Role of vimentin in CCL2 production in mast cells

Evidence that Formation of Vimentin/Mitogen-Activated Protein (MAP) Kinase Complex Mediates Mast Cell Activation Following FcεRI/CC Chemokine Receptor 1 Cross-talk

Masako Toda1#∫, Chuan-Hui Kuo1#, Satty K Borman3, Ricardo Micheler Richardson4, Akihito Inoko5, Masaki Inagaki5, Andrea Collins1, Klaus Schneider3§, Santa Jeremy Ono1, 2

1 Division of Allergy and Immunology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, and 2 University of Cincinnati College of Medicine, OH, USA
3 Department of Computational and Structural Chemistry, GlaxoSmithKline Research and Development, Gunnels Wood Road, Stevenage SG1 2NY, UK
4 Julius L. Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, NC 27707, USA
5 Division of Biochemistry, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan.

∫ Current address: Paul-Ehrlich-Institut, Langen 63225, Germany
§ Current address: Central Analytical Department, Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany
# These authors contributed equally to this study.

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To whom correspondence should be addressed: Masako Toda, Junior Research Group 1 "Experimental Allergology", Paul-Ehrlich-Institut, Paul-Ehrlich-Str. 59, 63225 Langen, Germany, Phone: +49 (0)6103-77 5407, Fax: +49 (0)6103-77 1258, E-mail: Masako.Toda@pei.de
Santa Jeremy Ono, Division of Allergy and Immunology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center; University of Cincinnati College of Medicine, 2614 McMicken Circle, 210 Van Wormer Hall, PO Box 210097, Cincinnati, OH 45221-0097, USA, Tel: 513-556-2588, Fax: 513-556-7861 E-mail: onosa@ucmail.uc.edu

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Background: CC chemokine ligand 2 (CCL2) recruits leucocytes in inflammatory tissues.

Results: Vimentin, a cytoskeletal protein, interacted with phosphorylated MAP kinases, was critical for CCL2 production in mast cells activated via FcεRI and a CC chemokine receptor.

Conclusion: Vimentin was involved in optimal CCL2 production in mast cells.

Significance: Understanding mechanisms for chemokine production in mast cells, which are therapeutic targets for allergic inflammation.

Summary

Accumulating evidence points to cross-talk between FcεRI and CC chemokine receptor (CCR)-mediated signaling pathways in mast cells. Here, we propose that vimentin, a protein comprising type III intermediate filament, participates in such cross-talk for CCL2/monocyte chemotactic protein 1 (MCP-1) production in mast cells, which is a mechanism for allergic inflammation. Co-stimulation via FcεRI, using IgE/antigen, and CCR1, using recombinant CCL3/macrophage inflammatory protein-1α (MIP-1α), increased expression of phosphorylated, disassembled and soluble vimentin in rat basophilic leukemia (RBL)-2H3 cells expressing human CCR1 (RBL-CCR1 cells) and bone marrow derived murine mast cells, both models of mucosal type mast cells. Furthermore, co-stimulation enhanced production of CCL2, as well as phosphorylation of MAP kinase. Treating the cells with p38 MAP kinase inhibitor SB203580, but not with MEK inhibitor PD98058, reduced CCL2 production, suggesting that p38 MAP kinase, but not ERK1/2, plays a critical role in the chemokine production. Immunoprecipitation analysis showed that vimentin interacts with phosphorylated ERK1/2 and p38 MAP kinases in the co-simulated cells. Preventing disassembly of the vimentin by aggregating vimentin filaments using beta,beta'-iminodipropionitrile reduced the interaction of vimentin with phosphorylated MAP kinases, as well as CCL2 production in the cells. Taken together, disassembled vimentin interacting with phosphorylated p38 MAP kinase could mediate CCL2 production in mast cells upon FcεRI and CCR1 activation.

Introduction

Mast cells play an important role in IgE-associated allergic disorders and immune responses to parasites, and FcεRI cross-linking is a key event in activating mast cells. Allergens binding IgE cross-linked to FcεRI triggers signaling cascades leading to activation of kinases, phosphatases, and GTPases, which subsequently induces a variety of events such as degranulation, cytoskeleton rearrangement, increased gene transcription, and cytokine/chemokine production in the activated mast cells (1,2).

