

Omalizumab inhibits acceleration of FcεRI-mediated responsiveness of immature human mast cells by immunoglobulin E

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ARTICLE INFO

Article history:

Received for publication October 19, 2011.

Received in revised form December 19, 2011.

Accepted for publication January 16, 2012.

ABSTRACT

Background: A large body of evidence has demonstrated that treatment with omalizumab is clinically effective for the management of moderate to severe allergic asthma, emphasizing the importance of IgE in the pathogenesis of allergic asthma. We hypothesized that IgE accelerates FcεRI-mediated responsiveness of “immature” human mast cells (MCs) and that omalizumab downregulates the acceleration.

Objectives: To examine when MC progenitors acquired the ability to degranulate following FcεRI aggregation, whether IgE accelerates the responsiveness of immature MCs following FcεRI aggregation, and whether omalizumab regulates such an acceleration.

Methods: Gene expression was examined using a microarray and quantitative reverse transcription polymerase chain reaction. Protein expression was investigated using FACS. Histamine release was examined using an EIA.

Results: The time-course analysis of the mRNA expression of MC-related genes, including FcεRI, in Kit⁺ sorted cells during the differentiation and histamine experiments revealed that the expression level of FcεRI in 5 week (w)-cultured MCs was not sufficient to induce degranulation following FcεRI aggregation but that 5 w-cultured MCs were fully responsive to calcium ionophore. By addition of IgE in culture medium FcεRI expression level and FcεRI-mediated histamine release of 5 w-cultured MCs were significantly increased compared with those without addition of IgE, whereas the expression level of tryptase and number of MCs was not affected. Omalizumab significantly inhibited IgE-dependent enhancement of FcεRI expression level and FcεRI-mediated histamine release.

Conclusions: High levels of IgE in the microenvironment in vivo may upregulate the responsiveness of immature MCs to allergens. Omalizumab may inhibit the IgE-mediated responsiveness of not only mature MCs, but also immature MCs.

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Introduction

Mast cells (MCs) play a central role in immunoglobulin E (IgE)-dependent allergic diseases such as asthma, and rhinoconjunctivitis through the release of a variety of vasoactive and bronchospastic

autacoid mediators and functionally diverse proteases, chemokines, and cytokines.¹ Mast cells normally do not mature before leaving the bone marrow (BM) but circulate through the vascular system as immature progenitors that then complete their development peripherally within connective or mucosal tissues.¹ Human CD34 positive (CD34⁺) progenitor cells derived from BM, umbilical cord blood (CB), or peripheral blood (PB) develop into tryptase⁺, Kit⁺, FcεRI⁺ MCs when cultured in the presence of human stem cell factor (SCF) and IL-6.^{2–4} Ishizaka et al described that human cultured MCs are recognized as mature MCs if they have amounts of histamine that are comparable to those of tissue-isolated MCs, contain tryptase, exhibit a morphologically mature appearance when observed using electron microscopy, and are capable of releasing histamine after the aggregation of FcεRI.⁵ In the culture system, BM-, or PB-derived MCs cultured for more than 10 weeks seem to be mature according to the definition.^{2–6} Although human

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Disclosures: Authors have nothing to disclose.

Funding Sources: This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (Project No. (C) 20591195 and (C) 23591470, awarded to Y.O., and (B) 22390202, awarded to C.R.), the Nihon University Multidisciplinary Research Grant for 2010 (awarded to Y.O.), and the Matching Fund Subsidy for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (to C.R.) and Novartis Pharma AG, Basel, Switzerland (to Y.O.).

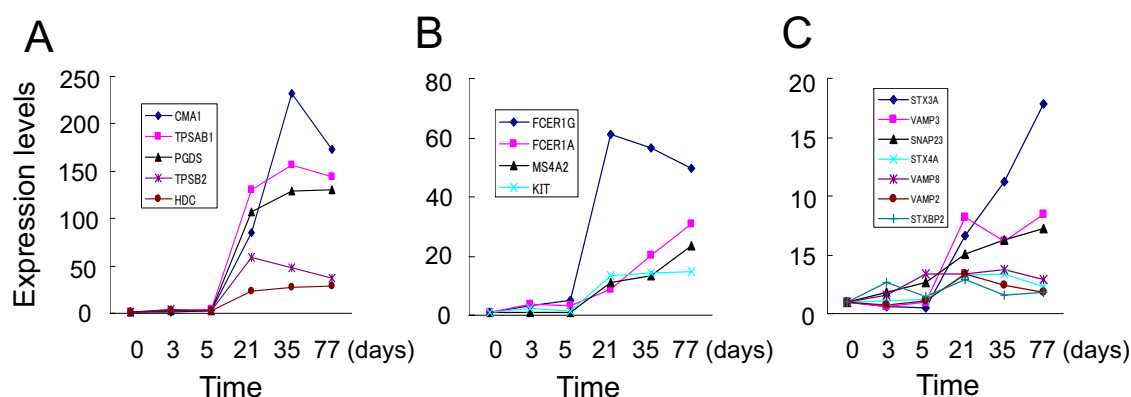


Fig. 1. Kinetics of mRNA expression of MC-related genes (A), Fc receptors and KIT (B), and SNARE proteins (C) during the culture of human CD34⁺ progenitor cells derived from bone marrow in the presence of stem cell factor. The Kit⁺ cells were isolated at the indicated time points, and mRNAs were extracted for DNA chip analysis. The expression levels shown on the vertical axis were obtained by normalizing the average difference values against the median value of 66,996 transcripts. The data are average of 3 different donors. The purities of MCs on days 21, 35, and 77 were 56.4, 76.6, and 89.7%, respectively (mean of 3 donors). The MCs were identified with Kimura's stain. Abbreviations: MCs; mast cells, SNARE; soluble N-ethylmaleimide-sensitive factor attachment protein receptors.

