

Osteopontin is produced by mast cells and affects IgE-mediated degranulation and migration of mast cells

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Osteopontin (OPN), originally discovered in bone as an extracellular matrix protein, was identified in many cell types in the immune system, presumably being involved in many aspects of pathogenesis of inflammatory and immune diseases. Mast cells are also involved in such pathological aspects by secreting multiple mediators. However, it has not been determined whether mast cells produce OPN and whether it affects their function. To test this, we used murine fetal skin-derived cultured mast cells (FSMC) and bone marrow-derived cultured mast cells. We found that OPN was spontaneously produced by FSMC and inducible by ionomycin and FcεRI aggregation in bone marrow-derived cultured mast cells. In the presence of mast cell growth factors, FSMC were similarly generated from both OPN-deficient (OPN^{-/-}) and -sufficient (OPN^{+/+}) mice without significant differences in yield, purity, granularity, and viability. Using OPN^{-/-} FSMC, we found that recombinant OPN augmented IgE-mediated degranulation and induced FSMC chemotaxis. Both effects were mediated by OPN receptors (*i.e.* CD44 and integrin αv). IgE-mediated passive cutaneous anaphylaxis was significantly reduced in OPN^{-/-} mice compared with OPN^{+/+} mice, indicating physiological relevance of OPN. These results indicate that OPN is a mast cell mediator, enhances mast cell responses to antigen, and thus may influence mast cell-related pathological conditions.

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Abbreviations: **BMMC:** bone marrow-derived cultured mast cells · **FSMC:** fetal skin-derived cultured mast cells · **HSA:** human serum albumin · **OPN:** osteopontin · **PCA:** passive cutaneous anaphylaxis · **r:** recombinant · **RA:** rheumatoid arthritis

Introduction

Mast cells are widely distributed throughout the body, being especially abundant at the environmental interfaces (*e.g.* skin, lung, and mucosal surfaces), where they are located mostly around blood vessels, lymph vessels, and peripheral nerves. Mast cells have long been known as major effector cells in acute allergic responses because cross-linking of IgE-bound high-affinity IgE receptors (FcεRI) on mast cells by multivalent antigens (Ag) triggers immediate degranulation and subsequent

release of a wide variety of chemical and lipid mediators from these cells [1–3]. Recent advances in mast cell biology revealed that mast cells are also equipped with potent capacities to participate in a wide array of pathological processes (e.g. innate and adaptive immune responses, autoimmune responses, tissue remodeling/wound repair, and tumor progression [2, 4–8]) by elaborating a huge array of products secreted by these cells and/or by their direct interaction with other cell types.

Osteopontin (OPN) is an arginine-glycine-aspartate-containing glycoprotein with pleiotropic properties expressed in many tissues and cell types [9]. OPN has been implicated in a wide variety of physiological and pathological conditions. For example, OPN has been reported to participate in bone remodeling [10], wound healing [11], dystrophic calcification, coronary restenosis, and tumor cell metastasis [9]. In addition to the involvement of OPN in the above-mentioned pathological conditions, accumulating evidence has revealed another importance of OPN in regulating inflammatory and immune responses. Among immune cells, macrophages, activated T cells, myeloid DC, plasmacytoid DC, and NKT cells have been reported to produce OPN, exhibiting many effects on the immune system [12–16].

For example, OPN, also called early T lymphocyte activation-1 (Eta-1), reportedly played an important role in efficient Th1 immune responses in many experimental settings. Working with OPN-deficient (OPN^{-/-}) mice, Ashkar *et al.* [17] demonstrated that OPN contributed to host defense against pathogens (e.g. herpes simplex virus and *Listeria monocytogenes*) and Th1-dependent granuloma formation. It functioned as an essential cytokine for an early step of effective Th1 immune responses by differentially favoring the expression of a Th1-driving cytokine, IL-12, while inhibiting a Th2-skewing cytokine, IL-10, secreted by macrophages. Recent studies further supported the involvement of OPN derived from DC and/or T cells in Th1 immune responses. Indeed, OPN induced the maturation of myeloid DC toward a Th1-promoting phenotype [13]. In addition, T-bet-dependent OPN expression by activated T cells skewed CD4⁺ and CD8⁺ T cells towards Th1 and Tc1 CD8⁺ T cells, respectively [14]. More recently, intracellular OPN expression by plasmacytoid DC was found to be an essential regulator for IFN- α production by these cells, which were involved in Th1 immunity to viral infections and anti-tumor immunity [15].

Another recently reported role of OPN is that NKT cell-derived OPN was a major mediator of concanavalin A-induced hepatitis by augmenting NKT cell activation and triggering neutrophil infiltration [16]. Furthermore, OPN has been implicated in several autoimmune diseases including multiple sclerosis (MS), experimental allergic encephalomyelitis (EAE), an

animal model for human MS [18, 19], rheumatoid arthritis (RA) [20], and animal RA models [21, 22]. Thus, OPN is now believed to be an important mediator or regulator for complex processes of many inflammatory and immune diseases.

Since OPN and mast cells have been separately reported to be involved in the same or similar inflammatory and immune diseases, we hypothesized that mast cells may produce OPN and it may affect their function and participate in such diverse pathological processes. To test this hypothesis, we used murine fetal skin-derived cultured mast cells (FSMC) generated by a culture method recently established by our laboratory [23]. In this method we cultured single-cell suspensions propagated from day-14 or -15 fetal skin in the presence of stem cell factor (SCF) and IL-3 for 2 wk, and obtained relatively large numbers of mast cells at high purity (>98%). FSMC maintained the important features of freshly isolated skin mast cells including their surface phenotype and cytokine and chemokine profiles [23].

In addition, we have found that FSMC possess many characteristic features of connective tissue-type mast cells. These include (1) higher content of histamine and heparin than bone marrow-derived cultured mast cells (BMMC), (2) their capacity for degranulation in response to cationic secretagogues (i.e. compound 48/80 and substance P), (3) the high expression of CD49b, and (4) cytokine and chemokine profiles [23]. Thus, FSMC, representing connective tissue-type mast cells, serve as an excellent model for *in vitro* studies of skin-derived mast cells or connective tissue-type mast cells. Indeed, we found distinct expression profiles of TLR and endothelin-1 receptors between FSMC and BMMC [24, 25]. Reflecting the differences, we observed contrasting impacts of TLR activators and endothelin-1 on these two mast cell types. Taking advantage of these two types of mast cell cultures, we here examined OPN production and the effects of OPN on these different cell systems.