Besides the classical FcεRI-mediated
mechanism, mast cells are also activated by chemokines (3,4). A superfamily of small, structurally related cytokine molecules, chemokines are characterized by their ability to affect trafficking of leukocytes. Some chemokines, such as CCL2/monocyte chemotactic protein 1 (MCP-1), CCL3/macrophage inflammatory protein-1 α (MIP-1α), CCL5/Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES), and CCL11/eotaxin-1, have been reported to activate mouse, rat, or human mast cells (3-6). Abundant expression of these CC chemokines and accumulation of leucocytes has also been observed in allergic inflammatory tissues (7-9). It is very likely that FceRI and CCR mechanisms occur either simultaneously or in relatively rapid succession in mast cells in vivo.

We previously found that CCL3 acts as a co-stimulator for FceRI-mediated degranulation in conjunctival mast cells using CCL3-deficient mice (10). Moreover, CCL3 synergistically enhanced FceRI-mediated degranulation and gene expression of cytokines and chemokines (e.g. IL-6 and CCL7/MCP-3) in a rat basophilic leukemia 2H3 cell line (RBL-2H3) expressing CC chemokine receptor 1 (CCR1), a receptor for CCL3, and bone marrow-derived murine mast cells (11-14). These observations indicate that (i) the simultaneous engagement of FceRI and CCR1 is important for optimal activation of mast cells in vitro and physiologically relevant levels of mast cell activation in vivo, and (ii) there is a cross-talk between the FceRI-mediated and CCR1-mediated signaling cascades.

In this paper, we also found that CCL3 synergistically enhanced FceRI-mediated CCL2 production in RBL-CCR1 cells and bone marrow derived murine mast cells (BMMCs). CCL2 is a chemo-attractant to induce migration of monocytes, T cells and eosinophils (3,9). Increased expression of CCL2 protein in inflammatory tissues of allergic patients has been observed (3,7-9,15). Targeting chemokine(s) is a strategy to establish new anti-inflammatory drugs for treatment of allergenic diseases. To better understand the molecular mechanisms for chemokine production involved in the complex response of mast cell activation, we investigated the proteins involved in the cross-talk between FceRI-mediated and CCR1-mediated signaling pathways.

Here, we identified phosphorylated vimentin, a cytoskeletal protein, as the major protein species up-regulated in the co-stimulated mast cells with IgE/Ag and CCL3. Interestingly, vimentin interacted with mitogen-activated protein (MAP) kinase, extracellular-signal-regulated kinase 1/2 (ERK1/2) and p38 MAP kinase. Furthermore, our findings suggest that vimentin is a component for optimal production of CCL2 in the FceRI- and CCR1-engaged mast cells.

**Experimental procedures**

*Cell culture*—Monolayer cultures of RBL-CCR1
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cells (rat basophilic leukemia RBL-2H3 cells expressing human CCR1) (16) were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 12% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin and 1 mg/ml of geneticin.

To generate primary bone marrow derived murine mast cells (BMMCs), low density mononuclear cells (LDMNCs) were isolated from BALB/c mice (Jackson Laboratories) and cultured in RPMI1640 containing 10%FBS, 4 mM glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 0.1 mM non-essential amino acids, and 50 µM 2-mercaptoethanol in the presence of 5 ng/ml of r-murine IL-3 (PeproTech) for 4 to 6 weeks (12,17). The purity of BMMCs exceeded 80%, which was determined by flow cytometric analysis after staining with c-kit and FcεRI on the cell surface. Tissue culture media and cell culture supplements were from Invitrogen.

