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Article

Suppression of IgE-independent degranulation of murine connective tissue-type mast cells by dexamethasone

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Abstract: Steroidal anti-inflammatory drugs are widely used for treatment of chronic cutaneous inflammation, such as atopic dermatitis, although it remains unknown how they modulate cutaneous mast cell functions. Murine connective tissue-type mast cells, which were sensitive to mast cell secretagogues, such as compound 48/80 and substance P, were generated by co-culture of bone marrow-derived mast cells with Swiss 3T3 fibroblasts in the presence of stem cell factor. This process was accompanied by up-regulation of α subunit of a trimeric G protein, G_{i1}, and several Mas-related G protein-coupled receptor (Mrgpr) subtypes. Secretagogue-induced degranulation and up-regulation of these genes were suppressed when they were cultured in the presence of a synthetic glucocorticoid, dexamethasone. The profiles of granule constituents were drastically altered by dexamethasone. Several Mrgpr subtypes were found to be expressed in the cutaneous tissues and their expression levels were decreased in response to topical application of dexamethasone. The numbers of degranulated cutaneous mast cells in response to compound 48/80 were decreased in mice treated with dexamethasone. These results suggest that mast cell-mediated IgE-independent cutaneous inflammation could be suppressed by steroidal anti-inflammatory drugs through down-regulation of $G_{\alpha i1}$ and several Mrgpr subtypes in mast cells.

Keywords: mast cell; dexamethasone; trimeric G protein; Mrgpr, skin, inflammation

1. Introduction

Glucocorticoid was found to have a potential to suppress inflammation in 1940s and synthetic glucocorticoids, steroidal anti-inflammatory drugs, have widely prescribed for the treatments of various chronic inflammatory diseases, such as atopic dermatitis and autoimmune disorders [1, 2]. A large part of glucocorticoid-mediated effects arises through its binding to glucocorticoid receptor (GR, Nr3c1). Because GR is ubiquitously expressed in a variety of cells, it is quite difficult to attribute the anti-inflammatory effects of glucocorticoids to the actions on the specific cell types. Steroidal anti-inflammatory drugs have been frequently used for therapy of various cutaneous inflammatory

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diseases, in some of which cutaneous mast cells were found to play critical roles, although it remains to be fully clarified how they act on cutaneous mast cells.

Mast cells originate in the hematopoietic stem cells in the bone marrow and undergo terminal differentiation in the tissue, in which they infiltrate from the circulation [3]. These findings indicated that tissue mast cells should have a greater diversity. We previously established a murine bone marrow-derived cultured mast cell model, which had similar characteristics with cutaneous mast cells, through modification of the previous models [4]. We found that this model could undergo degranulation in response to mast cell secretagogues, such as compound 48/80 and substance P. Sensitivity to mast cell secretagogues is one of the signatures of connective tissue-type mast cells. Because no suitable culture models have been developed, the signaling pathways involved in secretagogue-induced degranulation remained largely unknown [5]. Tatemoto et al. first demonstrated that Mas-related G protein-coupled receptor (Mrgpr) X2 should be involved in secretagogue-induced degranulation of mast cells. Recently, one of murine orthologues of MrgprX2, MrgprB2, was found to be responsible for pseudo allergic drug responses induced by mast cell degranulation using the gene-targeted mice, indicating that Mrgpr family should be involved in secretagogue-induced mast cell degranulation [6]. MrgprX2 was found to be up-regulated in the cutaneous mast cells of the patient with severe chronic urticaria [7]. Accumulating evidence suggests that IgE-independent activation of mast cells should play critical roles in a wide variety of cutaneous inflammatory diseases; about 20% of the patients with atopic dermatitis were reported to have no IgE-sensitization to environmental antigens and low serum IgE levels (intrinsic atopic dermatitis) [8], and about 50% of the patients with chronic urticaria were found to spontaneously develop the symptoms [9]. Steroidal anti-inflammatory drugs have been one of the primary therapeutic agents for these inflammatory diseases [10,11].

We here investigated the effects of a synthetic glucocorticoid, dexamethasone, on our connective tissue-type cultured mast cells and on the cutaneous vascular responses in mice in order to clarify how glucocorticoid modulate the functions of cutaneous mast cells.

2. Materials and Methods

Mice

Specific-pathogen-free, 8-10 week-old male BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan), and all mice were kept in a specific-pathogen-free animal facility at Okayama University. This study was approved by the Committee on Animal Experiments of Okayama University (Approved #OKU-2012218, 2015040, and 2015430).

Materials

The following materials were commercially obtained from the sources indicated: dexamethasone, *p*-nitrophenyl-β-*D*-2-acetoamide-2-deoxyglucopyranoside, compound 48/80, an anti-dinitrophenyl IgE antibody (clone SPE-7), *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, mitomycin C, substance P, and dinitrophenyl human serum albumin (DNP-HSA) from Sigma-Aldrich (St. Louis, MO), Toluidine blue, Safranin-O, and Evans blue from Wako Pure Chemical Industries (Osaka, Japan), an anti-trinitrophenyl IgE antibody (clone IgE-3) from BD Biosciences (San Diego, CA), trinitrophenyl bovine serum albumin (TNP-BSA) from LSL (Tokyo, Japan), H-*D*-Ile-Pro-Arg-*p*NA (S-2288) from Chromogenix (Milano, Italy), *N*-(4-Methoxyphenylazoformyl)-Phe-OH potassium salt (M-2245) from Bachem AG (Bubendorf, Switzerland), an anti-$G_{\alpha i2}$ antibody from Santa Cruz Biotechnology (Dallas, TX), an anti-pan-actin antibody (clone C4), an anti-$G_{\alpha i1}$ antibody, an anti-$G_{\alpha i3}$ antibody, and thapsigargin from Merck Millipore (Billerica, MA), and recombinant mouse IL-3 from R&D Systems (Minneapolis, MN). All other chemicals were commercial products of reagent grade.