Results

Impact of OPN on the development of FSMC

To explore the effects of OPN on the development of FSMC, we used OPN^{-/-} and OPN^{+/+} fetal skin derived from the same pregnant mouse to generate FSMC. Fourteen days after culturing dissociated skin cell suspensions derived from each fetus in the presence of SCF and IL-3, we then compared their yield, purity, viability, and granularity.

We could generate c-kit⁺ CD45⁺ FSMC derived from OPN^{-/-} fetal skin similar to FSMC derived from OPN^{+/+} fetal skin, with no significant differences in their yield ($3.7 \times 10^6 \pm 2.1 \times 10^6$ /flask vs. $3.4 \times 10^6 \pm 1.6 \times 10^6$ /

flask, $n=3$), purity ($>98\%$ for both FSMC), viability ($>98\%$ FSMC for both FSMC), and granularity (assessed by G-mean of side scatter by FACS; 332 ± 61 vs. 332 ± 50 , $n=3$). Since OPN was secreted by wild-type mice (Fig. 1), OPN was not required for the development of FSMC from fetal skin. Consistent with these results, we observed comparable numbers of mast cells in the dermis of skin in both $OPN^{-/-}$ and $OPN^{+/+}$ adult mice (248 ± 13 vs. 226 ± 16 cells/cm, $n=3$). Since it has been reported that other leukocytes (e.g. $CD4^{+}$ and $CD8^{+}$ T cells, B cells, $Gr-1^{+}$ cells, and $Ter119^{+}$ cells) were present in equal numbers in secondary lymphoid organs [17] or bone marrow [26] in both $OPN^{-/-}$ mice and their wild-type $OPN^{+/+}$ littermates, OPN may have a minimal impact on the development of a broad range of leukocytes.

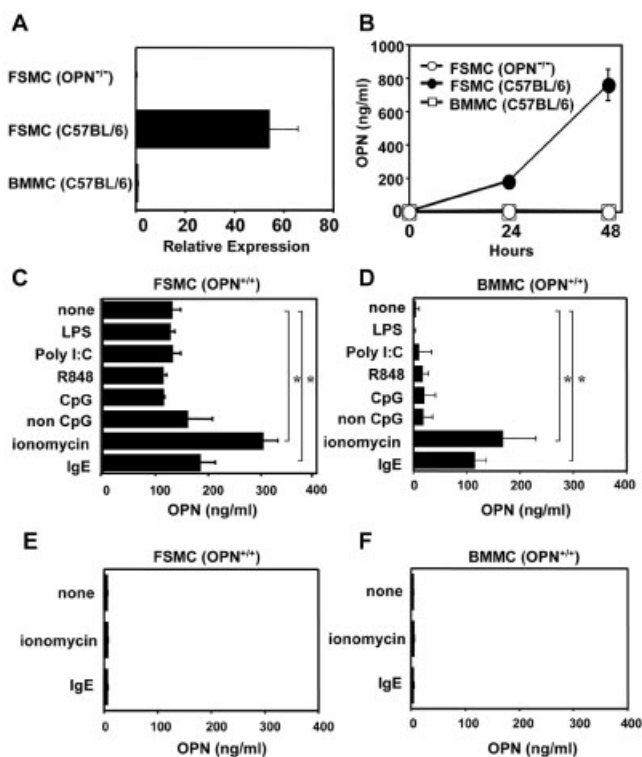


Figure 1. OPN production by mast cells. (A) mRNA purified from FSMC and BMMC was subjected to quantitative real-time RT-PCR using SYBR Green I for OPN. The relative gene expression levels were determined in triplicate by differences of cycle threshold numbers. Data are representative of two independent experiments. (B) Supernatants from FSMC or BMMC (1×10^6 cells/mL) cultured in their growth medium for 0, 24, or 48 h were tested for OPN protein using an OPN ELISA kit. Data are representative of three independent experiments, showing the means $\pm$ SD from triplicate cultures. (C–F) FSMC or BMMC were stimulated for 24 h (C, D) or 2 h (E, F) with or without LPS (100 ng/mL), poly(I:C) (100 μ g/mL), R848 (10 μ g/mL), CpG (0.1 μ M), non-CpG (0.1 μ M), ionomycin (1 μ M), and IgE plus Ag and then each supernatant was tested for OPN protein (ELISA).

Production of OPN by mast cells

We next determined whether mast cells would produce OPN. As shown in Fig. 1A, FSMC derived from C57BL/6 mice spontaneously expressed OPN mRNA, whereas BMMC from the same strain only marginally expressed its mRNA, and $OPN^{-/-}$ FSMC expressed no OPN mRNA at all, as expected. Corresponding to OPN mRNA expression by C57BL/6 mice, only FSMC from C57BL/6 mice secreted OPN protein in the culture supernatant in a culture period-dependent fashion, whereas BMMC from the same strain and $OPN^{-/-}$ FSMC did not secrete any detectable levels of OPN (Fig. 1B). In addition, we failed to detect OPN in the supernatant of $OPN^{+/+}$ FSMC immediately after their degranulation (data not shown). These results indicate that OPN is not a component of mast cell granules and FSMC secrete OPN in their culture medium.