Chemokine production assay—RBL-CCR1 cells (3.0x10^6 cells/ml) and BMMCs (1.0x10^6 cells/ml) were sensitized with 10 and 100 ng/ml anti-DNP IgE monoclonal antibody (SPE7, Sigma-Aldrich) overnight, respectively. These cells were then treated with, or without the p38 MAP kinase inhibitor SB203580 (Calbiochem) or MEK inhibitor PD98058 (Calbiochem) for 1 h, or beta,beta’-iminodipropionitrile (Sigma-Aldrich), inhibitor of soluble vimentin formation, for 1 h, and subsequently stimulated with DNP-conjugated human serum albumin (DNP-HSA from Sigma-Aldrich) and/or human rCCL3 (R&D Systems) in DMEM containing 2% FCS for 2 to 6 h. The concentrations of rat and murine CCL2 in the culture supernatant were measured by ELISA (Peprotech and eBioscience, respectively).

Measurement of phosphorylated ERK1/2 and p38 MAP kinase by ELISA—After sensitization with anti-DNP-IgE mAb, cells were stimulated with rCCL3 and/or DNP-HSA for 5 min, and lysed in 10 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na4P2O7, 2 mM Na3VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate. The level of total and phosphorylated ERK and p38 kinase in cell lysates were measured by ELISA (Invitrogen) following the manufacture’s instructions.

Two-dimensional gel electrophoresis—RBL-CCR1 cells (1.5x10^6 cells/ml) were sensitized with anti-DNP IgE mAb overnight and stimulated with 10 ng/ml of DNP-HSA and 100 ng/ml of rCCL3 for 5 min. The cells were washed with cold PBS and lysed in a buffer containing urea (8 M), CHAPS (4% w/v), DTE (65 mM), resolytes 3.5-10 (2% v/v), 2.5 µg/ml of DNase I, 2.5 µg/ml of RNase, 50 mM NaF, 1 mM Na3VO4, protease inhibitors (Complete, Roche Diagnostics), and a trace of bromophenol blue. The total lysates were loaded on the first dimensional separation with a
sigmoidal immobilized pH gradient (IPG) from pH 4.0 to 7.0. After equilibration, the IPG gel strips were transferred onto the second dimension vertical gradient slab gels and run with the Laemmli-SDS-discontinuous system. Proteins were detected using Coomassie staining. Two-dimensional gel electrophoresis and gel staining were performed by the Proteomics Core Facility at the University of Geneva, Switzerland.

**Protein Identification by MALDI-TOF MS**—Protein spots were excised from the polyacrylamide gel, reduced with dithiothreitol, treated with iodoacetamide (Sigma-Aldrich) for carboxymidation of the cysteine residues, and digested in-situ with trypsin (Sequencing grade, Roche), according to the method of Shevchenko et al. (18,19). An aliquot of the liquid surrounding the gel pieces was mixed with equal volume of matrix solution (60% (v/v) acetonitrile, 0.5% (v/v) trifluoroacetic acid (Applied Biosystems), 6 mg/ml a-cyano-4-hydroxycinnamic acid (Bruker Daltonics GmbH, Bremen, Germany). One microliter of the mixture was spotted immediately onto a 384-well stainless steel MALDI-TOF target and allowed to dry. Peptide calibration standard solution (Bruker Daltonics) was spotted in a similar manner adjacent to the samples.

The mass spectra were recorded on an Ultraflex I (Bruker Daltonics) MALDI mass spectrometer. Peptides were selected for fragmentation analysis by LIFT-MS/MS sequencing (20). The spectra were interpreted using FlexAnalysis and Biotools software (Bruker Daltonics) and the data searched against non-redundant protein sequence databases using the program MASCOT (21). Proteins were identified with 0.2Da accuracy and a minimum of four matching peptides.

**Extraction of soluble vimentin**—The method of Valgeirsdotter et al. for extracting soluble vimentin was slightly modified (22). Briefly, after washing with ice-cold PBS, cells were lysed in PBS (pH 7.4) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM NaF, 1 mM Na3VO4 and protease inhibitors. The lysates were diluted twenty-fold with PBS containing 1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4 and protease inhibitors. The lysates were incubated with protein A/G Plus-Agarose (Santa Cruz Biotechnology) and rabbit anti-vimentin mAb (clone H54, Santa Cruz Biotechnology). Proteins complexed to the agarose gels were recovered, re-suspended in sample buffer and analyzed by immunoblotting. For immunoprecipitation to detect proteins
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associated with vimentin, the cells were lysed in a buffer used for extracting soluble vimentin. The lysates were incubated with protein G-agarose (Santa Cruz Biotechnology) and monoclonal mouse anti-vimentin mAb V9 (Sigma-Aldrich).