MCs were reported to express Fc ϵ RI during an early stage of differentiation,⁷ the timing when MC progenitors acquire the ability to undergo degranulation after the aggregation of Fc ϵ RI and how IgE affects the Fc ϵ RI-mediated degranulation of immature MCs remains unknown.

Mouse IgE molecules by themselves display a wide spectrum of heterogeneity in their ability to induce the production and secretion of IL-6 and tumor necrosis factor alpha and to induce MC survival and growth⁸; those that do so are referred to as being cytokinergic. Highly cytokinergic IgE molecules can efficiently activate MCs to produce cytokines in the absence of antigen, whereas poorly cytokinergic IgEs are less effective.⁸ The culture of mouse BM-derived MCs in the presence of mouse

monomeric IgE reportedly results in an increased number of MCs and a reduction in the culture time required to generate more than 80% pure MCs, compared with cultures grown without IgE.⁹ Furthermore, the number of highly cytokinergic IgE-induced MCs were greater than those of poorly cytokinergic IgE-induced MCs.⁹ A human highly cytokinergic IgE (myeloma IgE, Calbiochem-Novabiochem, Gibbstown, New Jersey) alone promoted lung MC survival through the autocrine production of IL-6.¹⁰ Conversely, another highly cytokinergic IgE alone (myeloma IgE, Biodesign International) failed to enhance the survival of CB-derived cultured mature MCs after SCF withdrawal.¹¹ Therefore, how human IgE affects the proliferation of immature human MCs has not been well investigated.

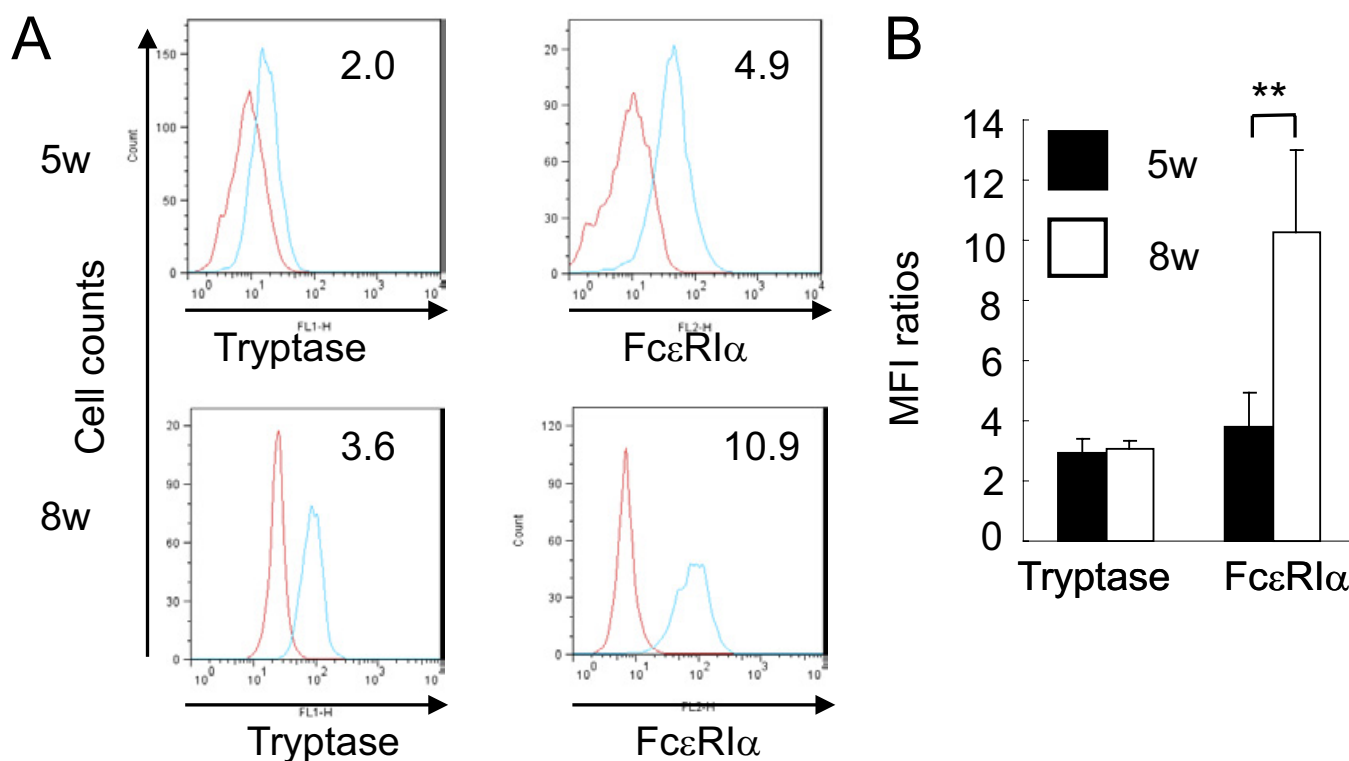


Fig. 2. Comparison of the expression levels of tryptase and cell surface Fc ϵ RI between 5 w- and 8 w-peripheral blood-derived cultured mast cells using FACS. The numbers in the histograms indicate the MFI ratios. The red and blue lines indicate isotype controls and tryptase or Fc ϵ RI α , respectively (A). (B) Statistical analysis of the expression levels shown in (A). The expression levels shown on the vertical axis are the MFI ratios. Data are expressed as the mean $\pm$ SEM of 4 experiments using 4 different donors. ** $P < .01$.