We next examined whether activation of mast cells induces OPN expression by FSMC and BMMC. To do so, FSMC or BMMC were treated with various stimuli for 24 h before measuring OPN content in the supernatant. As shown in Fig. 1C, ionomycin and Fc ϵ RI aggregation significantly augmented OPN production by $OPN^{+/+}$ FSMC, whereas TLR activators (i.e. LPS, poly(I:C), R848, and CpG) had no effect. Similarly, ionomycin and Fc ϵ RI aggregation significantly induced OPN production by BMMC, whereas the TLR activators did not (Fig. 1D). The augmentation or induction of OPN by FSMC or BMMC, respectively, when stimulated with ionomycin or Fc ϵ RI aggregation, was not observed at an earlier time point (e.g. 2 h after stimulation) (Fig. 1E, F). Freshly isolated murine peritoneal mast cells, which are frequently used as a model of connective tissue-type mast cells, were stained with both anti-OPN Ab and avidin, indicating that they expressed OPN (data not shown). Since we did not examine other types of mast cells, it is not clear at this moment whether OPN is constitutively produced by connective tissue-type mast cells and inducible in immature or mucous-type mast cells.

Effects of OPN on degranulation by FSMC

One of the functional hallmarks of mast cells is their degranulation in response to Fc ϵ RI aggregation triggered by IgE plus multivalent Ag and other stimuli (e.g. calcium ionophore, ionomycin). We first compared ionomycin- and Fc ϵ RI aggregation-induced degranulation between $OPN^{-/-}$ and $OPN^{+/+}$ FSMC, using a β -hexosaminidase release assay. As shown in Fig. 2A, Fc ϵ RI aggregation as well as ionomycin induced the degranulation of both cell types at comparable levels, indicating that the secretion machinery in $OPN^{-/-}$ FSMC is intact and works similarly in response to signals from

FcεRI compared to those of OPN^{+/+} FSMC, which were continuously exposed to secreted OPN. We then determined whether OPN would induce degranulation by mast cells, if added exogenously. Recombinant (r) OPN failed to induce β-hexosaminidase release from OPN^{-/-} FSMC and IgE-bound cells (Fig. 2B), indicating that OPN is not an inducer for mast cell degranulation,

even though mast cells are sensitized with monomeric IgE Ab.

We next examined whether rOPN would affect the degranulation induced by FcεRI aggregation. Before the aggregation of FcεRI by cross-linking cell-bound IgE with multivalent Ag, OPN^{-/-} FSMC were pretreated with rOPN. As shown in Fig. 2C, rOPN significantly up-regulated the degranulation induced by FcεRI aggregation in a dose-dependent fashion. Furthermore, this up-regulation was dependent on the Ag concentration (Fig. 2D). This effect of rOPN on FSMC degranulation was similarly observed in BMMC (Fig. 2E, F). Neutralizing Ab against OPN, but not control Ab, significantly inhibited this IgE-mediated degranulation by OPN^{-/-} FSMC or OPN^{+/+} FSMC (Fig. 3A), confirming that the

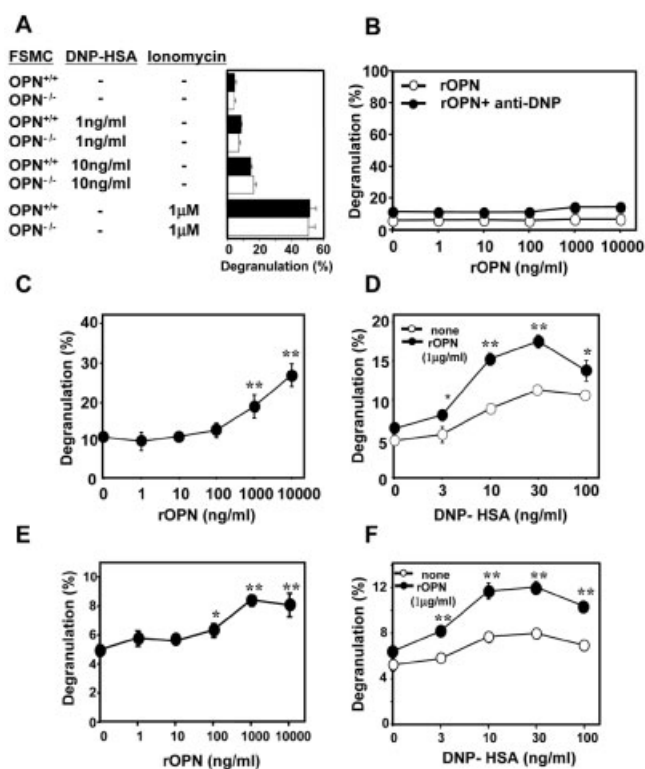


Figure 2. Effects of OPN on degranulation by mast cells. (A) OPN^{+/+} FSMC (filled bars) and OPN^{-/-} FSMC (open bars) were pretreated for 18 h with mouse IgE anti-DNP mAb (0.3 μg/mL). Cells were then stimulated with DNP-HSA (1 or 10 ng/mL) for 30 min, and degranulation assessed by β-hexosaminidase assay. For control, both types of cells were stimulated for 10 min with ionomycin (1 μM). Degranulation was then assessed by β-hexosaminidase assay. (B) OPN^{-/-} FSMC pretreated for 18 h in the presence (filled circles) or absence (open circles) of mouse IgE anti-DNP mAb (0.3 μg/mL) were stimulated for 30 min with rOPN at the indicated concentrations. Our preliminary experiment demonstrated that 30 min incubation with OPN is long enough to reach the plateau for the maximal augmentation in this assay condition (not shown). Degranulation was then assessed by β-hexosaminidase assay. (C) OPN^{-/-} FSMC pretreated for 18 h with mouse IgE anti-DNP mAb (0.3 μg/mL) were treated with rOPN at the indicated concentrations for 30 min. Cells were then stimulated with DNP-HSA (3 ng/mL) for 30 min, and degranulation was then assessed as above. (D) OPN^{-/-} FSMC pretreated for 18 h with mouse IgE anti-DNP mAb (0.3 ng/mL) were treated with rOPN (1 μg/mL) for 30 min. Cells were then stimulated with DNP-HSA at graded concentrations for 30 min followed as above. Data are representative of three similar independent experiments, showing the means ± SD from triplicate cultures; **p* < 0.05, ***p* < 0.01. (E, F) OPN^{-/-} BMMC were similarly tested.

