 of the 168 mast cells
had the morphology of anaphylactic degranulation. These results suggested that not only anaphylactic degranulation but also PMD might play some roles in the pathophysiology of AKC and VKC. In this study, we investigated the roles of mast cells and epithelial cells interactions, as well as the roles of PMD and CCL2 expression in mast cells, in the pathophysiology of AKC/VKC.

Material and Methods

Coculture model of mast cells and conjunctival epithelial cells. Human mast cell line LAD2 was provided by Dr. Arnold Kirchenbaum (NIH) and maintained as previously described. Human peripheral blood derived mast cells (p-mast) were raised and maintained as previously described. The human conjunctival epithelial cell line HCjE was provided by Prof. Ilene Gipson (Schepens Eye Research Inst., MA) and maintained as previously described. Coculture models of these there cells were made using Costar Transwell permeable supports (for 12-well culture dishes).

Antibody array analysis and ELISA analysis using culture supernatant. For antibody array analysis, the culture supernatant was incubated with Human Inflammation Array NO.3 (Ray Biotech Inc., Norcross, GA) according to the manufacturer’s protocol. The results were visualized and quantified using an LAS 3000 image ware (Fuji Film, Tokyo Japan). Human CCL2 ELISA was carried out using a Quantikine CCL2 ELISA kit
(R&D Systems, Minneapolis MN) according to the manufacturer’s protocol.

**Giant papillae and control conjunctivae samples.** Giant papillae were resected for therapeutic purposes from 5 patients, 3 with AKC and 2 with VKC, and control conjunctival tissue was biopsied from 8 conjunctivochalasis patients during resection surgery after obtaining written informed consent as previously described. Additional giant papillae were obtained from 2 AKC and 4 VKC patients for immunohistochemical analysis. All of the procedures were approved by the ethics committees of the Juntendo University School of Medicine and Kyoto Prefectural University of Medicine, and the study was conducted in accordance with the tenets of the Declaration of Helsinki. AKC was defined as a bilateral chronic inflammation of the conjunctiva and eyelids associated with atopic dermatitis, and VKC was defined as a chronic, bilateral, conjunctival inflammatory condition found in individuals as previously described.

**Reverse-transcription (RT) and real-time PCR.** Total RNA was extracted from the cultured cells and tissues of the giant papillae using a NucleoSpin II RNA isolation kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany), and cDNAs were prepared from 1 μg of total RNA using random primers and the RevaTra-Ace reverse transcriptase (both from Toyobo, Tokyo Japan) according to the manufacturer’s protocol. We used TaqMann® real-time PCR probes and primers specific for human CCL2...
(Hs00234140_ml) and 18SrRNA obtained from Applied Biosystems (Assay-on-Demand gene expression products; Applied Biosystems Inc., Foster City, CA). Real-time PCR analysis was carried out on a 7500 Real-Time PCR system (Applied Biosystems). For CCL2 mRNA expression, the comparative Ct method, which utilizes the 18SrRNA expression in the same cDNA as a control, was used. CCL4 and ccl2 mRNA were quantified using Fast SYBR green master mix (Applied Biosystems) and the following pairs of the primers forward 5’-CTGTGCTGATCCAGTGAATC-3’, and reverse 5’-TCAGTTCCAGTCCAGGTCTACAA-3’ (CCL4), forward 5’-AGCACAGGTGTCCCAAAGAAG-3’, reverse 5’-GCACAGCTCTCTCAGCTTG-3’ (ccl2). For CCL4 and ccl2 mRNA expression, the comparative Ct method, which utilizes the GAPDH/gapdh expression in the same cDNA as controls, was used.

**Immunohistochemical analysis.** The specimens form the giant papillae were immediately fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 3 hours. After washing with 30% sucrose in PBS, the tissues were frozen in Optimal Cutting Temperature (OCT, Sakura Finetek, Tokyo, Japan) compound using liquid nitrogen. Then 5μm frozen sections were made and air-dried. Immunohistochemical staining was performed according to the previously described methods.11 A mouse
anti-human CCL2 monoclonal antibody (R&D) was used as a primary antibody (10μg/ml). For mast cell staining, a rabbit anti-FcεRIβ antibody was prepared and used as previously described. Alexa 488-, and 594-conjugated donkey anti-mouse IgG, and anti-rabbit IgG (all from Invitrogen Corporation, Carlsbad, CA) were used as secondary antibodies. Negative control staining was carried out using isotype-matched IgG (normal mouse IgG1 obtained from BioLegend, San Diego, CA and normal rabbit IgG obtained from Santa Cruz Biotechnology, Santa Cruz, CA) as substitutes for the primary antibodies. A confocal laser scanning microscope (Olympus FV-1000, Tokyo, Japan) was used for imaging.