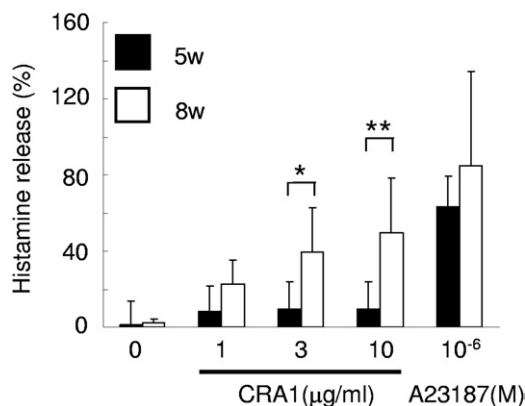


Fig. 3. Comparison of histamine release between 5 w- and 8 w-peripheral blood-derived cultured mast cells in response to CRA1 and A23187. Data are expressed as the mean $\pm$ SEM of 4 experiments using 4 different donors. * $P < .05$; ** $P < .01$.

Omalizumab is an anti-IgE recombinant humanized monoclonal antibody that decreases the concentration of IgE in the serum and also has been shown to reduce Fc ϵ RI expression on basophils, skin MCs, and dendritic cells.^{12–15} However, omalizumab treatment caused no reductions in tryptase⁺ cells in skin sections.¹³ The addition of omalizumab to the treatment of persistent allergic asthma inadequately controlled with inhaled corticosteroids has been demonstrated to improve asthma control by reducing the number of asthma exacerbations.^{16–18} However, the effect of omalizumab on immature MCs has not yet been studied.

Methods

Ethical considerations

The study was approved by the Ethics Committees of Nihon University; all the subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association.

Generation of adult BM and PB progenitor-derived MCs

The CD34⁺ BM cells were isolated from human BM cells (purchased from Cambrex, Charles City, Iowa) by CD34⁺ magnetic selection using anti-CD34 monoclonal antibody (mAb; clone QBEND/10; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The total number of CD34⁺ cells from 100 mL of PB mononuclear cells was normally 10⁴ to 10⁵. To avoid the loss of CD34⁺ cells by isolation, we used the lineage-negative selection instead of CD34⁺ positive selection in this study. Lineage-negative (CD4[−], CD8[−], CD11b[−], CD14[−], CD16[−], and CD19[−]) mononuclear cells were selected from PB mononuclear cells. The CD34⁺ BM cells or the lineage-negative mononuclear cells from PB mononuclear cells were cultured in serum-free Iscove's methylcellulose medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) and IMDM containing 200 ng/mL of recombinant human (rh) SCF (PeproTech EC Ltd., London, United Kingdom), 50 ng/mL of rh interleukin (IL)-6 (PeproTech EC Ltd.), and 1 ng/mL of rhIL-3 (PeproTech EC Ltd.), as previously described.³ On culture day 42, methylcellulose was

dissolved in phosphate-buffered saline (PBS), and the cells were resuspended and cultured in IMDM containing 100 ng/mL of rhSCF and 50 ng/mL of rhIL-6 with 2% fetal calf serum. For GeneChip analysis, Kit⁺ cells were isolated from cultured cells from CD34⁺ BM cells on 0, 3, 5, 21, 35, and 77 days of the culture by Kit⁺ magnetic selection using anti-CD117 mAb (clone AC126; Miltenyi Biotec, GmbH). The anti-CD117 mAb clone AC126 does not interfere with SCF. Mast cell responses to SCF (and anti-IgE) are unaffected by the presence of the anti-CD117 mAb clone AC126.¹⁹ The MC numbers and purity were determined by counting after metachromatic staining with toluidine blue (Kimura's stain)²⁰ or by fluorescence-activated cell sorting (FACS) with fluorescein isothiocyanate (FITC)-conjugated anti-tryptase mAb (clone AA1; Dako, Glostrup, Denmark).

Effect of IgE and omalizumab on cultured MCs

Myeloma IgE (1 μ g/mL; Calbiochem), microcentrifuged at approximately 14,000g for 20 minutes at 20°C to remove possible large aggregates, 10 μ g/mL of omalizumab (anti-IgE recombinant humanized mAb; donated by Novartis Pharma AG [Basel, Switzerland], isotype: human IgG1) or human IgG1 (Beckman Coulter Inc., Fullerton, California) were added to cultures of lineage-negative mononuclear cells on day 0. Additional IgE and omalizumab were added on day 14 and 28. The myeloma IgE used in the current study was poorly cytokinergic, because the treatment of IgE alone failed to induce degranulation (data not shown) and to upregulate messenger RNA (mRNA) for IL-8 in human CB-derived cultured mature MCs (eFig 1).

Activation of human MCs

The MCs were incubated with the indicated concentrations of mouse anti-human Fc ϵ RI α (clone CRA1), 1 μ M of calcium ionophore A23187 (A23187; Sigma-Aldrich, St. Louis, Missouri), or N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) alone at 37°C for 30 minutes.

Statistical Analysis

Differences between two groups of in vitro data were analyzed using an unpaired Student *t*-test and were considered significant at $P < .05$.

See eMethods for additional information about the experimental protocols (Isolation of human PB mononuclear cell, Isolation, purification and culture of human lung MCs, RNA isolation and real-time quantitative reverse transcription polymerase chain reaction [RT-PCR], GeneChip expression analysis, Flow cytometric analyses, Immunoblotting, and Histamine release assays).

Results

Kinetics of mRNA expression of MC-related genes, Fc receptors and KIT, and SNARE proteins during the culture of human CD34⁺ progenitor cells derived from BM in the presence of SCF

To examine the time-course of the mRNA expression of MC-related genes, such as *CMA1* (chymase 1), *TPSAB1* (tryptase α/β 1), *PGDS* (prostaglandin synthase), *KIT*, and *FCER1A* (Fc ϵ RI α), as well as

Table 1
The numbers of 5 w-PB-derived cultured MCs treated with or without IgE, in the presence or absence of omalizumab

	Control IgG	IgE + control IgG	IgE + omalizumab
Mean $\pm$ SEM	$5.0 \times 10^5 \pm 6.6 \times 10^4$	$6.5 \times 10^5 \pm 9.5 \times 10^4$	$6.9 \times 10^5 \pm 1.2 \times 10^5$
Fold	1	1.3 ± 0.5	1.4 ± 0.3
No. of experiments	16	16	10

Abbreviations: MCs, mast cells; PB, peripheral blood.