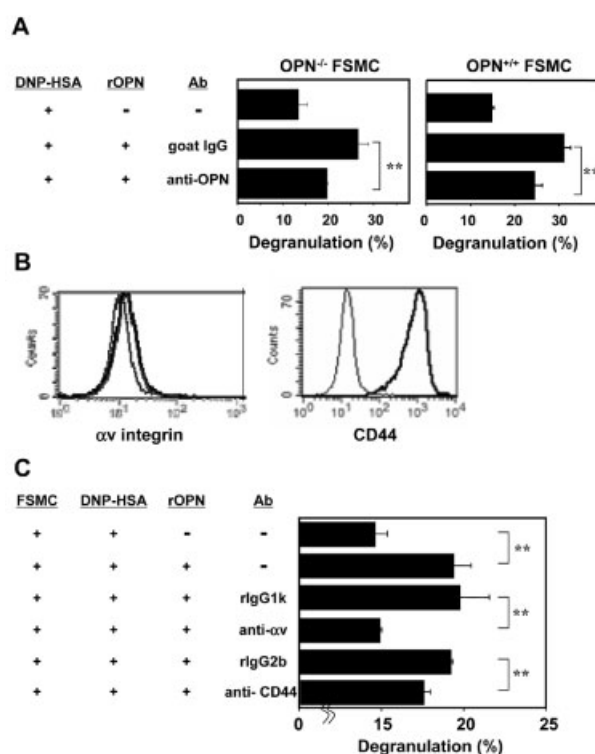


Figure 3. OPN-induced augmentation of IgE-mediated degranulation is dependent on CD44 and the integrin αv subunit on FSMC. (A) IgE-sensitized OPN^{-/-} FSMC or OPN^{+/+} FSMC were treated with rOPN (1 μg/mL) pre-incubated with anti-OPN Ab (10 μg/mL) or control Ab. Cells were then stimulated with DNP-HSA (10 ng/mL) for 30 min, and degranulation was assessed by β-hexosaminidase assay. (B) OPN^{-/-} FSMC were stained with anti-αv integrin mAb and anti-CD44 mAb (thick lines) or control Ab (thin lines) gated on CD45⁺ c-kit⁺ cell populations. (C) IgE-sensitized OPN^{-/-} FSMC were treated for 30 min with anti-αv mAb, anti-CD44 mAb (10 μg/mL) or control mAb, followed by the treatment with rOPN (1 μg/mL). Cells were then stimulated with DNP-HSA (3 ng/mL) for 30 min, and degranulation was assessed by β-hexosaminidase assay. Data are representative of three similar independent experiments, showing the means ± SD from triplicate cultures; ***p* < 0.01, brackets indicate groups being compared.

augmentation was mediated by added rOPN. These results clearly indicate that the binding of OPN on mast cells before Fc ϵ RI aggregation augments the degranulation triggered by Fc ϵ RI aggregation.

FSMC expressed both integrin α v and CD44 (Fig. 3B), indicating that these two receptors are putative OPN receptors expressed by FSMC. We next examined their functional role in OPN-induced augmentation of IgE-mediated degranulation. Anti- α v mAb (RMV7) completely blocked this OPN-mediated augmentation, whereas anti-CD44 mAb (IM7) partially blocked this effect (Fig. 3C). Both appropriate control mAb had no effect. These results indicate that integrin α v is more effectively involved in OPN-mediated augmentation of degranulation by FSMC than CD44.

FSMC migrate toward OPN

OPN has been reported to be a chemoattractant to a variety of cell types, including monocyte/macrophages and DC [22, 27, 28]. Since bone marrow-derived DC migrated toward OPN in both α v β 3- and CD44-dependent manners [28], we sought to determine whether OPN also induced the migration of FSMC. Using a transwell system, we observed that OPN^{-/-} FSMC migrated toward rOPN in a dose-dependent manner (by 100 ng/mL) with high-dose inhibition at 1000 ng/mL (Fig. 4A). The dose ranges of OPN-dependent migration were similar to those of SCF-dependent migration of FSMC (Fig. 4B), consistent with a previous report working with rat peritoneal mast cells [29]. FSMC migrated to rOPN in a manner consistent with migration to stimuli (chemotaxis) rather than random migration (chemokinesis), because increasing concentrations of rOPN in the upper chamber inhibited FSMC migration to rOPN (Table 1). This migration was almost completely blocked by anti-CD44 mAb and significantly inhibited by anti- α v mAb, indicating that OPN-induced FSMC migration is dependent on both CD44 and integrin α v (Fig. 4C).

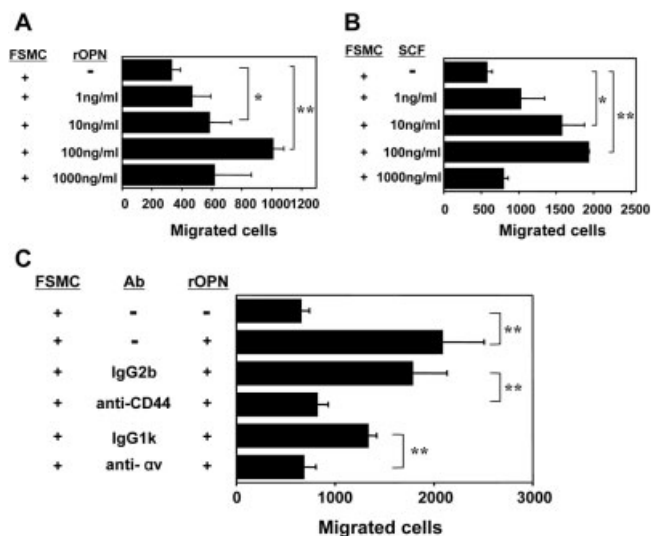


Figure 4. OPN-induced migration of FSMC is dependent on CD44 and integrin α v subunit on FSMC. The migration of FSMC toward rOPN was measured in a transwell chemotaxis assay. OPN^{-/-} FSMC were plated into the upper chamber. rOPN (A) or SCF (B) at the indicated concentrations were added in the lower chamber. After 6 h incubation, migration was measured by counting the numbers of cells harvested from the lower chamber. (C) OPN^{-/-} FSMC were treated for 1 h with anti- α v mAb, anti-CD44 mAb or control mAb (10 μ g/mL). After extensive washing, the cells were assessed for migration toward rOPN (100 ng/mL) as described in (A). Data are representative of three similar independent experiments, showing the mean of migrated cells $\pm$ SD ($n=3$); * $p<0.05$, ** $p<0.01$, brackets indicate groups being compared.