_Immunoblotting_—The lysates, or the proteins immunoprecipitated with anti-vimentin antibodies, were suspended in sample buffer, loaded onto 12% polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. The membranes were probed with primary antibodies, detected using appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology), and enhanced with a chemiluminescent kit (Pierce). Mouse anti-vimentin phospho Ser 55 mAb (4A4) (23), rabbit anti-vimentin phospho Ser71 mAb (TM71) (24), mouse anti-vimentin phospho Ser6 (MO6), or vimentin phospho Ser82 mAb (MO82) (25), rabbit anti-vimentin mAb (H54), rabbit anti-phospho ERK1/2 mAb (Cell signaling technology), and rabbit anti-phospho p38 MAP kinase mAb (Cell signaling technology) were used as the primary antibodies.

**Results**

_CCL2 production and MAP kinase activation are enhanced in FceRI- and CCR1-activated RBL-CCR1 cells_—CCL2 plays a critical role in activation and accumulation of leucocytes in allergic inflammatory tissues (3,7-9,15). To examine whether cross-talk between FceRI and CCR-mediated signaling pathways in mast cells is involved in expression of this chemokine, we used RBL-2H3 cells expressing human CCR1, a model cell line of mucosal type of mast cells.

We found that IgE/Ag (DNP-HSA) and rCCL3 co-stimulation synergistically enhanced CCL2 production in RBL-CCR1 cells (Fig. 1A). However, this CCL2 production stimulated with DNP-HSA and/or rCCL3 was attenuated by the p38 MAP kinase inhibitor SB203580 (Fig. 1B), but not by the MEK inhibitor PD98058, which inhibits activation of ERK1/2 (Fig. 1C). These results suggest that p38 MAP kinase but not ERK1/2 kinase plays a role in FceRI- and CCR1-mediated CCL2 production in RBL-CCR1 cells. Co-stimulation with IgE/Ag and rCCL3 also enhanced phosphorylation of p38 MAP kinase and ERK1/2 in the cells, but SB203580 and PD98058 abolished this stimulation, respectively (Fig. 2). The results indicate that cross-talk between FceRI- and CCR1-mediated signaling pathways synergistically enhance MAP kinase activation, as well as subsequent CCL2 production in RBL-CCR1 cells.

Protein expression analysis of FceRI- and CCR1-activated RBL-CCR1 cells revealed vimentin proteins—CCL2 is a therapeutic target in allergic diseases(9,26). To elucidate the molecular mechanisms for CCL2 production in mast cells, we set out to identify proteins involved in the
cross-talk between FcεRI- and CCR1-mediated signaling pathways. Therefore, we analyzed total cell lysates of RBL-CCR1 cells non-stimulated or co-stimulated with IgE/Ag plus rCCL3 by two-dimensional electrophoresis. We observed clear up-regulation of six protein species (Fig. 3), and identified rat vimentin (accession P31000) as the major component of all these spots using MALDI-TOF MS (Table 1). Vimentin has multiple phosphorylation sites, which would explain the various isoforms observed migrating in a horizontal line across the gel. It is likely that spot 6, with a lower molecular weight, is a truncated fragment of vimentin.

Vimentin was disassembled in FcεRI- and CCR1-activated RBL-CCR1 cells—Vimentin is a major structural component of intermediate filaments that create cell rigidity and shape (27). Upon phosphorylation, vimentin regulate the disassembly of these intermediate filaments (28-32). Vimentin also organizes signaling process in a phosphorylation-dependent manner (33,34). To obtain insights into the roles of vimentin in mast cell activation, we examined phosphorylation of vimentin in FcεRI and/or CCR1-activated RBL-CCR1 cells.