MCs were identified with Kimura's stain. The culture was started with approximately 10⁶ of lineage-negative (CD4[−], CD8[−], CD11b[−], CD14[−], CD16[−], and CD19[−]) mononuclear cells/well.

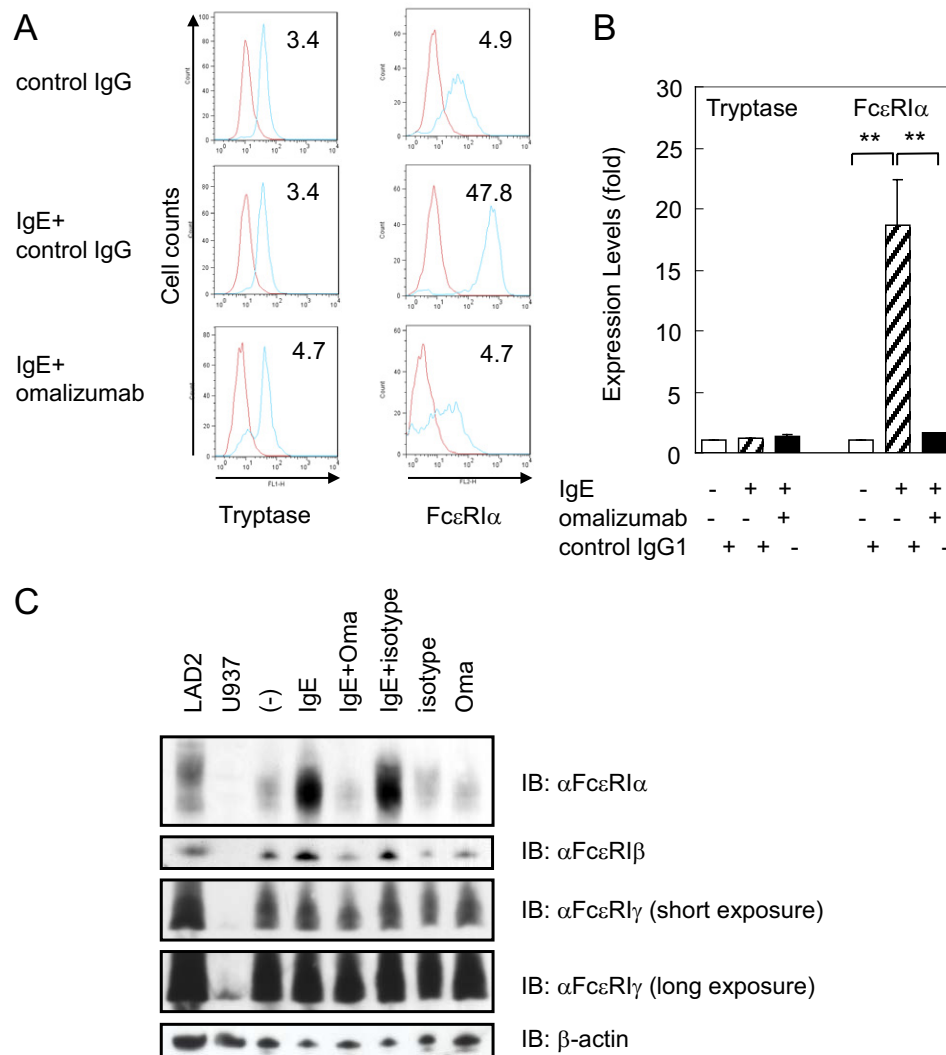


Fig. 4. Effect of IgE (1 μ g/mL) on FcεRI expression and effect of omalizumab (10 μ g/mL) on IgE-mediated upregulation of FcεRI expression in 5 w-peripheral blood-derived cultured mast cells. The numbers in the histograms indicate the MFI ratios. The red and blue lines indicate the isotype controls and tryptase or FcεRIα, respectively (A). (B) Statistical analysis of the expression levels shown in (A). The expression levels of tryptase and cell surface FcεRI in 5 w-cultured mast cells treated with control IgG1 alone (10 μ g/mL) were designated as 1. Data are expressed as the mean $\pm$ SEM of 7 experiments using 7 different donors. ** $P < .01$. (C) FcεRIα, FcεRIβ or FcεRIγ protein expression levels in human MCs treated with culture medium alone (-), IgE alone (IgE), IgE plus omalizumab (IgE + Oma), IgE plus control IgG (IgE + isotype), control IgG (isotype), or omalizumab alone (Oma) for 5 weeks were examined by immunoblotting. The data are representative of similar results obtained from 2 independent experiments using 2 different donors. LAD2 cells and U937 cells were used as a positive and a negative control of FcεRIα and FcεRIβ expression, respectively. Note that FcεRIγ of U937 is visible after long exposure of the same blots of short exposure.

soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins, such as the VAMP, STX (syntaxin), and SNAP-23 families, during the differentiation of human MCs, the gene expression profiles of Kit⁺ cells derived from CD34⁺ BM cells cultured in the presence of SCF for 0, 3, 5, 21, 35, and 77 days were investigated using a GeneChip analysis. Because the total number of CD34⁺ cells from 100 mL of PB mononuclear cells is approximately 10^4 to 10^5 , we used CD34⁺ BM cells for a GeneChip analysis. We found that the expression levels of CMA1, TPSAB1, PGDS, TPSB2 (tryptaseβ2), HDC (histidine decarboxylase), FCER1G (FcεRIγ), KIT, and SNARE proteins (VAMP2, VAMP3, VAMP8, SNAP23, STX4A, and STXBP2 [STX-binding protein2]) reached a plateau after 35 days (5 weeks) of culture (Fig 1A, 1B, and 1C). However, the expression levels of FCER1A and MS4A2 (FcεRIβ) in the 5w-cultured Kit⁺ cells were approximately 60% of those in 11w-cultured Kit⁺ cells (Fig 1B). The mRNA expression of a SNARE protein, STX3A, increased according to the culture period (Fig 1C).