FSMC secrete biologically active OPN

We next examined whether OPN in the culture supernatant of OPN^{+/+} FSMC had the same migratory activity. To test this, we added supernatant (collected from OPN^{+/+} FSMC cultured 24 h without SCF and IL-3) that was pretreated with a neutralizing OPN mAb or a control mAb in the lower chamber. As shown in Fig. 5A, the supernatant (containing 10 ng/mL OPN, assessed by OPN ELISA) induced FSMC migration, and

Table 1. Checkboard analysis of rOPN as a chemoattractant^{a)}

rOPN in lower chambers (ng/mL)	Number of migrated cells		
	rOPN in upper chambers (ng/mL)		
	0	20	100
0	543.8 $\pm$ 129.1	581.3 $\pm$ 70.4	662.5 $\pm$ 102.3
20	1216.7 $\pm$ 80.4	603.0 $\pm$ 74.0	579.9 $\pm$ 96.9
100	2112.5 $\pm$ 291.3	1295.0 $\pm$ 256.5	714.3 $\pm$ 149.7

^{a)} rOPN was added in the lower chamber at the indicated concentrations. OPN^{-/-} FSMC were added into the upper chamber with indicated concentrations of rOPN. After 6 h incubation, cells in the lower chamber were collected for counting cell numbers. Results are representative of mean $\pm$ SD ($n=3$) of two independent experiments.

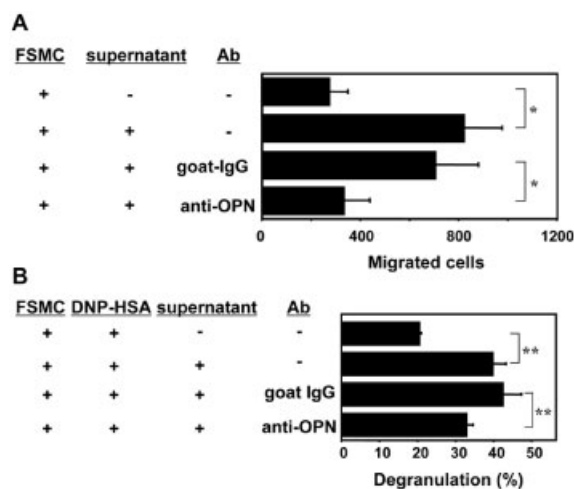


Figure 5. FSMC-derived OPN is biologically active. The biological activity of OPN in the supernatant of OPN^{+/+} FSMC was evaluated by a migration assay (A) and degranulation assay (B). (A) OPN^{-/-} FSMC were plated into the upper chamber. The culture supernatant of OPN^{+/+} FSMC pretreated for 1 h with anti-OPN Ab (10 µg/mL) or control Ab was added in the lower chamber. After 6 h incubation, migration was measured by counting the numbers of cells harvested from the lower chamber. (B) IgE-sensitized OPN^{-/-} FSMC were treated for 30 min with the supernatant of OPN^{+/+} FSMC pre-incubated with anti-OPN Ab (10 µg/mL) or control Ab. Cells were then stimulated with DNP-HSA (3 ng/mL) for 30 min, and degranulation was assessed by β-hexosaminidase assay. The supernatant of OPN^{+/+} FSMC was collected 48 h after the cells were cultured (1 × 10⁶ cells/mL) in phenol red-free medium, and one fourth of final volume was added for the assay. Data are representative of two independent experiments, showing the mean of migrated cells ± SD (n=3); *p<0.05, **p<0.01, brackets indicate groups being compared.

this migration was almost completely blocked by anti-OPN mAb, but not by control mAb. We also tested whether OPN in culture supernatant affects the IgE-mediated degranulation. As shown in Fig. 5B, the supernatant (containing 178 ng/mL OPN, assessed by OPN ELISA) augmented the degranulation triggered by FcεRI aggregation. This augmentation was significantly, but modestly blocked by anti-OPN mAb, but not by control mAb. These results indicate that OPN secreted from wild-type FSMC is biologically active.

Passive cutaneous anaphylaxis reactions are diminished in OPN^{-/-} mice

To assess the *in vivo* relevance of OPN-induced augmentation of IgE-mediated degranulation, we used an IgE-mediated passive cutaneous anaphylaxis (PCA) reaction, in which normal tissue-resident mast cells are involved. As shown in Fig. 6, the PCA reaction in OPN^{-/-} mice is significantly reduced, compared with those in OPN^{+/+} mice, suggesting that OPN may be tuning the magnitude of IgE-mediated mast cell degranulation

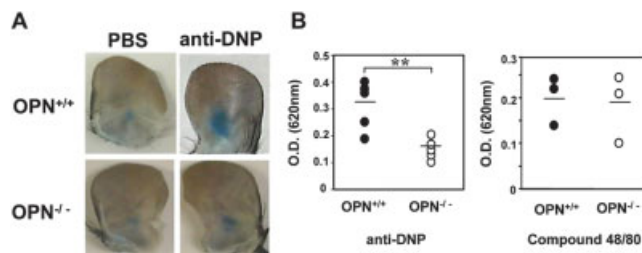


Figure 6. PCA reaction is significantly reduced in OPN^{-/-} mice compared with OPN^{+/+} mice. (A) Representative Ag-induced PCA reactions in OPN^{-/-} and OPN^{+/+} mice. (B) Quantification of Ag-induced (left) and compound 48/80-induced (right) reactions. For Ag-induced reactions, the right ear and left ear of mice (n=5) were intradermally injected with IgE anti-DNP mAb or PBS, respectively. After 24 h, DNP-HAS in 1% Evans' blue solution (100 µL) was injected intravenously. Thirty minutes after the injection, the ears were examined for the dye amounts. For compound 48/80-induced reactions, compound 48/80 was injected intradermally in the ears, immediately after the intravenous injection of 1% Evans' blue solution. Twenty minutes after the injection, the ears were examined for the dye amounts. Data are representative of two similar independent experiments, showing the absorbency (620 nm) of the dye extracted from each ear (n=5 for Ag-induced and n=3 for compound 48/80-induced reaction); **p<0.01, brackets indicate groups being compared.

in vivo. In a sharp contrast, a mast cell secretagogue, compound 48/80, induced comparable levels of PCA reactions in both OPN^{-/-} and OPN^{+/+} mice, supporting that mast cells in dermal tissues are equivalent in both mice, both in terms of their numbers and degranulation capabilities.