Preparation of bone marrow-derived cultured mast cells

Preparation of IL-3-dependent bone marrow-derived cultured mast cells (BMMCs) and connective tissue type mast cell-like cultured mast cells (CTMC-like MCs) was performed as

described [4]. Briefly, bone marrow cells obtained from male BALB/c mice were cultured in the presence of 10 ng/ml IL-3 for ~30 days. Greater than 95% of the cells exhibited metachromasy by the acidic toluidine blue staining and were FcεRI^{+}c-kit^{+} on the flow cytometry. CTMC-like MCs were obtained through 16-days of co-culture of BMMCs with mitomycin C-treated Swiss 3T3 fibroblasts in the presence of 100 ng/ml recombinant murine stem cell factor (SCF). Greater than 90% of the cells were confirmed as mature mast cells by Safranin-O staining on Day-16. Dexamethasone (final concentration, 1 μM) was added to the culture medium simultaneously with SCF with 48 hr of interval thorough the co-culture period.

Measurement of degranulation

Cultured mast cells were suspended in 25 mM PIPES-NaOH, pH 7.4 containing 125 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 5.6 mM glucose, and 0.1% bovine serum albumin, and then stimulated for 30 min at 37°C. Degranulation was evaluated by measuring enzyme activity of a granule enzyme, β-hexosaminidase using the specific substrate, *p*-nitrophenyl-β-*D*-2-acetoamide-2-deoxyglucopyranoside.

Measurement of histamine and IL-6

The amount of histamine was determined by the fluorometrical method with *o*-phthalaldehyde [13]. Tissues were homogenized using a Polytron homogenizer (Kinematica AG, Schweiz, Switzerland) in phosphate buffered saline containing 2 M NaCl and the resultant homogenate was treated with 0.5% Triton X-100. The soluble fraction was subjected to histamine assay. The amount of IL-6 in the medium was measured using the ELISA system (BD Biosciences) according to the manufacturer's instruction.

Measurement of granule protease activities

Three categories of granule protease activities were measured using their specific substrates basically as described previously [4]. In this study, *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA was used as the substrate for chymase instead of S-2586.

Quantitative PCR analysis

Messenger RNA levels of various granule proteases and *Mrgpr* gene family were analyzed by quantitative reverse transcription (RT)-PCR with DNase-treated total RNAs. Total RNAs were prepared using NucleoSpin RNA kit (TaKaRa Bio, Kusatsu, Japan). PCR was performed using StepOne Plus (Thermo Fisher Scientific, Waltham, MA) with KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) or Fast SYBR Green Master Mix (Thermo Fisher Scientific) the specific primer pairs (forward, reverse); *Mcpt4*, (5′-CCT TAC ATG GCC CAT CT-3′, 5′-CTT CCC CGG CTT GAT A-3′), *Mcpt5*, (5′-AGA ACT ACC TGT CGG C-3′, 5′-GTC GTG GAC AAC CAA AT-3′), *Mcpt6*, (5′-CTT TGA ACC GGA TCG T-3′, 5′-CTC GTC ATT ATC AAT GTC GC-3′), *Mcpt7*, (5′-AGC TAT GAC ACG AGA AGG-3′, 5′-GCT TAC GGA GCT GTA CT-3′), *Cpa3*, (5′-GAT GTC TCG TGG GAC T-3′, 5′-GCC GTA GAT GTA ACG GG-3′), *Mrgpra4*, (5′-CCT GTG TGC TGT GAT CTG GT-3′, 5′-TCA CGG TTA ATC CAG GGC AC-3′), *Mrgprb1*, (5′-GAC ACA GAG CAA ATT ACC ATC TTC-3′, 5′-CAA GGT TGA GGA TGT AGA CAG AG-3′), *Mrgprb2*, (5'-TGC TTG TCT GTA ATA TGG CCC-3', 5'-GTC ACA TAC AGC CTG GTC ATA G-3'), *Mrgprb10*, (5′-CCC AGG TTG GTG GAA CTG TT-3′, 5′-GCC AGA AGC CTG ACA GTA GG-3′), *Mrgprc11*, (5′-CTA GCA TCC ACA ACC CCA G-3′, 5′-TGT TTC CTG CCA GTC CAA C-3′), *Mrgpre*, (5′-AGA ACT ACC TGT CGG C-3′, 5′-TTG CCT TCT GGC AGT GAT-3′) and *Gapdh*, (5′-TGT GTC CGT CGT GGA TCT GA-3′, 5′-TTG CTG TTG AAG TCG CAG GAG-3′).

Immunoblot analyses

Immunoblot analyses were performed as described previously [14]. SDS-PAGE was performed using 10% slab gels and PVDF membrane transfer was carried out by the semi-dry blotting method. Immunoreactive bands were detected by horseradish peroxidase-conjugated secondary antibodies and were visualized by ECL Western Blotting Detection Reagents (GE healthcare, Chicago, IL).

Gene expression analysis by next generation sequencing

Sequence reads from each group were individually aligned to the mm10 genome assembly (GRCm38) using TopHat v2.1.0 [15] with default parameters. Aligned read counts were calculated using HTSeq version 0.6.1 [16] with the RefSeq gene annotations obtained from the UCSC Genome Browser [17]. Read counts were then analyzed using DESeq version 1.20.0 [17] to detect differential expression genes (DEGs). Briefly, DESeq normalizes the raw read counts for each sample using size factors, which are calculated from the median of the ratio of observed count to geometric mean for each gene across all samples, and then infers DEGs based on the negative binomial distribution with estimated dispersion and mean linked by local regression. For without biological replicates situation, DESeq estimates dispersion using the samples from the different conditions as replicates. Three comparisons were made between BMMCs and CTMC-like MCs, between BMMC and CTMC-like MCs treated with dexamethasone, and between CTMC-like MCs and CTMC-like MCs treated with dexamethasone. The detected DEGs for each comparison were filtered to those having a false discovery rate (FDR) < 0.1, and divided into up-regulated genes and down-regulated genes based on the logarithmic fold change of normalized counts.