Comparison of surface FcεRI expression and FcεRI-mediated histamine release between immature (5 w-cultured) and mature (8 w-cultured) MCs

We next compared the protein levels of tryptase and cell surface FcεRIα in 5 w- and 8 w-cultured MCs using FACS (Fig 2A and 2B). The results showed that the tryptase levels in 5 w-cultured MCs were not significantly different from those in 8 w-cultured MCs, but the expression levels of cell surface FcεRI on the 5 w-cultured MCs were significantly lower than those on the 8 w-cultured MCs (Fig 2B, $P < .01$, $n = 4$ donors). Next, the histamine releases by 5 w- and 8 w-cultured MCs in response to anti-FcεRIα mAb (CRA1) or calcium ionophore A23187 (A23187) were compared (Fig 3). Both MCs released comparable levels of histamine in response to A23187, but the amounts of histamine release from the 5 w-cultured MCs in response to CRA1 (3 and 10 μ g/mL) were significantly smaller than those from the 8 w-cultured MCs ($P < .05$ or $.01$).

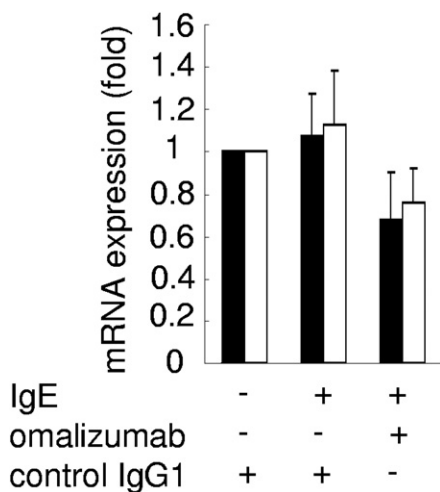


Fig. 5. FcεRIα mRNA (closed bars) and FcεRIβ mRNA (open bars) expression levels in 5 w-peripheral blood-derived cultured mast cells treated with or without IgE in the presence or absence of omalizumab. The expression levels of FcεRIα and FcεRIβ mRNA, which were analyzed using real-time RT-PCR, in 5 w-cultured mast cells treated with control IgG1 alone, were designated as 1. Data are expressed as the mean ± SEM of 5 experiments using 5 different donors.

Effect of IgE and omalizumab in the culture medium on the numbers of 5 w-PB-derived cultured MCs

We next examined the effect of IgE on the number of MCs by determining the presence of IgE. The IgE was added to the culture medium from the beginning of the culture. Additional IgE was added on day 14 and 28. The number of MCs was not significantly affected by the presence of IgE and omalizumab (Table 1).

Effect of IgE on FcεRI expression and effect of omalizumab on IgE-mediated upregulation of FcεRI expression in 5 w-PB-derived cultured MCs

To test whether monomeric IgE accelerates the responsiveness of immature MCs to CRA1, the effect of IgE on FcεRI-mediated MC activation was investigated by comparing FcεRI cell surface expression, tryptase expression, and FcεRI-mediated histamine release between 5 w-cultured MCs treated with IgE and those treated without IgE. By the addition of IgE to the culture medium, the cell surface expression level of FcεRI on the 5 w-cultured MCs significantly increased to 18.7 ± 3.8 -fold, compared with cells cultured without the addition of IgE ($P < .01$, Fig 4A and 4B), whereas the expression level of tryptase was not significantly affected (1.1 ± 0.1 -fold). To examine the effect of omalizumab on the IgE-dependent enhancement of cell surface FcεRI expression, omalizumab was added to the culture medium with IgE. Omalizumab significantly inhibited the IgE-dependent enhancement of cell surface FcεRI expression ($P < .01$, Fig 4A and 4B). To confirm the changes of FcεRI protein expression by IgE and omalizumab, we performed immunoblotting analysis using anti-FcεRIα, anti-FcεRIβ, and anti-FcεRIγ Abs (Fig 4C). The results revealed that IgE upregulated both FcεRIα and FcεRIβ protein expressions. Omalizumab downregulated IgE-mediated upregulation of both FcεRIα and FcεRIβ expressions. Omalizumab alone did not affect both FcεRIα and FcεRIβ expression levels. The expression of FcεRIγ appeared not to be affected by IgE and omalizumab.

FcεRIα mRNA and FcεRIβ mRNA expression levels in 5 w-PB-derived cultured MCs treated with or without IgE in the presence or absence of omalizumab

We confirmed that the mRNA expression levels of FcεRIα and β, which were analyzed using real-time RT-PCR, were not affected by the presence of IgE and omalizumab (Fig 5).

Effect of IgE on histamine release from 5 w-PB-derived cultured MCs in response to CRA1 and A23187 and effect of omalizumab on IgE-mediated enhancement of histamine release by CRA1

CRA1 (1 and 3 μg/mL)-induced histamine release from IgE-treated, 5 w-cultured MCs was significantly higher than that from MCs without the addition of IgE ($P < .05$ or $< .01$, Fig 6). However, the amounts of histamine release from the MCs in response to A23187 were not affected by pretreatment with IgE (Fig 6). Omalizumab significantly inhibited the IgE-dependent enhancement of FcεRI-mediated histamine release ($P < .05$ or $< .01$, Fig 6).