Discussion

Working with FSMC, we have revealed that OPN is capable of regulating various functions of mast cells. To recapitulate the essence of our observations, OPN was found to (1) be spontaneously produced by FSMC and be inducible in BMMC in response to stimuli (e.g. FcεRI aggregation and ionomycin), (2) significantly augment mast cell degranulation by FcεRI aggregation, and (3) promote the migration of mast cells. Our results have also revealed that OPN secreted by FSMC was biologically active and had no effect on the development of FSMC.

With respect to the development of leukocytes in OPN-null mice, it has been reported that OPN^{-/-} mice had no major defects in T cell, B cell, and NKT cell development in secondary lymphoid tissues [16, 17]. Furthermore, a recent study demonstrated that OPN deficiency had minimal impact on the numbers of lineage marker-positive mature blood cells (B cells, T cells, granulocytes, and erythrocytes) in bone marrow,

although hematopoietic stem cells were significantly increased in OPN^{-/-} mice compared with their wild-type littermates, implicating OPN as a component of the hematopoietic stem cell niche that negatively regulates stem cell pool size [26]. We observed that OPN deficiency had no significant effect on the *in vitro* generation of FSMC from their precursors in fetal skin, or on the mast cell numbers that reside in the adult dermis (Fig. 1). Our observations provide additional information on the unimpaired development of another type of leukocytes, mast cells, in the context of OPN deficiency.

Our decision to examine OPN production by mast cells was prompted by independent lines of evidence indicating mast cells and OPN to be involved in a wide array of same or similar pathological conditions. For example, growing evidence suggests now that mast cells participate in the inflammatory process and subsequent demyelination in MS [30]. Indeed, elevated numbers of mast cells in MS plaques have been reported [31], and this observation was supported by gene expression profiling of MS plaques demonstrating the elevated expression of FcεRI and a mast cell-specific mediator, tryptase [32]. Using mast cell-knock-out and -knock-in mice, it has been proven that mast cells were essential for the development of EAE, an animal model for human MS [33]. On the other hand, an abundance of OPN transcripts was identified when analyzing a large-scale sequencing of cDNA libraries derived from MS plaques. Microarray analysis of spinal cords from EAE rats also revealed increased OPN transcripts. In addition, OPN^{-/-} mice were resistant to progressive EAE, compared to wild-type mice, and myelin-reactive T cells from OPN^{-/-} mice produced more IL-10 and less IFN-γ than those from wild-type mice [18]. Thus, OPN seems to be central for the pathogenesis of this Th1-mediated demyelinating disease.

Another example of an important role played by mast cells and OPN in the pathogenesis of inflammatory diseases was found in inflammatory arthritis, including RA. Increased numbers of mast cells have been detected in the synovial tissues and fluids of RA and other inflammatory arthritis patients, presumably serving as a driving force in shaping microenvironments for inflammatory cycles by recruiting other inflammatory and immune cells and/or by elaborating proteolytic and cytokine activities [34, 35]. A recent study more clearly demonstrated an essential role of mast cells in the pathogenesis of K/BxN serum-induced arthritis using mast cell-knock-out and -knock-in mice [36]. On the other hand, OPN has also been shown to be involved in the pathogenesis of human RA [20] and animal RA models by its multiple effects [21, 22]. One may consider that mast cell-derived OPN may participate in the processes of inflammatory arthritis. Likewise, mast cell-

derived OPN may participate in other pathological processes such as angiogenesis, tissue remodeling, atherosclerosis, and tumor progression in which both mast cells [8, 37–39] and OPN [9, 40, 41] have been separately reported to be involved. Since many immune cells produce OPN, it is important to evaluate whether or not mast cell-derived OPN may be involved in complex processes of the above-mentioned pathological conditions.

Cross-linking of IgE bound to FcεRI with Ag induces mast cell degranulation by promoting the aggregation of FcεRI, thereby releasing many chemical mediators from these cells. Thus, mast cells have long been considered to be involved in allergic responses. We found in the present study that OPN augmented IgE plus Ag-mediated degranulation, suggesting that OPN may have a potential to control the magnitude of IgE-mediated degranulation. Corresponding to this *in vitro* finding, we observed that PCA reactions in OPN^{-/-} mice are significantly diminished, compared with OPN^{+/+} mice. Mast cells may be more sensitive to Ag when OPN levels at inflammatory sites are up-regulated.

Since cross-linking of IgE bound to FcεRI with Ag induced OPN production by FSMC and BMMC at a delayed phase, but not at an acute phase (Fig. 1), mast cell-derived OPN may have more impacts on the delayed phases of allergic diseases. In this scenario, it would be interesting to explore the role of OPN in Th2 cell-mediated immune diseases (*i.e.* atopic diseases including atopic dermatitis and asthma), in which mast cells participate. Indeed, Katagiri and Arakawa [42] recently reported that OPN-transgenic mice producing a large amount of OPN from the liver spontaneously developed late-onset (approximately at 5 month of age) atopic dermatitis-like skin lesions with high levels of serum IgE and enhanced eosinophil infiltrates in the lung in a model of asthma. Xanthou *et al.* [43] have very recently found opposing effects of OPN on Th2-mediated immune responses depending on the phase of the disease, using a mouse model of allergic airway inflammation. They demonstrated that OPN enhanced Th2 responses during antigenic sensitization by inhibiting the migration of plasmacytoid DC to draining lymph nodes, whereas it repressed Th2 responses during secondary antigenic challenge by inhibiting the migration of conventional DC to draining lymph nodes. Therefore, further studies are required to determine how OPN influences mast cells presumably participating in Th2 responses under these circumstances.