We therefore extracted total proteins of un-stimulated and stimulated cells using a lysis buffer containing 2% SDS and 10% glycerol, and diluted it using a buffer containing 1% Nonidet P-40 for immunoprecipitation. We then analyzed the phosphorylation status of vimentin by immunoprecipitating the protein and detecting different phospho-species by immunoblotting with specific anti-phosphotyrosine, phosphothreonine phosphoserine antibodies. The total tyrosine phosphorylation of vimentin in RBL-CCR1 cells was enhanced in rCCL3 concentration dependent manner, when the cells were co-stimulated with the chemokine and IgE/Ag. In contrast, high levels of serine and threonine phosphorylation were observed in the cells co-stimulated with 10 ng/ml of rCCL3 and/or 10 ng/ml of IgE/Ag (Fig. 4). The highest levels of phosphorylation at serines 55, 71 and 82 in the N-terminus of vimentin were detected when the cells were stimulated with 10 ng/ml of rCCL3 and 10 ng/ml of IgE/Ag. Interestingly, appreciable levels of phosphorylation at Ser 6 required co-stimulation with 100 ng/ml of rCCL3 and 10 ng/ml of IgE/Ag (Fig. 4).

Serine phosphorylation at the N-terminus appears to regulate the disassembly of vimentin filaments (28-32). It has also been shown that disassembly of vimentin filaments convert the protein into a soluble form (29-32). Vimentin reportedly interacts with phosphorylated MAP kinase in neurons and adipocytes (35-37). Hence, we hypothesized that (i) FcεRI and CCR1 activation induces vimentin disassembly, and that (ii) the disassembled and soluble vimentin interacts with activated MAP kinases, and is thus involved in CCL2 production in mast cells. To test
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these hypotheses, we then examined levels of vimentin filament disassembly by detecting amounts of soluble vimentin in activated RBL-CCR1 cells. Cell lysates were prepared with lysis buffer containing 1% Nonidet P-40, which extracts soluble forms of vimentin (22). Indeed, costimulation with rCCL3 and IgE/Ag enhanced formation of soluble vimentin in RBL-CCR1 cells (Fig. 5A). Taken together, FcɛRI- and CCR1-engagement induces vimentin phosphorylation, which in turn increases soluble vimentin levels in these activated RBL-CCR1 cells.

Vimentin interacted with phosphorylated MAP kinases in FcɛRI- and CCR1- activated RBL-CCR1 cells—Next, we examined whether vimentin interacts with MAP kinases, and is involved in CCL2 production in RBL-CCR1 cells upon FcɛRI and CCR1 stimulation. Immunoprecipitation with anti-vimentin Ab were performed using extracts of the cells stimulated with IgE/Ag and/or rCCL3, which contained soluble vimentin. The immunoprecipitated samples were separated on one-dimensional SDS-PAGE followed by Western blotting with anti-phospho p38 MAP kinase, or anti-phospho ERK1/2 mAb. We detected association of vimentin with both phosphorylated p38 kinase and ERK1/2 in the extract from the stimulated cells (Fig. 5B). These results suggest that vimentin may interact with phosphorylated p38 MAP kinase and ERK1/2 in activated RBL-CCR1 cells.

CCL2 production in CCR1- and FcɛRI- activated RBL-CCR1 cells was decreased by preventing formation of soluble vimentin—To further examine whether vimentin filaments are required for the phosphorylation of MAP kinases and production of CCL2, RBL-CCR1 cells were treated with beta,beta'-iminodipropionitrile (IDPN), which induces aggregation of vimentin intermediate filaments and prevents formation of soluble vimentin (37,38). The IDPN-treated cells were then stimulated with rCCL3 and/or IgE/Ag. As expected, soluble vimentin amounts decreased in the stimulated RBL-CCR1 cells after treatment with IDPN (Fig. 5B). Notably, FcɛRI and CCR1 stimulated phosphorylation of p38 MAP kinase and ERK1/2 was not reduced in the IDPN-treated RBL-CCR1 cells (Fig. S1). Due to low amount of soluble vimentin, the levels of associated MAP kinases with the vimentin were lower in the IDPN treated cells (Fig. 5B). Furthermore, IDPN treatment reduced CCL2 production in FcɛRI- and CCR1-activated RBL-CCR1 cells (Fig. 6A), suggesting involvement of soluble vimentin in the chemokine production.