Effect of IgE and omalizumab in the culture medium on the 5 w-human lung tissue-derived cultured MCs

The number of lung MCs in the culture medium with control IgG, with IgE and control IgG, and with IgE and omalizumab were 3.65×10^5 , 3.71×10^5 , and 3.43×10^5 , respectively, suggesting that the number of lung MCs was not affected by the presence of IgE and omalizumab. By the addition of IgE to the culture medium, the cell surface expression level of FcεRI on the 5 w-cultured lung MCs increased to 3.3-fold, compared with cells cultured without the addition of IgE (Fig 7A), whereas the expression level of tryptase was not affected (0.97-fold). Omalizumab downregulated the IgE-dependent enhancement of cell surface FcεRI expression. The mRNA expression levels of FcεRIα and β in 5 w-cultured lung MCs were not affected by the presence of IgE and omalizumab (Fig 7B). CRA1 (0.3 and 1 μg/mL)-induced histamine release from IgE-treated, 5 w-cultured lung MCs was higher than that from MCs without the addition of IgE, and its acceleration was inhibited by addition of omalizumab (Fig 7C).

Discussion

In this study, we found that the expression level of FcεRI in 5 w-cultured MCs was not sufficient to induce degranulation after FcεRI aggregation but that 5 w-cultured MCs were fully responsive to A23187. Immunoglobulin E accelerates the responsiveness of immature (5 w-cultured) MCs to CRA1, and omalizumab inhibits the IgE-mediated acceleration. These data suggest that high levels of IgE in the microenvironment in vivo may upregulate the responsiveness of immature MCs to allergens. Omalizumab may inhibit the IgE-mediated responsiveness of not only mature MCs but also immature MCs.

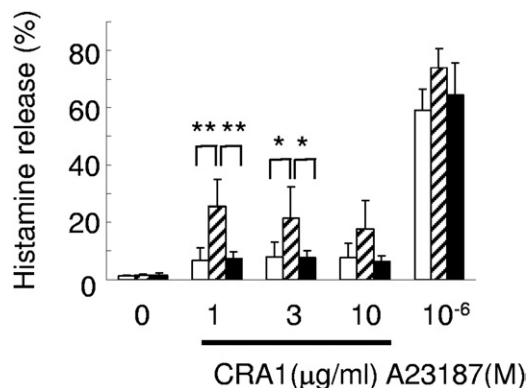


Fig. 6. Effect of IgE on histamine release from 5 w-peripheral blood-derived cultured mast cells in response to CRA1 and A23187 and effect of omalizumab on IgE-mediated enhancement of histamine release by CRA1. Open, hatched, and closed bars indicate mast cells treated with control IgG1 alone, those treated with IgE and control IgG1, and those treated with IgE and omalizumab, respectively. Data are expressed as the mean ± SEM of 5 experiments using 5 different donors. * $P < .05$; ** $P < .01$. Treatment of mast cells with omalizumab alone did not induce histamine release (data not shown).

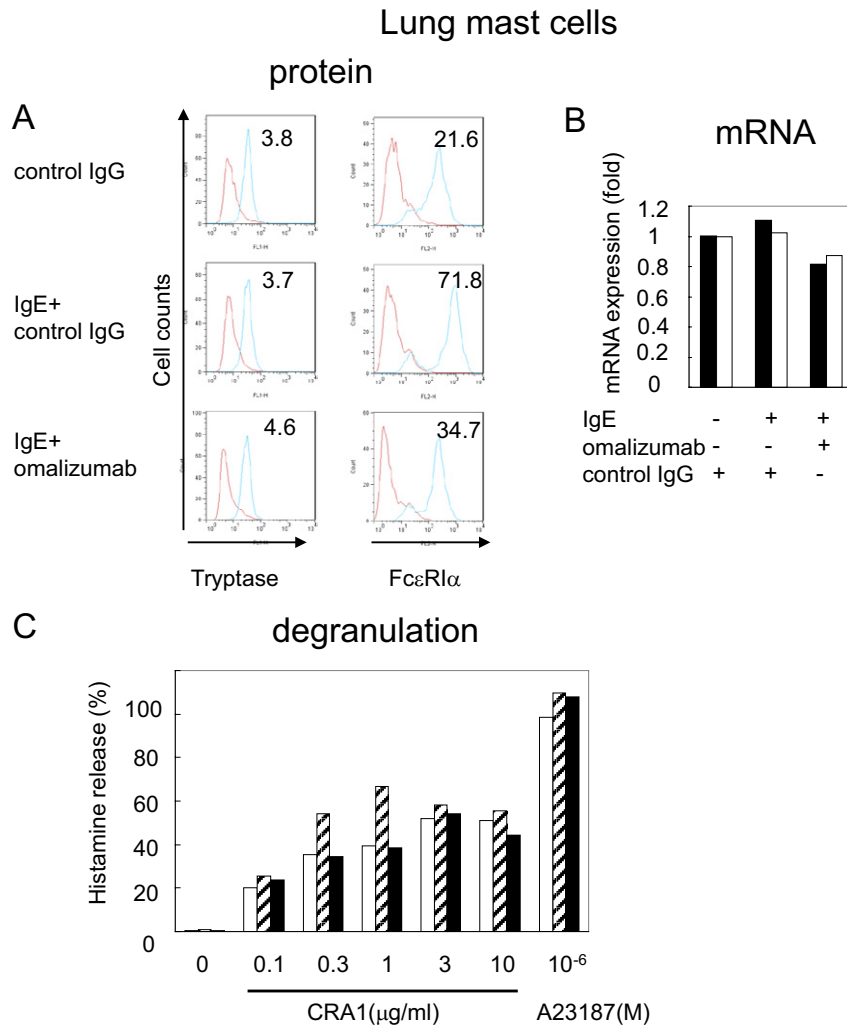


Fig. 7. Effect of IgE and omalizumab in the culture medium on the 5 w-human lung tissue-derived cultured MCs. (A) Effect of IgE (1 μg/mL) on FcεRI expression and effect of omalizumab (10 μg/mL) on IgE-mediated upregulation of FcεRI expression in 5 w-lung tissue-derived cultured mast cells. The numbers in the histograms indicate the MFI ratios. The red and blue lines indicate the isotype controls and tryptase or FcεRIα, respectively. (B) FcεRIα mRNA (closed bars) and FcεRIβ mRNA (open bars) expression levels in 5 w-lung tissue-derived cultured mast cells treated with or without IgE in the presence or absence of omalizumab. (C) Effect of IgE on histamine release from 5 w-lung tissue-derived cultured mast cells in response to CRA1 and A23187 and effect of omalizumab on IgE-mediated enhancement of histamine release by CRA1. Open, hatched, and closed bars indicate mast cells treated with control IgG1 alone, those treated with IgE and control IgG1, and those treated with IgE and omalizumab, respectively. Data are expressed as the mean of 2 experiments using 2 different donors. The donors of experiments (A) and (B) are 2 different donors. The 2 donors of experiment (C) are the same as the donors of experiments (A) and (B).