**Ultrastructural analysis of human cultured mast cells.** P-mast cells were stimulated with 20ng/ml recombinant human CCL2 (purchased from Peprotech, London, UK) for 3 min and fixed with 2.5% glutaraldehyde and postfixed with 2% osmic acid. HCjE cocultured p-mast cells were also prepared for ultrastructural analysis after 24-hour coculture experiments. For negative control, recombinant human CXCL8 (Peprotech; 20ng/ml, for 3min) stimulated p-mast cells, and crosslinked p-mast cells using human IgE (1μg/ml, purchased from Chemicon/Millipore, Billerica, MA) and rabbit anti-IgE (1μg/ml, from Dako Japan, Kyoto, Japan) were prepared. The samples were embedded in epoxy resin and ultrathin sections (60-80nm) were made. The ultrathin sections were then
examined using a transmission electron microscope (7000-100; Hitachi High-Technologies, Tokyo, Japan).

**Alum ragweed induced experimental allergic conjunctivitis**

Alum ragweed (RW)-induced experimental allergic conjunctivitis models were prepared as previously described using male BALB/C mice at the age of 10-12 weeks (SLC, Hamamatsu, Japan). The expression of ccl2 mRNA was quantified for mouse conjunctival tissue 24 hours after final RW challenge. For comparison, a single-challenge RW eye drop model and 4-challenge RW eye drop model were used. Ultrastructural analysis was carried out for conjunctival and eye-lid samples after final RW challenge (4-challenge model). All the animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**RESULTS**

**Mast cell HCjE interaction induces CCL2 expression in mast cells.** The coculture experiment showed synergistic increases of CCL2 and CCL4 protein in the supernatant of the mast cell (LAD2) HCjE coculture model (Figure 1A-1C). These results were also confirmed by ELISA analysis (Figure 1D). The LAD2 HCjE coculture model induced 2-fold higher CCL2 mRNA expression in LAD2 cells than in LAD2 cells cultured by themselves. The p-mast HCjE coculture model using also showed a significant increase
of CCL2 mRNA (Figure 2A). For CCL4 mRNA expression, only HCjE-cocultured LAD2 showed a CCL4 mRNA increase; no change was observed in HCjE-cocultured p-mast cells. (Figure 2B).

**Epithelial cells and infiltrating cells in VKC/AKC tissue express CCL2 protein in situ.** Immunohistological analysis of the tissue samples from the giant papillae showed positive CCL2 immunostaining in epithelial cells (Figure 3). FcεRIβ-immunopositive mast cells were also observed within and around CCL2-positive epithelial cells (Figure 3B, 3C). We confirmed the specificity of the CCL2 immunostaining by using isotype-matched normal mouse IgG1 instead of the CCL2 antibody (Figure 3F). The specificity of FcεRIβ immunostaining was already demonstrated in our previous report\(^1\) and we always run negative controls specimens in our experiments (data not shown).

CCL2-positive infiltrating cells were also observed in the substantia propria of the tissue (Figure 4A). Higher magnification of the figure revealed the CCL2/ FcεRIβ double-positive mast cells (arrows in Figure 4B and 4C), as well as CCL2+/ FcεRIβ-infiltrating cells (arrowheads). The results of CCL2 immunohistochemical staining are summarized in Table 1.

**Significantly higher CCL2 mRNA expression was observed in tissues from giant papillae than in conjunctivochalesis tissue samples.** Five samples from giant papillae
and eight conjunctivochalasis samples (Table 2) were collected and cDNA was prepared. Real-time PCR analysis showed significantly increased CCL2 mRNA in the samples from giant papillae compared to the conjunctivochalasis samples. (Figure 5)

**Mast cells in the giant papillae showed PMD and recombinant CCL2 could induce PMD.** Three-minute treatment of p-mast cells with recombinant CCL2 (20ng/ml) could induce PMD (Figure 6B, 6F) compared to naïve p-mast cells (Figure 6A, 6E). P-mast HCjE coculture (for 24 hours) also induced PMD morphology (Figure 6C, 6G). IgE/anti-IgE crosslinking treatment induced anaphylactic degranulation in p-mast cells (Figure 6D). In contrast to CCL2 treatment, CXCL8 (IL-8) treatment did not induce PMD (Figure 6H). Ultrastructural analysis of giant papillae showed that intraepithelial mast cells had PMD morphology (Figure 7A) as well as anaphylactic degranulation morphology (Figure 7B).