Finally, we verified the involvement of vimentin in FcɛRI/CCR1 stimulated CCL2 production in BMMCs, a model of primary mast cells. Stimulating BMMCs with IgE/Ag plus rCCL3 enhanced formation of soluble vimentin, phosphorylation of MAP kinases, and CCL2 production (Fig. 6B and S2).
Furthermore, pre-treatment of BMMCs with IDPN reduced CCL2 production upon IgE/Ag and rCCL3 stimulation (Fig. 6B).

**Discussion**

Here, we identified vimentin as a component involved in the cross-talk between FcεRI- and CCR1-mediated signaling pathways in mast cells. FcεRI and/or CCR1 activation of RBL-CCR1 cells induced phosphorylation and subsequent disassembly of vimentin. Importantly, the disassembled and soluble vimentin interacted with phosphorylated ERK1/2 and p38 MAP kinases in the stimulated RBL-CCR1 cells. P38 MAP kinase plays a role in FcεRI- and CCR1-mediated CCL2 production in RBL-CCR1 cells. Conversely, inducing aggregation of vimentin filaments by IDPN reduced the levels of disassembled vimentin, and hence the interactions with phosphorylated MAP kinases and production of CCL2 in mast cells. These results suggest that vimentin could play a role in optimal CCL2 production in mast cells and basophiles.

Vimentin has been shown to interact with phosphorylated ERK1/2 in several cells such as neurons and adipocytes (35,37). Perlson et al observed that vimentin binds directly to phosphorylated ERK1/2, but not to phosphorylated p38 MAP kinase and non-phosphorylated forms of these MAP kinases (36). We observed that soluble vimentin interacted not only with phosphorylated ERK1/2 but also with phosphorylated p38 MAP kinase in FcεRI- and CCR1-activated RBL-CCR1 cells. Since vimentin is capable of interacting with a variety of proteins involved in cell signaling cascades (31,39), phosphorylated p38 MAP kinase might indirectly interact with vimentin by binding to other vimentin-binding proteins. In neurons, it has been shown that the complex of vimentin and phosphorylated ERK1/2 moves from the cytoplasm to the nucleus (35). Translocation of MAP kinases into the nucleus is a mechanism for the gene expression of cytokines and chemokines. Disassembled vimentin, which is generated by FcεRI and/or CCR1 stimulation, may act as a shuttle protein for the MAP kinases to enter to the nucleus in mast cells, as observed in neurons (see hypothetical model in Fig. 7).

p38 MAP kinase has been shown to play a critical role in calcium ionophores-, cytokine-, or FcεRI-mediated CCL2 production in cultured mast cells such as BMMC and a human leukemic mast cell line (HMC)-1 (40-42). Consisted with these previous studies, p38 MAP kinase is apparently involved in FcεRI- and CCR1-mediated CCL2 production in RBL-CCR1 cells, since inhibiting p38 MAP kinase reduced CCL2 production. Co-stimulation with rCCL3 and IgE/Ag enhanced phosphorylation of ERK1/2 and p38 MAP kinase in RBL-CCR1 cells and BMMCs. Such enhanced phosphorylation of p38 kinase and the association of the activated kinase with the disassembled vimentin could lead to its translocation into the nucleus and the enhanced CCL2 production we
observed. Although vimentin interacts with phosphorylated ERK1/2, only p38 MAP kinase is required for CCL2 production in RBL-CCR1 cells. This may be due to enhancer and promoter regions in the CCL2 gene that only p38 MAP kinase may regulate. It has shown that ERK1/2 and p38 MAP kinase activate a different set of substrates and transcription factors (43). Future studies on enhancer/promoter region in CCL2 gene would elucidate a detailed molecular mechanism for p38 MAP kinase-mediated chemokine production in mast cells.