OPN-induced augmentation of the degranulation triggered by FcεRI aggregation on mast cells was mediated through integrin αv and CD44, which are known to be OPN receptors. It has been reported that extracellular matrix proteins (*i.e.* fibronectin, fibrinogen, and collagen) affect degranulation by mast cells in

addition to their adhesion, migration, and inflammatory responses by binding a wide array of integrins expressed by mast cells [44–46]. OPN-induced migration by FSMC was virtually blocked by Ab against either CD44 or integrin α v (Fig. 4), suggesting that these receptors may be co-activated by OPN for mast cell migration. Indeed, CD44-mediated OPN binding to a tumor cell line reportedly required β 1-containing integrins for cell motility and chemotaxis [47]. Therefore, it was not surprising that OPN, a ligand for integrins, affected degranulation by mast cells and promoted their migration. In the microenvironment of inflamed tissue, where integrin-activating mediators including OPN are abundantly present, OPN derived from mast cells and/or other cells (e.g. macrophages) may promote the accumulation of mast cells, and such microenvironments may allow mast cells to be more sensitive to lower levels of antigenic or presumably non-antigenic stimulation.

In summary, this study provides the first evidence that mast cells produce OPN and this affects mast cell function. In addition to our findings, accumulating evidence of the involvement of OPN in the pathogenesis of many inflammatory and immune diseases may provide a key to elucidate a link between mast cells and those diseases. However, it is important to note many key questions that remain to be clarified.

First, which cell types are the major sources of OPN that affect the functions of mast cells *in vivo*? Second, we must examine the involvement of mast cell-derived OPN in each pathological process (e.g. MS, RA, and asthma). The degree of contribution of mast cell-derived OPN in each pathological process must be determined. For example, does OPN production by mast cells significantly change plasma OPN levels or local OPN levels enough to have a biological impact on each pathological process? Third, it should be determined whether mast cells are new targets for potential modulation of inflammatory and acquired immune responses by OPN receptor antagonists or neutralizing OPN.

These questions will need to be addressed in future studies to clarify a link between mast cell-derived OPN and the above-mentioned diseases. Nevertheless, we believe that the present study will provide new insights into our understanding of the role of OPN as a pleiotropic mediator that regulates many functions of mast cells, presumably participating in multiple aspects of inflammatory and immune diseases.

Materials and methods

Animals

C57BL/6 (SLC Japan, Hamamatsu, Japan) and OPN-deficient (OPN^{-/-}) mice [48] backcrossed 11 times to C57BL/6 mice at the Institute for Genetic Medicine, Hokkaido University, were used [16]. All the experimental procedures using mice were approved by the Institutional Review Board at the University of Yamaguchi.

Preparations of FSMC and BMMC

(OPN^{-/-}/C57BL/6) F1 mice were mated to generate wild-type (OPN^{+/+}) or OPN^{-/-} fetuses. FSMC were prepared from fetal skin of each fetus of F1 $\times$ F1 pregnant mice (on day 14 or 15 of pregnancy) as described previously [23–25]. At the same time, the OPN genotype of each fetus (i.e. OPN^{+/+}, OPN^{+/-}, or OPN^{-/-}) was determined by analyzing DNA isolated from fetal tissues using PCR as described previously [49]. Only FSMC derived from OPN^{+/+} and OPN^{-/-} fetuses were used.

Briefly, single-cell suspensions were prepared from excised trunk skin specimens by limited trypsinization. The crude cells (5×10^4 cells/mL) were cultured in complete RPMI in the presence of mast cell growth factors, murine rIL-3 (10 ng/mL) and rSCF (10 ng/mL) (PeproTech, Boston, MA). Complete RPMI 1640 contained 10% heat-inactivated FCS (Sigma, St. Louis, MO), 2 mM L-glutamine, 10 mM nonessential amino acids, 1 % (v/v) penicillin/streptomycin, 10 mM sodium pyruvate, 25 mM HEPES, and 50 μ M 2-mercaptoethanol. Non-adherent cells and loosely adherent cells were collected 14–21 days after this initial culture, and enriched for mast cells by density gradient centrifugation in 1.05196 g/mL Percoll (Amersham Life Science, Arlington Heights, IL). The resulting mast cell preparations, containing >98% CD45⁺ CD117⁺ (c-kit⁺) cells, were used as FSMC without further purification. These cells stained red with alcian blue and safranin red, indicating the predominance of heparin, which is characteristic of connective tissue-type mast cells [23].

BMMC were prepared from bone marrow cell suspensions from C57BL/6 mice as described previously [24, 25]. Briefly, crude bone marrow cells (4×10^4 cells/mL) were cultured in complete RPMI in the presence of murine rIL-3 (10 ng/mL) and rSCF (10 ng/mL). Non-adherent cells were recovered twice a week, and further expanded in fresh growth medium for 4 wk. The resulting cell preparations, containing >96% CD45⁺ CD117⁺ cells, were used as BMMC without further purification. In contrast to FSMC, these mast cells stained blue with alcian blue and safranin red, indicating the predominance of chondroitin sulfate, which is a characteristic feature of immature mast cells [23]. The other phenotypic and functional features of both mast cells are described elsewhere [23–25].

Quantitative real-time PCR

Total RNA was isolated from FSMC and BMMC using Trizol (Invitrogen, San Diego, CA), followed by treating total RNA for 2 h at 25°C with RNase-free DNase (Promega, Madison, WI) and heparinase (Sigma) to digest contaminated genomic DNA and heparin, respectively [24]. RNA was then reverse-

transcribed to cDNA using the Superscript system (Invitrogen). mRNA was purified from total RNA preparations using Oligotex mRNA isolation columns (Qiagen, Valencia, CA), and then cDNA was synthesized using the Superscript system.