**Ccl2 expression in alum-RW induced mouse experimental allergic conjunctivitis**

Increased ccl2 mRNA expression (Figure 8A) was observed in RW-induced allergic conjunctivitis (4-challenge RW eye drop model) compared to PBS-challenged conjunctivae. The single-challenge RW eye drop model did not show a significant ccl2 mRNA increase compared to the PBS-challenged conjunctivae. PMD morphology was observed in the conjunctival mast cells of RW conjunctivitis. (Figure 8B)
DISCUSSION

In this study we showed that mast cell-conjunctival epithelial cell interaction induced CCL2 expression at the mRNA and at protein levels. We used both LAD2 cells (a cell line) and p-mast cells (primary-cultured cells) as the source of mast cells. Initial coculture experiments, including antibody array experiments, were carried out using LAD2, and confirmatory experiments were carried out with primary p-mast cells due to their limited availability. Increased concentrations of CCL2 and CCL4 (MIP-1β) protein were observed in LAD2 HCjE coculture supernatant (Figure 1), and upregulation of both CCL2 mRNA and CCL4 mRNA was confirmed in LAD2 cells in the coculture model. We then tried to replicate the results using p-mast cells. CCL2 mRNA upregulation was observed in cocultured p-mast cells, but no CCL4 mRNA upregulation was observed (Figure 2A, 2B), so we focused at the role of CCL2 for further studies.

To examine the relevance to the pathophysiology of AKC/VKC, we next evaluated CCL2 expression in the tissues of giant papillae tissue obtained from patients.

Immunohistochemical analysis showed CCL2-positive staining of conjunctival epithelial cells (Figure 3). Double immunohistochemical staining with a mast cell marker (FceRIβ) showed mast cells within and beneath the CCL2-positive conjunctival epithelial cells (Figure 3A, 3B, 3C). We also found positive CCL2 immunostaining at the substantia
propria of the tissue from the giant papillae (Figure 4). Abu El-Asrar et al. found increased number of CCL2-positive staining of cells infiltrating the substantia propria of the limbal tissue of VKC. 13 Although they reported negative CCL2 expression in the conjunctival epithelium in limbal VKC tissue, we clearly detected CCL2-positive immunostaining of the conjunctival epithelium tarsal form of giant papillae (Figure 3). Giustizieri et al. demonstrated CCL2 mRNA expression in the epithelial cells of the lesional skin of atopic dermatitis patients by in situ hybridization. 14 Gordon reported increased CCL2 expression in both epidermal cells and dermal cells in a dinitrophenyl serum albumin-induced mouse passive cutaneous anaphylaxis (PCA) model, using immunohistological analysis. 15 Mercer et al. reported positive CCL2 immunostaining at the epithelial cells of human idiopathic pulmonary fibrosis tissue but not at the epithelial cells of lung tumor tissue (control tissue). 16 These three reports on CCL2 expression are supportive for our results for positive CCL2 immunostaining of epithelial cells. Since we did not examined the expression of CCL2 in the limbal VKC tissue, the reason for the difference between our results and those of Abu El-Asrar is unknown. We speculate that there may be a difference between the limbal and tarsal forms of VKC for the epithelial expression of CCL2.

We found few CCL2/FceRIβ double-positive mast cells (Figure 4B) by
immunohistochemical analysis. Although the main CCL2 mRNA-producing cells were mast cells (Figure 2A), CCL2 protein was also secreted from mast cells as we found in culture supernatant samples (Figure 1D). Therefore we hypothesize that continuous CCL2 secretion from mast cells was the reason we found few CCL2/FcεRIβ double-positive mast cells. This discrepancy between abundant CCL2 mRNA expression and poor CCL2 retention in mast cells was reported previously.\textsuperscript{15, 17}

We obtained tarsal giant papilla tissues from refractory AKC/VKC patients, all of whom were treated with topical dexamethasone eye drops for at least 4 weeks (Table 1), so treatment may have downregulated the CCL2 expression as reported previously.\textsuperscript{18} Nonetheless, significantly higher CCL2 mRNA expression in samples from giant papillae than in conjunctival tissues obtained from conjunctivochalesis patients was observed by real-time PCR analysis (Figure 5).