Mast cell-related genes such as (a) *CMA1*, *TPSAB1*, *PGDS*, *TPSB2*, and *HDC* and (b) *FCER1G*, *FCER1A*, *MS4A2*, and *KIT* as well as SNARE proteins such as (c) *STX3A*, *STX4A*, *VAMP2*, *VAMP3*, *VAMP8*, *SNAP23*, and *STXBP2* were selected according to previously reported data.^{9,21} The granule-localized vesicle SNARE, *VAMP8*, and the plasma membrane-associated Syntaxin 4 and SNAP-23 target SNARE are reported to be involved in the secretion of preformed mediators in MCs after the aggregation of FcεRI.^{22–25} Granule-localized Syntaxin 3 and relocated SNAP-23 may play a role in the granule–granule fusion observed in MCs.^{26–28} The expression levels of *CMA1*, *TPSAB1*, *PGDS*, *TPSB2*, *HDC*, *FCER1G*, *KIT*, and SNARE proteins (*VAMP2*, *VAMP3*, *VAMP8*, *SNAP23*, *STX4A*, and *STXBP2*) reached a plateau after 5 w of culture (Fig. 1). Although the expression levels of *MS4A2*, *FCER1A*, and *STX3A* in 5 w-cultured MCs were much lower than those in 11 w-cultured MCs, 5 w-cultured MCs fully acquired the ability to degranulate in response to A23187, but not to after the aggregation of FcεRI. These data suggested that the expression levels of FcεRI may not be sufficient to enable a response to CRA1 in the 5 w-cultured MCs. We further investigated whether 4 w-cultured MCs release histamine in response to A23187. Although 4

w-cultured MCs were able to degranulate in response to A23187, and cell surface FcεRI expression in 4 w-cultured MCs was enhanced by the presence of IgE, IgE-treated, 4 w-cultured MCs released a marginal amount of histamine after FcεRI aggregation (data not shown). These data indicate that the enhancement of surface FcεRI expression on 4 w-cultured MCs was not sufficient to induce degranulation after the aggregation of FcεRI, suggesting that some of the FcεRI-mediated signaling molecules are not fully functional in 4 w-cultured MCs. Therefore, we decided to use 5 w-cultured MCs to examine whether IgE accelerates the responsiveness to CRA1. The surface expression levels of FcεRI on 5 w-cultured MCs were almost half of those of 8 w-cultured MCs (Fig 2B). Ability of degranulation in response to A23187 and intracellular tryptase level in 5 w-cultured MCs are comparable to those in 8 w-cultured MCs (Figs 2B and 3). According to Ishizaka et al's definition,⁵ 5 w-cultured MCs are immature, because the FcεRI expression level was very low, and the MCs failed to induce histamine release after aggregation of FcεRI. Thus, our data indicate that FcεRI-related maturation occurs during the latter phase of this MC culture system. Shimizu et al¹⁶ previously reported that total tryptase levels

(mean $\pm$ standard error of the mean [SEM], pg/mast cell) in human BM-derived cultured MCs at 2, 4, 8 and 12 weeks of culture were 4.5 ± 2.6 , 5.9 ± 0.8 , 5.1 ± 1.3 and 14 ± 9 , respectively. Chymase levels (mean $\pm$ SEM, pg/mast cell) in human BM-derived cultured MCs at 4, 8, and 12 weeks of culture were 1.6 ± 1.6 , 3.8 ± 1.4 , and 5.6 ± 0.9 , respectively.⁶ Histamine levels in human BM-derived cultured MCs at 4, 8, and 12 weeks of culture were not changed.⁶ Thus, 8w- and 11w-cultured MCs are mature, and 11w-cultured MCs contain high levels of chymase.

Immunoglobulin E accumulates surface Fc ϵ RI without upregulation of Fc ϵ RI α and Fc ϵ RI β mRNA expression. This is agreement with previous findings in mouse MCs,²⁹ human CB-derived MCs,³⁰ skin MCs,¹⁵ and basophils.³¹ Xia et al.³² showed that IgE increases the percentage of Fc ϵ RI⁺ cells developing from human fetal liver-derived progenitors. The findings suggest that IgE stabilizes Fc ϵ RI on the cell surface and inhibits its internalization in immature MCs, similar to its effect on mouse MCs, human CB-derived MCs, and basophils.³¹ Omalizumab downregulates and prevents IgE-induced Fc ϵ RI augmentation. This result also agrees with previous findings observed in human skin MCs.¹⁵ Gomez et al.¹⁵ reported that the increased expression of Fc ϵ RI enhanced degranulation when Fc ϵ RI was aggregated at low concentrations of anti-Fc ϵ RI mAb. The same tendency was observed in immature MCs (Fig 6).