Vimentin seems to be involved in mast cell degranulation (39). It was hypothesized that phosphorylation of vimentin induces disassembly of the intermediate filaments, subsequently increasing the mobility of granules (39,44,45). Supporting this hypothesis, degranulation of BMMCs was enhanced by vimentin deficiency (39). In this study, we found that the levels of vimentin disassembly (i.e. levels of soluble vimentin) were not associated with degranulation levels in FcεRI and/or CCR1-activated RBL-CCR1 cells (not shown). Degranulation of RBL-CCR1 cells was previously shown to be synergistically enhanced by rCCL3 and IgE/Ag stimulation in a CC chemokine dose-dependent manner: around 55% or 80% degranulation were induced by 10 or 100 ng/ml of rCCL3 together with IgE/Ag (11). However, the level of soluble vimentin in the stimulated cells was almost same (Fig. 5A). These results suggest that the disassembly of vimentin filaments do not determine the level of degranulation in mast cells.

Vimentin has many phosphorylation sites (27,28,31). FcεRI and CCR1 co-stimulation in RBL-CCR1 cells enhanced phosphorylation of tyrosine, serine and threonine residues of vimentin. It has been shown that phosphorylations at serines 55, 71, 72 and 82 of vimentin are coordinated by several kinases (i.e. Rho kinase, Aurora B) and induce disassembly of the protein filaments in mitotic cells (9,23-25,33). Here, co-stimulation of the cells with 10 ng/ml of rCCL3 plus 10 ng/ml of IgE/Ag induced the highest levels of phosphorylation at serines 55, 71 and 82. Interestingly, the level of soluble vimentin in the cells stimulated with 10 or 100 ng/ml of rCCL3 plus 10 ng/ml of IgE/antigen were comparable. These results suggest that not only serines 55, 71, 82, but other phosphorylation sites could regulate the disassembly of vimentin in activated RBL-CCR1 cells. Further study will be necessary to elucidate the mechanisms and roles of vimentin phosphorylation in the signaling cascades and function of mast cells and basophils.

In summary, our results suggest that vimentin could be a component inducing optimal CCL2 production in mast cells. Since increased expression of CCL2 has been observed in tissues of allergic patients, our findings could provide clues to unraveling detailed molecular mechanisms underlying allergic inflammation.
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Footnotes

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Figure legends

Fig. 1 Synergistic CCL2 production in RBL-CCR1 cells co-stimulated via FcεRI and CCR1. A. After sensitization with anti-DNP-IgE mAb, RBL-CCR1 cells were stimulated with 10 ng/ml of DNP-HSA and/or various concentrations of rCCL3 for 2, 4, or 6 h, before determining CCL2 production by ELISA. B. To inhibit p38 MAP kinase activation, the cells were incubated with 10 or 50 µM SB203580 for the last 1 h of sensitization. C. To inhibit ERK1/2 activation, the cells were incubated with 10, or 50 µM PD98058 for the last 1 h of sensitization. The cells were then stimulated with 10 ng/ml DNP-HSA and/or 100 ng/ml of rCCL3 for 6 h. The plotted data of mean CCL2 concentrations in culture supernatants measured by ELISA are representative of three independent experiments. * \( p<0.01 \)

Fig. 2 Increased phosphorylation of p38 MAP kinase and ERK1/2 in RBL-CCR1 cells stimulated via FcεRI and CCR1. After sensitization with anti-DNP-IgE mAb, RBL-CCR1 cells were stimulated with 10 ng/ml DNP-HSA and/or 100 ng/ml of rCCL3 for 5 min. To inhibit p38 kinase and ERK1/2 activation, the cells were incubated with 50 µM SB203580, or 50 µM PD98058 for the last 1 h of sensitization. Levels of phosphorylated and total p38 kinase (A) and ERK1/2 (B) protein in total cell lysates were measured by ELISA. The data are representative for three independent experiments. nd, not detectable; \(<1.0 \text{ U/ml} \). * \( p<0.01 \) ** \( p<0.05 \)

Fig. 3 Differential protein expression analysis in RBL-CCR1 cells stimulated via FcεRI and CCR1. After sensitization with anti-DNP-IgE, RBL-CCR1 cells were stimulated with or without 10 ng/ml of DNP-HSA and 100 ng/ml of rCCL3 for 5 min. A. Total cell lysates of the unstimulated cells, or B. total cell lysates of the stimulated cells were subjected to 2-D PAGE and CMB-stained. Protein spots up-regulated in a gel are shown with the red arrows. MW, molecular weight.