Interestingly, the report of Gordon also showed that CCL2 expression in a PCA model was dependent on mast cells because of significantly reduced CCL2 expression in the skin of mast cell-deficient mice (\textit{W/W\textsuperscript{v}}) during the PCA reaction.\textsuperscript{15} Their results suggesting that interaction between mast cells and other components of conjunctival cells (including conjunctival epithelial cells) could upregulate CCL2 expression during allergic reactions agreed with our results in this study.
To further elucidate the role of CCL2 protein in the pathophysiology of AKC/VKC, we examined the activation pattern of mast cells with special reference to PMD. We found that recombinant CCL2 stimulation (Figure 6B and 6F) as well as HCjE coculture procedures (Figure 6C and 6G) could induce PMD in cultured mast cells in vitro. We also tried to inhibit the effect of CCL2 by adding a CCR2 inhibitor (RS504393 from TOCRIS Bioscience) to the coculture model, and found partial inhibition of the PMD phenomenon (data not shown). Consistent with the results of a previous report\textsuperscript{19}, p-mast cells stimulated with another chemokine (CXCL8) did not show PMD morphology (Figure 6H). This result also supported the specificity of the CCL2-induced PMD phenomenon. Although we could not deny the possibility of other conjunctival epithelial cell-derived mast cell activators, CCL2 in the coculture medium played some roles in PMD. Continuous studies are ongoing in our laboratory to elucidate possible additional activators. We also found PMD in the intraepithelial mast cells of a VKC patient, showing the relevance of PMD to the pathophysiology of VKC (Figure 7). In our previous study, 20% of the mast cells in the giant papillae samples showed the PMD morphology and 17% of the mast cells in the giant papillae samples showed anaphylactic degranulation in VKC patients.\textsuperscript{6} These results suggested the importance of PMD and subsequent slow/persistent mediator release\textsuperscript{20} during chronic allergic keratoconjunctivitis. The
magnitude of inflammation with PMD seems to be smaller than with anaphylactic
degranulation; however, the PMD reaction lasts longer without IgE crosslinking by the
antigen\textsuperscript{5}. On the other hand, the mediator release from mast cells is not long lasting in the
case of anaphylactic degranulation because it needs some time to regain the cytoplasmic
granules\textsuperscript{4}.

We also confirmed \textit{Ccl2} mRNA upregulation and PMD of mast cells in the RW-induced
mouse experimental allergic conjunctivitis model. After 4 RW eye drop challenges, but
not after a single RW eye drop challenge, increased \textit{ccl2} mRNA expression compared to
PBS-challenged control conjunctival tissue and PMD morphology were observed in the
RW-challenged conjunctival tissue (Figure 8A). This result suggested that chronic
antigen stimuli were \textit{ccl2} mRNA-inducing factors. Although there are no appropriate
mouse models for AKC/VKC, and RW-induced mouse experimental allergic
conjunctivitis is considered to be an animal model of seasonal allergic conjunctivitis\textsuperscript{21},
RW-induced allergic conjunctivitis can be used as a model of chronic allergic
inflammation induced by repeated antigen stimuli in which eosinophil infiltration and T
cell activation\textsuperscript{22,23} are observed.

A study by Miyazaki et al. showed that CCL2 protein was expressed in the conjunctival
epithelium of mouse experimental allergic conjunctivitis and CCL2 subconjunctival
injection induced mast cell degranulation. Our results are consistent with their findings. They also reported that blocking the CCL2-CCR2 signaling cascade could attenuate signs and symptoms of the acute phase of experimental allergic conjunctivitis. Further experiments analyzing RW-induced experimental allergic conjunctivitis using mast cell-deficient mice to clarify mast cell-conjunctival epithelial cell interactions are now ongoing.