The MC progenitors differentiate and complete their development peripherally. Immature MCs do not respond after aggregation of Fc ϵ RI. Therefore, the immature MCs may not be involved in the pathogenesis of allergic asthma. In our human culture system, the poorly cytokinergic IgE, which we used, did not increase the number of MCs. If IgE accelerates Fc ϵ RI-mediated responsiveness, the number of MCs that are ready to be activated by allergens may increase to an extreme degree. Indeed, the in vitro studies, especially those based on long-term culture, suffer of several variables rendering them difficult to transfer to patients automatically. However, the number of degranulated MCs increased beneath the epithelium (where the MCs can easily access allergens) in samples from patients with asthma, compared with normal controls.³³ At least, we confirmed our data performed in peripheral blood-derived-cultured MCs by using human lung cultured MCs (Fig 7). Our data demonstrate that omalizumab downregulates the IgE-mediated responsiveness of immature MCs. Thus, our data support the strong evidence demonstrating that treatment with omalizumab is well tolerated and clinically effective for the management of moderate to severe allergic asthma.^{16–18} Our data suggest that the downregulation of Fc ϵ RI expression on immature MCs by omalizumab may be one explanation for the clinical stability of patients' asthma conditions with omalizumab therapy.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.anai.2012.01.009.

eMethods

Isolation of human PB mononuclear cells

Human PB mononuclear cells were isolated by the centrifugation of whole blood on a Ficoll-Isopaque density gradient (Nycomed, Oslo, Norway).

Isolation, purification, and culture of human lung MCs

Macroscopically normal human lung tissue was obtained during surgery. Lung cells were dispersed from chopped lung specimens by an enzymatic procedure, as described previously.¹ The dispersed lung cells were cultured in serum-free Iscove's methylcellulose medium as previously described.² The purity of human MCs was assessed with metachromatic staining.

RNA isolation and real-time quantitative RT-PCR

Total cellular RNA was isolated from the MCs and real-time quantitative PCR was performed as previously described.³ The primers used for the TaqMan analysis were as follows: FcεRIα, sense primer (5'-TGGAATCCCCTACTCTACTGTGTGA-3'), antisense primer (5'-CCTTAGGTTTCTGAGGGACTGC-3'), and probe (5'-FAM-CCTTACTGTTCTTCGCTCCAGATGGCGTGT-TRAM-3'). Human gene-specific primers and probe sets for FcεRIβ and glyceraldehyde 3-phosphate dehydrogenase were designed using the Assays-on-Demand service (Applied Biosystems, Tokyo, Japan).

GeneChip expression analysis

Human genome-wide gene expression was assessed using the Human Genome U133 Plus 2 probe array (GeneChip, Affymetrix, Santa Clara, California), which contains an oligonucleotide probe set for approximately 66,996 full-length genes and ESTs (expressed sequence tags), according to the manufacturer's protocol (Expression Analysis Technical Manual) and as in previous reports.³ The data obtained were analyzed using Genespring software, version GX 7.3 (Silicon Genetics, San Carlos, California). To normalize the staining intensity variations among the chips, the average difference values for all the genes on a given chip were divided by the median of all the measurements on that chip. Based on these normalized values, the genes were classified as upregulated or downregulated.

Flow cytometric analyses

Flow cytometric analysis of the MCs was performed as described previously.¹ PE-conjugated anti-FcεRIα mAb (clone: CRA1, eBioscience, San Diego, California), PE-conjugated isotype control mouse IgG2b, FITC-conjugated anti-tryptase mAb (clone AA1), or FITC-conjugated isotype control mouse IgG1 were used.

Immunoblotting

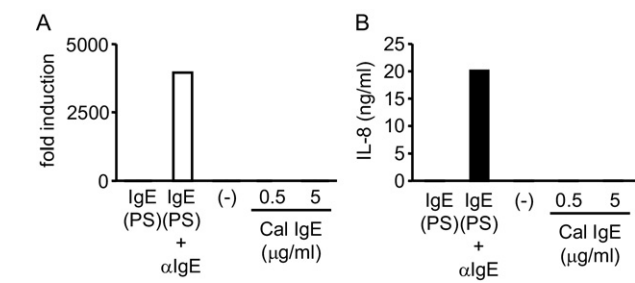
Cell lysates were prepared, and proteins were separated as described previously.⁴ To probe whole-cell lysates for FcεRIβ, FcεRIα, FcεRIγ, and β-actin, cell lysates were probed with anti-human FcεRIβ,⁵ anti-FcεRIα (Upstate Biotechnology), anti-FcεRIγ (Upstate Biotechnology), and anti-β-actin (clone C4, Santa Cruz Biotechnology, Inc., Santa Cruz, California), respectively.

Histamine release assays

The degree of MC degranulation was monitored by measuring the histamine released into the extracellular fluid after activation using an enzyme immunoassay kit (Immunotech A Beckman Coulter Co., Marseille, France). The percentage of histamine release was calculated from the ratio of each sample to the total histamine level.

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eFig. 1. Effect of IgE alone on IL-8 mRNA expression and IL-8 production. Human CB-derived cultured MCs (12 w-cultured) are incubated with 10 ng/mL of rhIL-4 for 24 h, then the cells were washed and incubated with 0 (PBS [-]), 0.5, or 5 μg/mL of myeloma IgE (Calbiochem [Cal]), or 1 μg/mL of IgE (PS) for 2 hours. The PS-IgE-preincubated MCs were washed and then activated with anti-IgE (IgE(PS) + αIgE) for 2h. IL-8 and GAPDH mRNA levels were measured by quantitative RT-PCR. The amount of IL-8 mRNA was normalized against GAPDH mRNA, and the relative value in PBS-treated cells was set at 1. (B) Interleukin-8 protein was measured using an enzyme-linked immunosorbent assay kit (BD PharMingen). Immunoglobulin E (PS) from the multiple myeloma patient PS was a gift from Dr. Kimishige Ishizaka, La Jolla Institute for Allergy and Immunology, La Jolla, California. Human CB-derived MCs express low levels of FcεRI on their cell surface.⁶ Previous studies showed that incubation of human CB-derived cultured MCs with IL-4 enhances FcεRI expression⁶ and renders the cells sensitive to stimulation with human IgE, which induces the production of chemokines, IL-8, and monocyte chemoattractant protein 1.⁷ Therefore, we used IL-4-preincubated human CB-derived cultured MCs in this study.