Dexamethasone treatment

Dexamethasone (20 μl/site, dissolved in acetone) was daily applied to the surface of the ear tissues of mice for 6 days. A series of experiments were performed 24 hr after the last application of dexamethasone.

Evaluation of cutaneous extravasation induced upon IgE-dependent passive cutaneous anaphylaxis, and treatment with compound 48/80 or histamine

Mice were intracutaneously sensitized with IgE (30 ng/site, clone SPE-7) in the ear tissues 24 hr before the challenge with intravenous injection of 60 μg DNP-HSA in 0.2 ml saline containing 1 mg Evans blue. The ear tissues were collected 30 min after the challenge and lysed in 3 N KOH. The amounts of Evans blue dye were determined by measuring the value of OD_{620}. Extravasation responses induced by IgE-independent stimulus were determined by monitoring the dye leakage as described above when non-sensitized male BALB/c mice were intracutaneously injected with compound 48/80 (30 μg/site) or histamine (30 μg/site).

Histological evaluation of cutaneous mast cells

IgE-mediated antigen challenge and compound 48/80 stimulation were performed as described above without injection of Evans blue dye and the ear tissues were collected 3 min after the stimulation. Cutaneous mast cells were visualized by the acidic toluidine blue staining (pH 3.3) and the numbers of degranulated and intact mast cells were respectively counted.

Statistical analysis

Data are presented as the means ± SEM. Statistical significance for comparisons was determined using one-way ANOVA. Additional comparisons were made with Dunnett multiple comparison test for comparison with the control groups or Tukey-Kramer multiple comparison test for all pairs of column comparison. Two-tailed unpaired Student's *t* test was used for comparison between two populations.

3. Results

3.1. Characteristic changes of the co-cultured mast cells induced by prolonged treatment with dexamethasone

We first investigated the effects of dexamethasone on proliferation of BMMCs when they were co-cultured with Swiss 3T3 fibroblasts in the presence of SCF. The number of mast cells was increased during the co-culture period, whereas it remained unchanged in the presence of

dexamethasone (Fig. 1a). A slight decrease in the number of mast cells cultured in the presence of dexamethasone resulted from the loss during the repeated subculture processes. The cell viability was unchanged during the co-culture periods (> 95%). The granule maturation was monitored by the Safranin staining, which reflects the amount of hypersulfated proteoglycans, such as heparin, stored in the granules. Dexamethasone did not affect the percentages of Safranin-positive cells and the granule number and morphology (Fig. 1b). We previously reported that granule protease activities were drastically increased during the co-culture periods [4]. Prolonged treatment with dexamethasone abolished the induction of chymotryptic activity but enhanced the carboxypeptidase A activity in CTMC-like MCs (Fig. 2a and 2c). The tryptic activity was not changed until Day-12, but was significantly decreased at Day-16 in the cells co-cultured in the presence of dexamethasone (Fig. 2b). Expression of *Mcpt4*, *Mcpt6*, and *Mcpt7* were all up-regulated in CTMC-like MCs and were suppressed in the presence of dexamethasone, indicating that dexamethasone should affect the granule protease expression at the transcriptional levels, whereas no significant changes were observed in the expression levels of *Mcpt5* and *Cpa3* (Fig. 2d). Cellular histamine content was drastically increased in the presence of dexamethasone (> 8 fold at Day-16, Fig. 3a), which is consistent with a previous study that exhibited the dexamethasone-mediated induction of histidine decarboxylase (HDC), which is the rate-limiting enzyme for histamine synthesis, in a mouse mastocytoma, P-815 [18]. We, indeed, confirmed that the enzymatic activity of HDC was increased 5-fold in the cells cultured in the presence of dexamethasone (HDC activity at Day-4 (nmol/min/mg protein), Control, 0.42 ± 0.138, +Dexamethasone, 2.11* ± 0.424, *P<0.05, n=3). Unexpectedly, enzymatic activity of β-hexosaminidase, a lysosomal enzyme, which might play critical roles in bactericidal actions [19] and is often used for monitoring degranulation levels, was significantly up-regulated in CTMC-like MCs obtained in the presence of dexamethasone (Fig. 3b).

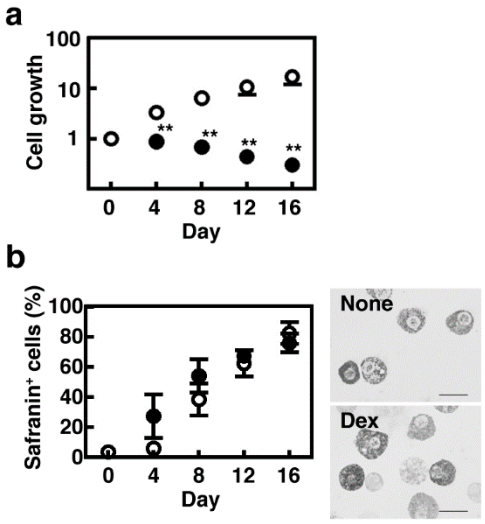

Figure 1. BMMCs were co-cultured with Swiss 3T3 fibroblasts in the presence (closed circles) or absence (open circles) of 1 μM dexamethasone for 16 days as described in Materials and Methods. (a) The numbers of the cultured mast cells were counted on Day-0, 4, 8, 12, and 16. Values were presented as the means ± SEMs (n=4). (b) The ratios of the Safranin-positive cells were determined. Values were presented as the means ± SEMs (n=4).