Relative expression of OPN mRNA between FSMC and BMMC was determined by real-time PCR using the ABI Prism 5500 Sequence Detection Systems (PE Biosystems, Foster City, CA) with SYBR Green I dye (Qiagen) according to the manufacturers' instructions. Primers corresponding to OPN and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed using Primer Express software (PE Biosystems) as follows: 5'-TCTAAGGAGTCCCTCGATGTC-ATC-3' and 5'-TGCCCTTTCCGTTGTTGTC-3' for OPN and 5'-CGTGTTCCTACCCCAATGT-3' and 5'-TGTCATACTGGC-AGGTTTCT-3' for GAPDH. Cycle threshold numbers (Ct) were derived from the exponential phase of PCR amplification. Fold differences in the expression of gene x in the cell population y and z were derived by 2^k , where $k = (Ct_x - Ct_{GAPDH})_y - (Ct_x - Ct_{GAPDH})_z$.

OPN production by mast cells

Supernatants from FSMC and BMMC that were cultured at 1×10^6 cells/mL from 24 through 72 h in fresh growth medium were tested for OPN production using a mouse OPN enzyme immunoassay kit (Immuno-Biological Laboratories, Japan) according to manufacturer's instruction. In some experiments, cells were stimulated for 24 h with LPS (100 ng/mL), poly(I:C) (100 µg/mL), R848 (10 µg/mL), CpG (0.1 µM), non-CpG (0.1 µM), ionomycin (1 µM). For IgE stimulation, cells were pre-incubated with 0.3 µg/mL of mouse IgE anti-DNP mAb (clone SPE7), and then washed extensively. The IgE-sensitized cells were then cultured for 2 or 24 h with 3 ng/mL of DNP-human serum albumin (HSA). Each supernatant was tested for OPN protein by ELISA. Each source of the above reagents was described before [24].

Degranulation assay

Mast cells pretreated with or without 0.3 µg/mL mouse IgE anti-DNP mAb for 18 h were plated at 2×10^4 cells/100 µL in Tyrode's buffer (Sigma) into 96-well U-bottom plates and allowed to equilibrate for 10 min at 37°C prior to the addition of rOPN (R&D Systems, Minneapolis, MN) or ionomycin at indicated concentrations for 30 min. The plates were then spun at $290 \times g$ for 5 min at 4°C. In Ab blocking experiments, cells were treated for 1 h with anti-OPN Ab (R&D Systems), anti- αv mAb (BD Pharmingen, San Diego, CA), anti-CD44 mAb (BD Pharmingen) or each control mAb (10 µg/mL), respectively. The cells were then stimulated with the indicated concentrations of DNP-HSA for 30 min after washing five times with Tyrode's buffer.

β -Hexosaminidase activity of the culture supernatants was determined as described previously [24]. Briefly, aliquots (50 µL) of the supernatants were transferred in new plates together with 100 µL of 2.5 mM p-nitrophenyl-N-acetyl β -D-glucosaminide (Sigma) solubilized in 0.04 M citrate buffer adjusted with disodium phosphate to pH 4.5. After incubation for 90 min at 37°C, the reactions were terminated by the addition of 50 µL of 0.4 M glycine, adjusted with sodium

hydroxide to pH 10.7. The colored products were measured using an ELISA plate reader (Molecular Device Corporation, Sunnyvale, CA) at 405 nm using a reference filter of 570 nm. The percentage of β -hexosaminidase release was determined as: (the ratio of activities in the supernatant of tested cells and in the frozen-thawed cell lysates) $\times$ 100.

OPN receptor expression

FSMC were examined for CD44 and CD51 (integrin αv) expression within CD45⁺ c-kit⁺ populations using FACSCalibur (BD Immunocytometry Systems, San Jose, CA). The Ab were purchased from BD Pharmingen.

Ear sections

Mast cell number per light field of standard microscope of $\times 100$ magnification was determined as follows. Ears from each of three mice per group (OPN^{-/-} and OPN^{+/+} wild-type mice) were fixed in formalin overnight and embedded in paraffin. Tissue sections of 6 µm from each ear were fixed on glass slides and stained with toluidine blue. Mast cell numbers in each section were randomly counted in five different fields. The average number of mast cells in five fields was determined, and the mean number of mast cells per 1 cm in each ear was calculated.

Passive cutaneous anaphylaxis

PCA was performed as previously described with slight modifications [50]. The right and left ear were injected with IgE anti-DNP mAb (50 ng/50 µL/ear) or PBS (the same volume), respectively. After 24 h, 200 µg DNP-HAS in 1% Evans' blue solution (100 µL) was injected intravenously. The mice were sacrificed 30 min after the injection. The amount of the dye extracted from the ear in formamide was determined from the absorbency at 620 nm. For compound 48/80-induced reactions, compound 48/80 (300 ng/50 µL) or PBS (50 µL) was injected intradermally in the left or right ear, respectively, immediately after the intravenous injection of 1% Evans' blue solution (100 µL). Twenty minutes after injection, the dye amounts were determined as described above.

Migration assay

Migration assays were performed in 24-well Transwell plates (Corning Costar, Cambridge, MA) with 8 µm pore size. OPN^{-/-} FSMC (1×10^5 cells/100 µL) suspended in complete RPMI without growth factors were added into the upper chamber and rOPN at the indicated concentrations in complete RPMI (600 µL) was added into the lower chamber. For checkerboard analysis, rOPN was added in both upper and lower chambers at the indicated concentrations. In some experiments, cells were pretreated for 1 h with anti- αv mAb, anti-CD44 mAb or each control mAb (10 µg/mL) before adding to the upper chamber. To evaluate biological activity of the culture supernatant of OPN^{+/+} FSMC, cells were extensively washed to remove OPN carryover, and then were cultured at 1×10^6 cells/mL for 24 h in complete RPMI without their growth factors (i.e. IL-3 and SCF) to collect the supernatant. The supernatant, pretreated

for 1 h with anti-OPN Ab or control Ab (10 µg/mL), was added into the lower chamber. After 6 h incubation at 37°C, cells in the lower chamber were collected for counting cell numbers.

Statistical analyses

Comparisons between two groups were performed using Student's *t*-test. Each experiment was repeated at least twice to assess reproducibility.

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