Fig. 4 Phosphorylation of vimentin in RBL-CCR1 cells stimulated via FcεRI and CCR1. After sensitization with anti-DNP-IgE mAb, RBL-CCR1 cells were stimulated with 10 ng/ml of DNP-HSA and/or various concentration of rCCL3 for 5 min. Total cell lysates of non-stimulated or the stimulated cells were subjected to immunoprecipitation with anti-vimentin mAb. The immunoprecipitated proteins were analyzed by immunoblotting using antibodies against phosphotyrosine (Tyr), phosphothreonine (Thr), phosphoserine (Ser), phosphorylated vimentin at Ser 6, 55, 71, or 82, or vimentin. The data are representative of two independent experiments.
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Fig. 5 Interaction of vimentin and phosphorylated MAP kinases in RBL-CCR1 cells stimulated via FcεRI and CCR1. After sensitization with anti-DNP-IgE, RBL-CCR1 cells were stimulated with 10 ng/ml of DNP-HSA and/or various concentration of rCCL3 for 5 min. A. After the stimulation, soluble form of vimentin were extracted in cell lysates prepared with 1% Nonidet P-40 lysis buffer and analyzed by immunoblotting using anti-vimentin Abs. B. The cells were incubated with beta,beta’-iminodipropionitrile IDPN (+) to promote aggregation of vimentin intermediate filaments for the last 1 h of sensitization. Soluble forms of vimentin in the cells were extracted and subjected to immunoprecipitation using anti-vimentin mAb and analyzed by immunoblotting using anti-phosphorylated ERK1/2 mAb, anti-phosphorylated p38 kinase mAb, or anti-vimentin Abs. The data are representative of three independent experiments.

Fig. 6 Reduced CCL2 production by beta,beta’-iminodipropionitrile treatment in RBL-CCR1 cells and BMMCs stimulated via FcεRI and CCR1. RBL-CCR1 cells and BMMCs were sensitized with anti-DNP-IgE and were incubated with IDPN for the last 1 h of sensitization. The cells were then stimulated with 10 ng/ml of DNP-HSA and/or 10, or 100 ng/ml of rCCL3. Culture supernatants were harvested after stimulation. Concentrations of CCL2 in the supernatant were measured by ELISA. The plotted CCL2 concentration data are representative of three independent experiments. * p<0.01

Fig. 7 Hypothetical model for FcεRI and CCR1 mediated signaling pathways in mast cells. FcεRI and CCR1 activation synergistically induces phosphorylation of ERK1/2, p38 MAP kinase and vimentin in mast cells. Phosphorylation of vimentin induces disassembly of the filament protein. The disassembled vimentin interacts with phosphorylated ERK1/2 directly, or with phosphorylated p38 MAP kinase indirectly via other vimentin-binding protein(s) (protein X). Vimentin could then act as a shuttle protein for the activated MAP kinases to translocate them into the nucleus.
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## Table 1. Identified proteins in RBL-CCR1 cells co-stimulated via FcεRI and CCR1

<table>
<thead>
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Figure 1 Toda et al
Figure 2 Toda et al.
Figure 3 Toda et al
Figure 4 Toda et al
Figure 5 Toda et al

A

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Vimentin

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IDPN (-)  
Vimentin  
Phospho-ERK1/2  
Phospho-p38k

IDPN (+)  
Vimentin  
Phospho-ERK1/2  
Phospho-p38k
Figure 6 Toda et al
Figure 7 Toda et al
Evidence that formation of Vimentin/Mitogen-Activated Protein (MAP) kinase complex mediates mast cell activation following Fc?RI/CC chemokine receptor 1 cross-talk
Masako Toda, Chuan-Hui Kuo, Satty K. Borman, Ricardo Micheler Richardson, Akihito Inoko, Masaki Inagaki, Andrea Collins, Klaus Schneider and Santa Jeremy Ono

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