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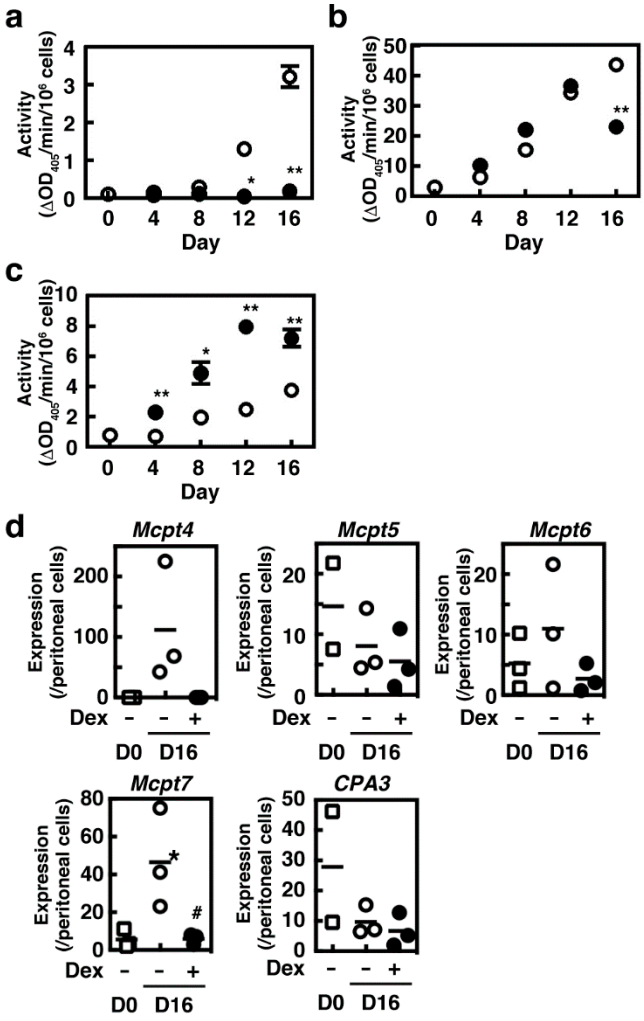

Figure 2. BMMCs were co-cultured with Swiss 3T3 fibroblasts in the presence (closed circles or columns) or absence (open circles or columns) of 1 μM dexamethasone for 16 days as described in Materials and Methods. (a-c) Enzymatic activities of three kinds of granule proteases (a; chymotryptic activity, b; tryptic activity, and c; carboxypeptidase A activity) were measured. Values with $*p < 0.05$ and $**p < 0.01$ are regarded as significant. (d) Expression levels of granule protease genes (*Mcpt4*, *5*, *6*, *7*, and *CPA3*) were determined by quantitative RT-PCR analyses. Values with $*p < 0.05$ (vs. D0) and $\#p < 0.05$ (vs. D16, (-)Dex) are regarded as significant.

3.2. Suppression of Gi-mediated degranulation in mast cells cultured in the presence of dexamethasone

BMMCs co-cultured with Swiss 3T3 fibroblasts were found to undergo degranulation in response to basic secretagogues, such as compound 48/80 and substance P, which is one of the characteristics of CTMCs and is mediated by pertussis toxin-sensitive Gi proteins [5]. Degranulation induced by these secretagogues was abolished in the cells co-cultured in the presence of dexamethasone, whereas that upon IgE-mediated antigen stimulation remained unchanged (Fig. 3c and 3d). Suppressive effects of dexamethasone on the Gi-dependent degranulation were not observed when dexamethasone was added 24 hr before stimulation (Fig. 3f), indicating that dexamethasone-mediated suppression of Gi-dependent degranulation should require long-term characteristic changes.

Previous studies demonstrated that treatment with dexamethasone down-modulated the surface expression of FcεRI and thereby suppressed antigen-induced degranulation in mast cells [20,21]. However, no significant changes were observed in the levels of degranulation of activated BMMCs, which were treated with dexamethasone 24 hr before the stimulation (Fig. 3g and 3h).

Surface expression levels of FcεRI were comparable between the control and dexamethasone-treated BMMCs (mean fluorescent intensity; Control, 47.2 ± 4.01, +Dexamethasone, 41.8 ± 0.768, n=3), whereas those of c-kit were significantly decreased in the dexamethasone-treated cells (mean fluorescent intensity; Control, 104 ± 3.55, +Dexamethasone, 81.5* ± 0.379, n=3, *p < 0.05). In contrast to unchanged levels of degranulation, treatment with dexamethasone for 24 hr significantly suppressed antigen-induced IL-6 production whereas Ca^{2+} influx-induced IL-6 production was unchanged in the presence of dexamethasone (Fig. 3i and 3j).