In conclusion, we showed that mast cell-conjunctival epithelial cell interaction could induce higher CCL2 expression and PMD in cultured human mast cells, which also observed in situ samples of chronic allergic conjunctivitis. These results suggested that suppression of CCL2-CCR2 signaling cascades might be useful for alternative therapy for severe chronic allergic conjunctivitis.

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References
13. Abu El-Asrar AM, Struyf S, Al-Kharashi SA, Missotten L, Van Damme J,


**Figure Legends**

**Figure 1. Mast cell conjunctival epithelial cell interaction induces CCL2 expression in mast cells.** Antibody arrays were used to analyze HCjE (A), LAD2 (mast cell; B), and LAD2 HCjE coculture (C) supernatants. Synergistic increases of CCL2 (red circles) and CCL4 protein (red arrows) in the coculture supernatant (C). Blue squares are positive control samples for array reactions. The synergistic increase of CCL2 in the supernatant of a coculture sample was also confirmed by ELISA analysis (D).

**Figure 2. Mast cell HCjE coculture induces CCL2 mRNA expression.** The mast cell HCjE coculture model induced significantly increased CCL2 mRNA expression in both LAD2 and p-mast cells compared to that in mast cells cultured alone (A). On the other hand, CCL4 mRNA induction was observed only with HCjE-cocultured LAD2, and no change was observed in HCjE-cocultured p-mast cells (B).

**Figure 3. CCL2 expression in the conjunctival epithelial cells of giant papillae**

Perpendicular (sagittal, A and B) and horizontal (coronal, C and D) sections of tissues from giant papillae showed positive CCL2 immunostaining (green) in epithelial cells. FcεRIβ-positive mast cells (red) were also observed within and around CCL2-positive epithelial cells (arrows in A and B, arrowhead in C). The FcεRIβ single staining image of (C) is also shown in (D). Specificity of CCL2 immunostaining was shown by anti-CCL2 staining (E) and control mouse IgG₁ antibody staining using two adjacent sections (F).
Original magnification (A, C and D: x200, B, E and F: x400).

**Figure 4. CCL2 expression in the substantia propria of giant papillae**

CCL2 immunohistochemical staining of the substantia propria of giant papillae is shown. CCL2/ FcεRIβ double-positive mast cell (arrow) and CCL2+/ FcεRIβ- infiltrating cells (arrowheads) are observed in the substantia propria of giant papillae tissue. (B) is a high magnification image of (A), (C) is the FcεRIβ single staining image of (B). Original magnification (A: x200, B and C: x400).

**Figure 5. Increased CCL2 mRNA expression in the tissues from giant papillae**

Five samples from giant papillae and eight control (conjunctivochalesis) samples were analyzed. Real-time PCR analysis showed significantly higher CCL2 mRNA expression in the samples from giant papillae than in the control samples. *P<0.05, Mann-Whitney’s U-test.

**Figure 6. Recombinant CCL2 stimulation could induce PMD morphology.**

CCL2-(20ng/ml, for 3mins) stimulated p-mast cells (B, F) show PMD morphology compared to naïve p-mast cells (A, E). P-mast HCjE coculture also induced PMD morphology (C, G). Anaphylactic degranulation morphology of a p-mast cell (D) induced by IgE/anti-IgE crosslinking is shown (D). A CXCL8-(20ng/ml, for 3mins) stimulated p-mast cell (H) is shown as a negative control.
Figure 7. Intraepithelial mast cells show PMD morphology *in situ*.

Ultrastructural analysis of a giant papilla obtained from a VKC patient shows
intraepithelial mast cells (M) with PMD morphology (A) and with anaphylactic
degranulation morphology (B). Empty granule chambers (arrows) in a mast cell with
PMD morphology and released granules (arrowheads) and labyrinth formation (asterisk)
in a mast cell with anaphylactic degranulation morphology are shown. Ep: conjunctival
epithelial cells.

Figure 8. *Ccl2* expression in mouse experimental allergic conjunctivitis

Alum-RW-induced mouse experimental conjunctivitis shows increased ccl2 mRNA (A)
PMD morphology (arrow) and anaphylactic degranulation (arrowheads) are observed in
the mast cells of conjunctival tissue (B, C). *: $P<0.05$ by Student’s $t$-test. The nucleus of a
degranulated mast cell is shown (M).
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Dex, 0.1% dexamethasone eyedrop; CsA, 0.1% cyclosporine eye drop.
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