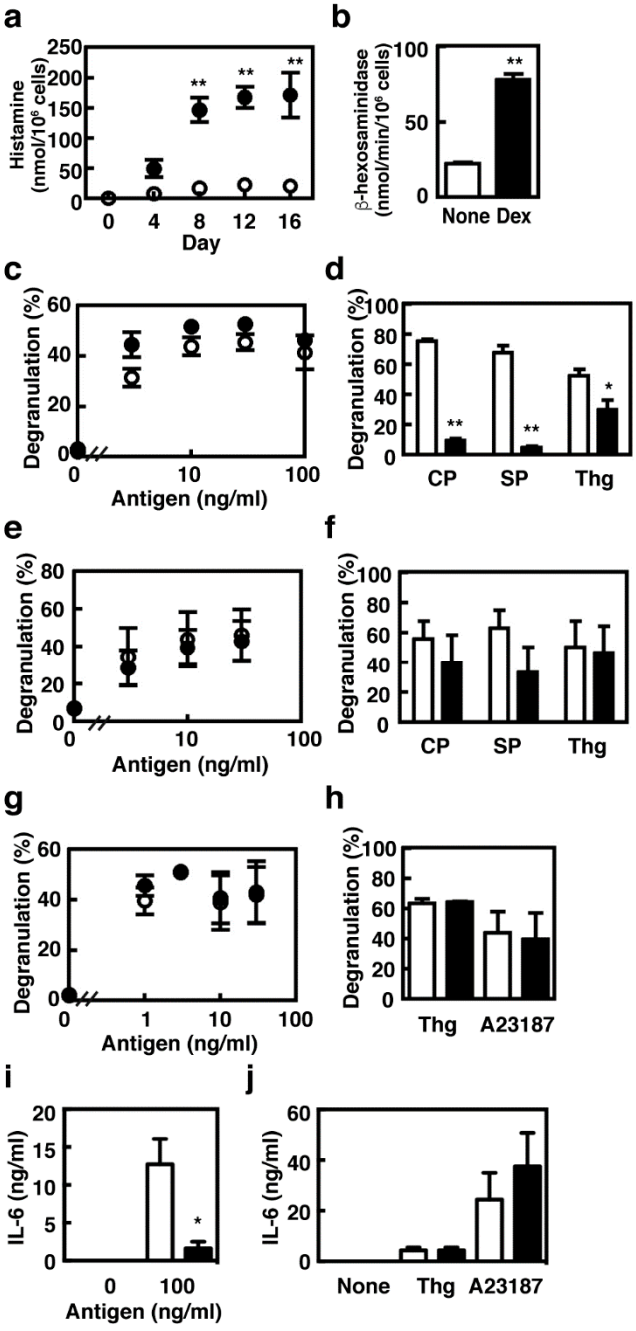

Figure 3. (a, b) The cellular histamine contents and enzymatic activities of -hexosaminidase in the mast cells co-cultured for 16 days in the presence (closed circles) or absence (open circles) of 1 μM dexamethasone were measured. (c-f) The co-cultured mast cells were sensitized with IgE (1 μg/ml, clone IgE-3) for 3 hr and then stimulated with the indicated concentrations of the antigen, or stimulated with compound 48/80 (CP, 10 μg/ml), substance P (SP, 100 μM), or thapsigargin (Thg, 300 nM) without sensitization. Degranulation upon IgE-mediated antigen stimulation (c) and treatment with compound 48/80, substance P, or thapsigargin (d) was measured in the mast cells co-cultured

for 16 days in the presence (closed circles or columns) or absence (open circles or columns) of 1 μM dexamethasone. (e, f) BMMCs were co-cultured for 16 days and were treated with 1 μM dexamethasone during the last 24 hr (closed circles and columns). Degranulation was then measured as described above. (g-j) BMMCs were treated without (open circles or columns) or with 1 μM dexamethasone (closed circles or columns) for 24 hr. The cells were then sensitized with 1 μg/ml IgE (clone IgE-3) for 3 hr and stimulated with the indicated concentrations of the antigen or stimulated with thapsigargin (Thg, 300 nM) or A23187 (A23187, 1 μM). Degranulation (g, h) and IL-6 release (i, j) were measured. The degree of degranulation was determined by measuring β-hexosaminidase activity. Values were presented as the means ± SEMs (n=3). Values with $*p < 0.05$ and $**p < 0.01$ are regarded as significant.

Because $G_{\alpha i1}$ protein was found to be up-regulated during the co-culture period [4], we then investigated the effects of dexamethasone on the expression of three subtypes of α subunit of trimeric G_i. Expression of $G_{\alpha i1}$ protein was exclusively induced during the co-culture period as previously reported and significantly suppressed by prolonged treatment with dexamethasone, whereas no obvious changes in the other $G_{\alpha i}$ protein expression were observed (Fig. 4a-4d). Recently, the possible candidates for the receptors of mast cell secretagogues have been identified; various secretagogues were found to act as the agonists of MRGPRX2 [6], and one of its murine orthologues, MrgprB2, was identified as the primary receptor for various mast cell secretagogues using the gene targeted mice [7]. We investigated the expression levels of 6 murine Mrgpr family genes based on the results obtained through the next generation sequencing analysis. Messenger RNA expression of *Mrgpra4*, *b1*, *b2*, *b10*, and *e* were detected in the cultured mast cells and murine peritoneal cells. *Mrgprc11* was not detected in murine peritoneal cells, but expressed in the cultured mast cells. *Mrgprb1*, *b2*, *b10*, and *c11* were found to be up-regulated during the co-culture period whereas *Mrgpra4* and *e* were down-regulated (Fig. 5). Overall, the presence of dexamethasone during the co-cultured period suppressed mRNA expression of these *Mrgpr* genes.

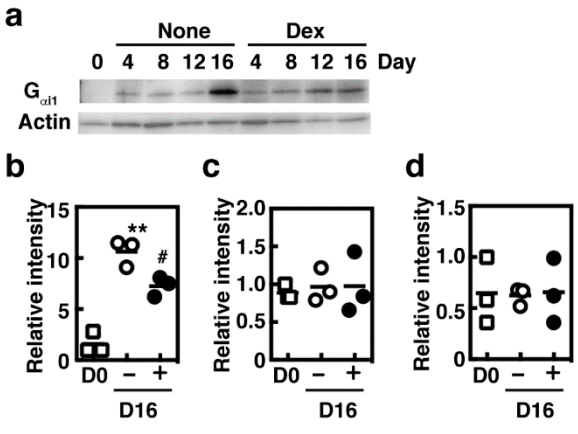

Figure 4. BMMCs were co-cultured with Swiss 3T3 fibroblasts in the presence (Dex) or absence (None) of 1 μM dexamethasone for 16 days as described in Materials and Methods. (a) Expression of G_{i1} was visualized by immunoblot analyses using an anti-$G_{\alpha i1}$ antibody. Expression of actin was measured as the loading control. (b-d) Expression levels of various $G_{\alpha i}$ proteins (b; $G_{\alpha i1}$, c; $G_{\alpha i2}$, and d; $G_{\alpha i3}$) in BMMCs (D0), and the Day-16 co-cultured mast cells prepared in the presence (+) or absence (-) of 1 μM dexamethasone were densitometrically determined. Values with $**p < 0.01$ (vs. D0) and $^{\#}p < 0.05$ (vs. D16, (-)Dex) is regarded as significant.

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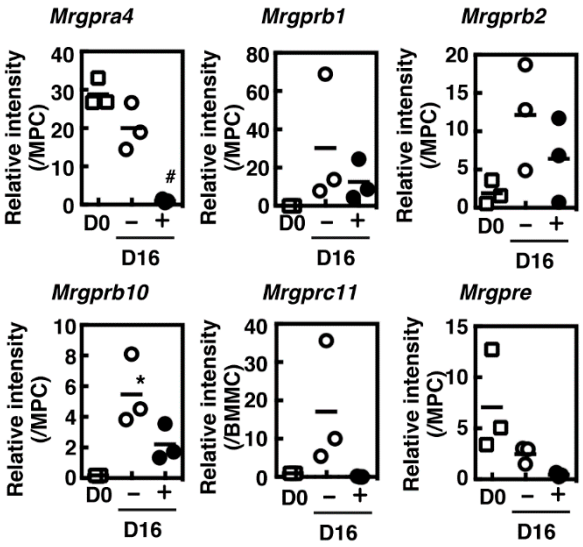

Figure 5. BMMCs were co-cultured with Swiss 3T3 fibroblasts in the presence (Dex) or absence (None) of 1 μM dexamethasone for 16 days as described in Materials and Methods. Expression levels of *Mrgpr* family genes in BMMCs (D0), and the Day-16 co-cultured mast cells prepared in the presence (+) or absence (-) of 1 μM dexamethasone were measured using quantitative RT-PCR. Relative expression levels were calculated based on the expression levels in mouse peritoneal cells (MPC) or those in BMMCs (BMMC). Values with $^{*}p < 0.05$ (vs. D0) and $^{\#}p < 0.05$ (vs. D16, (-)Dex) are regarded as significant.

3.3. Effects of dexamethasone on gene expression profiles of cultured mast cells

We then investigated the gene expression profiles by the next generation sequencing analysis. 376 genes were extracted as the up-regulated genes (FDR < 0.1) in CTMC-like MCs in comparison with BMMCs. This population included the characteristic genes of CTMCs, which we previously identified by the microarray analyses [4], such as *Bgn*, *Cd81*, *Gnai1*, *Icam1*, *Mcpt4*, *Ptges*, *Ptgis*, *Ptgs2*, *Thbs1*, and *Tpsab1*. Among them, 113 genes were not induced in the cells cultured in the presence of dexamethasone (Fig. 6, Group I and III). These dexamethasone-sensitive groups included the genes, such as *Cd81*, *Gnai1*, *Icam1*, *Mcpt4*, *Mmp9*, *Nfkbia*, and *Tpsab1*. The number of genes, which were up-regulated in CTMC-like MCs but were insensitive to dexamethasone, were 263 (Fig. 6, Group II). They included the genes involved in arachidonic acid metabolism, such as *Ptges*, *Ptgs2*, and *Ptgis*.

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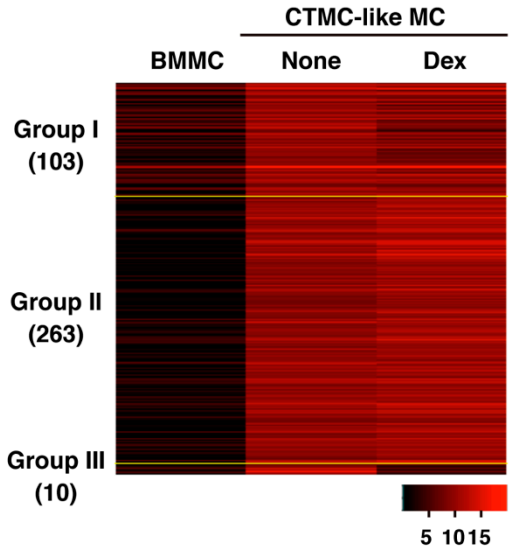

Group I

Gene	FDR
Ifit3	3.400E-6
Mx2	3.669E-5
Rtp4	3.799E-5
Slfn4	1.884E-4
Csf2	2.038E-4
Nr4a3	2.038E-4
Ptprn	2.202E-4
Cxcl11	4.376E-4
Mcpt2	6.484E-4
Csf1	6.752E-3
Nfkbia	0.01274
Mmp9	0.06755
Cd81	0.06771
Gnai1	0.08686
Icam1	0.08833

Group II

Gene	FDR
Cxcl1	2.060E-10
Bgn	3.989E-10
Cxcl10	4.692E-8
Ccl1	2.913E-5
Ccl5	2.705E-5
Cxcl5	4.097E-5
Ptges	3.571E-4
Ptgs2	1.784E-3
Thbs1	2.463E-3
Ccl7	3.166E-3
Ptgis	0.09743

Group III

Gene	FDR
Mcpt4	2.254E-10
Tpsab1	6.042E-7
Mcpt1	3.689E-6
Ccl1	2.913E-5
Irf7	8.312E-5
Krt16	3.271E-4
Slco2b1	1.934E-3
U90926	1.995E-3
Cd3e	0.01426
Ifih1	0.03939

Figure 6. Each RNA sample was collected from the cultured mast cells (BMMCs, CTMC-like MCs, and CTMC-like MCs prepared in the presence of Dex) and gene expression analyses were performed by the next generation sequencing. Differentially expressed genes between BMMCs and CTMC-like MCs were extracted with the false discovery rate (FDR) < 0.1. The heat map presents the expression profiles of the genes, of which expression were increased in CTMC-like MCs. The extracted genes are classified into three clusters based on the expression patterns.

3.4. Effects of dexamethasone on murine cutaneous vascular responses

We then investigated the effects of dexamethasone on cutaneous vascular responses. Six days of topical application of dexamethasone did not affect the number of cutaneous tissue mast cells, whereas cutaneous histamine content was significantly increased (Fig. 7a and 7b). Four *Mrgpr* genes, *Mrgpra4*, *b2*, *b10*, and *e*, were expressed in the ear tissues whereas expression of *Mrgprb1* and

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c11 could not be detected by RT-PCR (Fig. 7c). Prolonged treatment with dexamethasone down-regulated all *Mrgpr* genes expressed in the ear tissues.

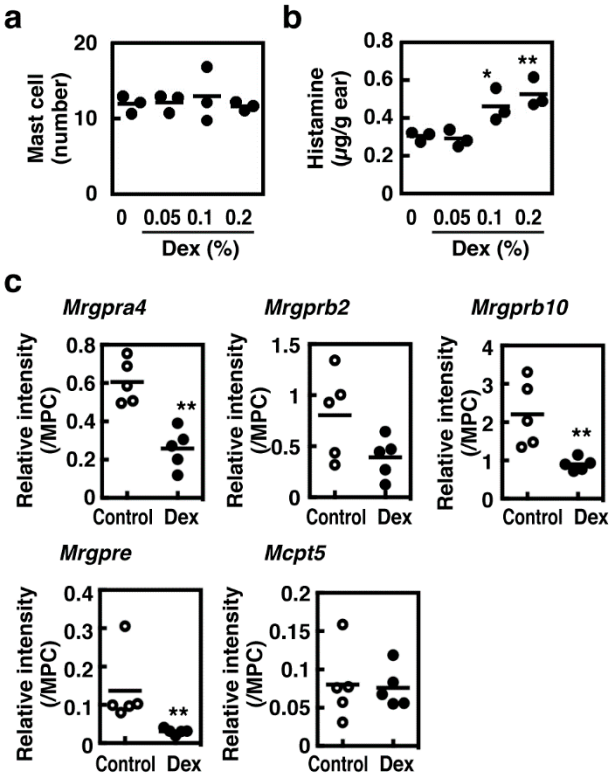

Figure 7. (a, b) The indicated concentrations of dexamethasone (20 μl/site, dissolved in acetone) were daily applied to the surface of the ear tissues for 6 days. The ear tissues were collected 24 hr after the last administration and the numbers of cutaneous mast cells (a) and tissue histamine content (b) were measured. Values with $*p < 0.05$ and $**p < 0.01$ are regarded as significant. (c) Expression levels of *Mrgpr* family genes and *Mcpt5* in the ear tissues were measured using quantitative RT-PCR. Relative expression levels were calculated based on the expression levels in mouse peritoneal cells (MPC). Values with $**p < 0.01$ and $*p < 0.05$ are regarded as significant.

Extravasation responses evaluated by Evans blue dye leakage upon IgE-mediated antigen stimulation or compound 48/80 were significantly attenuated in the mice daily treated with 0.05% dexamethasone (Fig. 8a and 8b). We then investigated the sensitivity to histamine, which is the major vasoactive mediator derived from mast cells, and found that histamine-induced extravasation responses was also attenuated in the dexamethasone-treated mice (Fig. 8c). These findings implied that decreased dye leakages observed in the dexamethasone-treated mice might result from impaired vascular responses to histamine rather than impaired degranulation. Previous studies demonstrated that glucocorticoids could augment the functions of tight junctions of vascular endothelial cells [22,23]. We, therefore, assessed the frequencies of degranulation of tissue mast cells by histological analyses with the acidic toluidine blue staining. In the mice daily treated with 0.05% dexamethasone, the number of degranulated mast cells was slightly but significantly increased upon IgE-mediated antigen stimulation, whereas that was significantly decreased upon the compound 48/80 application (Fig. 8d and 8e). Suppression of compound 48/80-induced degranulation was more pronounced in the mice daily treated with 0.2% dexamethasone, whereas antigen-induced degranulation was also suppressed under this condition (Fig. 8f and 8g).

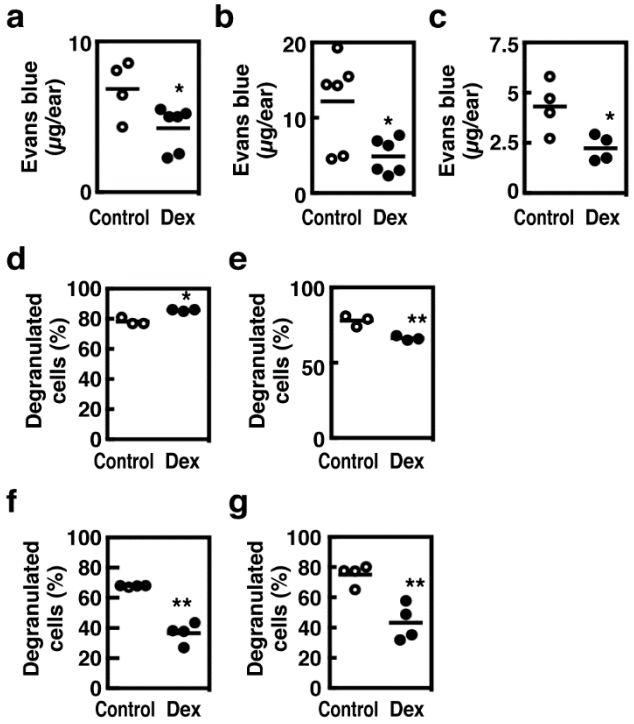

Figure 8. (a-c) After the daily application of 0.05% dexamethasone to the ear tissues for 6 days, extravasation in the ear tissues was evaluated by Evans blue dye leakage 24 hr after the last application. Mice were subjected to IgE-dependent passive cutaneous anaphylaxis (PCA) reactions (a), and intracutaneous injections of compound 48/80 (b, 30 μg/site) or histamine (c, 30 μg/site). Values with $*p < 0.05$ are regarded as significant. (d-g) Mice were daily treated without (Control) or with 0.05% (d and e) or 0.2% (f and g) of dexamethasone (Dex, 20 μl/site, dissolved in acetone) on the surface of ear tissues for 6 days. Mice were then subjected to IgE-dependent PCA reactions (d and f) or an intracutaneous injection of compound 48/80 (e and g, 30 μg/site). The ear tissues were collected 3 min after the stimulation and the degrees of degranulation of cutaneous mast cells were determined based on the acidic Toluidine blue staining. Values with $*p < 0.05$ and $**p < 0.01$ are regarded as significant.

4. Discussion

We demonstrated here that prolonged treatment with a synthetic glucocorticoid, dexamethasone, could suppress secretagogue-induced degranulation of murine mast cells. Because glucocorticoid receptor is ubiquitously expressed, it remains unknown how glucocorticoid suppress cutaneous inflammation. Our findings strongly suggest that down-regulation of *Gnai1* and *Mrgpr* family in mast cells should be involved in impaired secretagogue-induced degranulation. A large part of secretagogue-induced degranulation was found to be sensitive to pertussis toxin [5]. Aridor et al. suggested that G_{i3} should be involved in secretagogue-induced degranulation of rat peritoneal mast cells [24]. It is likely that cooperation between several Mrgpr subtypes and G_{i1} should be responsible for secretagogue-induced degranulation of murine mast cells. This mechanism might account at least in part for the therapeutic effects of steroidal anti-inflammatory drugs on IgE-independent cutaneous inflammation including intrinsic atopic dermatitis and contact dermatitis.

The effects of glucocorticoids on degranulation induced by IgE-mediated antigen stimulation have been extensively investigated. A majority of studies demonstrated that dexamethasone could significantly suppress degranulation of mast cells upon antigen stimulation [20,21,25-27]. It remains to be determined how dexamethasone should act on the signaling pathways involved in degranulation. Some of these studies suggested that the surface expression of FcεRI was decreased in the presence of dexamethasone. We observed here that prolonged treatment with dexamethasone did not affect antigen-induced degranulation of cultured mast cells but significantly suppressed that of cutaneous mast cells. We could not reproduce the previously reported findings that 24-hours of

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treatment with dexamethasone suppressed antigen-induced degranulation of BMMCs [20,21]. Antigen-induced IL-6 release was abolished by 24-hours of treatment with dexamethasone, excluding the possibility that BMMCs should be insensitive to glucocorticoids in our system. We have no good explanation for this discrepancy, although the methods for preparation of BMMCs are slightly different from our model.

It remains controversial how glucocorticoid affect the number of mast cells. Eklund et al. reported that dexamethasone suppressed proliferation of murine BMMCs induced by SCF or IL-3, which is consistent with our findings [28]. Methylprednisolone could deplete intestinal mast cells but did not affect the number of connective tissue-type mast cells in rats [29]. In cutaneous tissues, the effects of glucocorticoids on fibroblasts might be involved in regulation of the mast cell number. Chronic topical treatments with fluocinonide decreased the number of cutaneous mast cells through down-regulation of SCF in the fibroblasts, which induced apoptosis of the mast cells [30]. It is likely that the expression levels of SCF in Swiss 3T3 were down-regulated in the presence of dexamethasone in our system. However, the presence of a large amount of soluble exogenous SCF may prevent apoptic cell death of CTMC-like MCs. Clinical studies demonstrated that prolonged treatment with clobetasol-17-propionate and fluocinonide could down-modulate the number of cutaneous mast cells [31,32]. Because no changes in the number of cutaneous mast cells were observed in our system, dexamethasone might not affect the viability of them in the range of concentrations used here. We used Swiss 3T3 fibroblasts as the feeder cells to prepare CTMC-like MCs, raising the possibility that dexamethasone should affect the phenotype of mast cells indirectly through the effects on Swiss 3T3 cells. It is quite difficult to distinguish the direct effects from those through the fibroblasts. Kusunose et al. reported that treatment of murine fibroblasts with mitomycin c should attenuate the nuclear translocation of GR [33]. Because Swiss 3T3 cells were pretreated with mitomycin c before the co-culture in our system, the effects of dexamethasone on them may be relatively small.

We observed here that granule enzymes, such as carboxypeptidase A and β-hexosaminidase, and histamine contents were significantly up-regulated in the cultured mast cells generated in the presence of dexamethasone. Increase in histamine content was also observed in the cutaneous tissues of mice treated with dexamethasone. Accumulating evidence suggests that glucocorticoids could not only suppress inflammatory responses but also enhance the innate immune responses [34,35]. Carboxypeptidase A and a granule proteoglycan core, serglycin, were found to be up-regulated in BMMCs treated with dexamethasone [28]. Mast cells were found to contribute to wound healing through release of their mediators including histamine [36]. Dexamethasone-induced up-regulation of the granule mediators may be associated with its therapeutic effects.

We found that a variety of murine Mrgpr family was expressed in cultured mast cells in addition to *Mrgprb2* and that their expression levels were dynamically changed. Although McNeil et al. highlighted the critical roles of MrgprB2 using the gene targeted mice [7], it is plausible that the other Mrgpr family should be involved in IgE-independent degranulation of mature mast cells in cooperation with MrgprB2. Mrgpr family may respond to various secretagogues through its heterodimerization. We also detected mRNA expression of Mrgpr family in murine cutaneous tissues, the levels of which were decreased in the presence of dexamethasone. Because previous studies indicated that Mrgpr family was expressed exclusively in the sensory nerve, of which cell body is localized in the dorsal root ganglion, except that mast cells expressed a part of it, mRNA expression of Mrgpr subtypes in the cutaneous tissues might indicate that cutaneous mast cells should express them. Characterization of these Mrgpr subtypes in addition to MrgprB2 is necessary for understanding the mechanism of IgE-independent inflammatory responses.

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