

Regulation of IgE-Dependent Zinc Release from Human Mast Cells

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Key Words

Human mast cell · Immunoglobulin E · Zinc

Abstract

Background: Zinc (Zn) affects many aspects of immune function, including thymic development and the activities of immune cells. Zn is also involved in many steps of high-affinity IgE receptor (FcεRI)-induced mast cell (MC) activation, which is required for degranulation and cytokine production. Intracellular Zn levels increase in mouse MCs after FcεRI stimulation. We previously reported that Zn distribution in a human MC line, LAD2, changed dramatically following FcεRI aggregation with synchrotron radiation microbeams. However, the kinetics of Zn distribution and the underlying mechanisms following FcεRI cross-linking remain unknown. **Methods:** We used cord-blood-derived MCs and LAD2 cells. Degranulation was assessed by β-hexosaminidase (β-hex) release. Extracellular Zn levels were determined by inductively coupled plasma atomic emission spectrometry or

based on the fluorescence intensity of a Zn indicator. We also used RNAi to knockdown ZnT1 expression. mRNA expression levels were determined by real-time RT-PCR. **Results:** Zn was rapidly released from human MCs after FcεRI aggregation. The kinetics and optimal conditions for FcεRI cross-linking for Zn release were different from those for degranulation. Treating LAD2 cells with an intracellular Ca²⁺ chelator significantly inhibited IgE-mediated β-hex release but not Zn release. We investigated IgE-mediated β-hex and Zn release with specific inhibitors of signaling pathways. Zn and β-hex release were partly correlated with but also partly independent of IgE. Knockdown of the Zn efflux transporter, ZnT1, significantly inhibited Zn release from human MCs. **Conclusions:** Our results indicate that IgE-dependent Zn release from human MCs involves signaling cascades that are distinct from those of degranulation. Thus, Zn may have a unique function as a mediator of allergic inflammation.

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Introduction

Zinc (Zn) is an essential nutrient, and its deficiency causes growth retardation, immunodeficiency and neuronal degeneration [1, 2]. The functions of many enzymes, signaling molecules and transcription factors are critically dependent on and/or regulated by Zn. However, by itself, Zn is cytotoxic; it induces T and B cell apoptosis [3] and neuronal cell death [4]. Thus, Zn homeostasis is tightly regulated by Zn transporters, which control the movement of Zn into, out of and within cells [5, 6], and by binding proteins such as metallothioneins [7]. Zn transporters are believed to play a crucial role in maintaining the cellular balance between apoptosis and cell growth and thus disease prevention.

Mast cells (MCs) are the primary responding cells in allergic reactions, most of which are triggered by cross-linking of the high-affinity IgE receptor FcεRI [8, 9]. Following stimulation, MCs exert their biological effects by releasing preformed and de novo synthesized mediators [9]. During the 1960s, electron microscopic studies demonstrated that MC granules are rich in Zn [10]. Recently, FcεRI stimulation has been reported to trigger the formation of microtubules in a calcium-independent manner [11]. Zn is also required for FcεRI-induced translocation of granules to the plasma membrane [12]. In addition, Zn is essential for the plasma membrane translocation of protein kinase C and the subsequent nuclear translocation of NF-κB, leading to the production of cytokines such as IL-6 and TNF-α.

More recently, it has been reported that FcεRI cross-linking induces the intracellular release of free Zn from the perinuclear area in MCs, including the endoplasmic reticulum [13]. The intracellular increase in Zn was dependent on calcium influx and mitogen-activated protein kinase/extracellular signal-regulated kinase activation. We previously reported that Zn distribution in a human MC line, LAD2, was dramatically changed and that Zn translocated to the extracellular region following FcεRI aggregation with synchrotron radiation microbeams [14]. Zn accumulation in MCs was believed to be the sum of Zn influx and efflux via transporter proteins. However, the kinetics of Zn influx and efflux and the underlying mechanisms following FcεRI cross-linking remain unknown.

In this study, we analyzed the kinetics of intracellular and extracellular Zn distributions in human MCs following FcεRI aggregation. We found that Zn was released from MCs to the extracellular region both with common and distinct signaling cascades of anti-IgE-induced degranulation.

Materials and Methods

Cell Lines

LAD2 cells were kindly provided by Dr. Kirshenbaum, NIAID, NIH, Bethesda, Md., USA [15]. These cells were maintained as described previously [15].

Cord-Blood-Derived MCs and Adult

Peripheral-Blood-Derived MCs

Cord blood (CB) samples were purchased from the Cell Engineering Division of RIKEN Bioresource Center (Tsukuba, Ibaraki, Japan). Human peripheral-blood mononuclear cells (PBMNCs) were separated from venous blood samples of normal volunteers. All subjects provided written informed consent, and the study was approved by the ethical review board of each hospital. Human CBMNCs and PBMNCs were isolated and maintained as described previously [16].

Activation of Human MCs

LAD2 cells were preincubated with 1 μg/ml anti-4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP)-IgE (AbD Serotec, Raleigh, N.C., USA) for 24 h and then stimulated with NIP-BSA (Biosearch Technologies Inc., Novato, Calif., USA). CB-derived cultured MCs were preincubated with 1 μg/ml hIgE (Chemicon, Temecula, Calif., USA) and 10 ng/ml rhIL-4 (R&D Systems Inc., Minneapolis, Minn., USA) for 48 h and then stimulated with anti-IgE (Dako, Kyoto, Japan). MCs were also treated with 1 μg/ml ionomycin (Sigma-Aldrich, St. Louis, Mo., USA) or culture medium alone at 37°C for varying times.

Confocal Laser Scanning Microscopy

MCs in PBS were cytocentrifuged onto slides using Shandon Cytospin 2 (Fisher Scientific, Mississauga, Ont., Canada) at 400 g for 10 min. The cells were then air-dried and fixed in 99% ethanol for 10 min at room temperature. Dansylaminoethyl-cyclen (DAEC) (Dojindo Laboratories, Kumamoto, Japan) was used for Zn staining. Cell images were obtained by Leica TCS SP2 AOBs laser scanning confocal microscopy.

Measurements of Extracellular Zn Released from MCs

Cells at 2×10^5 cells/ml were stimulated. Cell supernatants were collected after centrifugation at 500 g for 5 min. Extracellular Zn levels were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES). These analyses were performed at the Kobelco Research Institute Inc. (Hyogo, Japan). We also measured extracellular Zn levels using the fluorescent Zn probe DAEC. CB-derived cultured MCs and LAD2 cells were incubated in HEPES buffer (pH 7.4) containing no Zn. Cell supernatants were collected following stimulation. The supernatants were incubated with DAEC (final concentration, 5 μM) for 30 min at 37°C. DAEC fluorescence intensities were determined using GENios, an optimal multidetector (Tecan AG, Switzerland).

β-Hexosaminidase and Histamine Release Assays

The extent of MC degranulation was monitored by measuring β-hexosaminidase (β-hex) and histamine release into the supernatants following FcεRI aggregation or ionomycin stimulation using a colorimetric assay and an enzyme immunoassay kit (MBL Co. Ltd., Nagoya, Japan), respectively. The net percentage of β-hex or histamine release was calculated as follows:

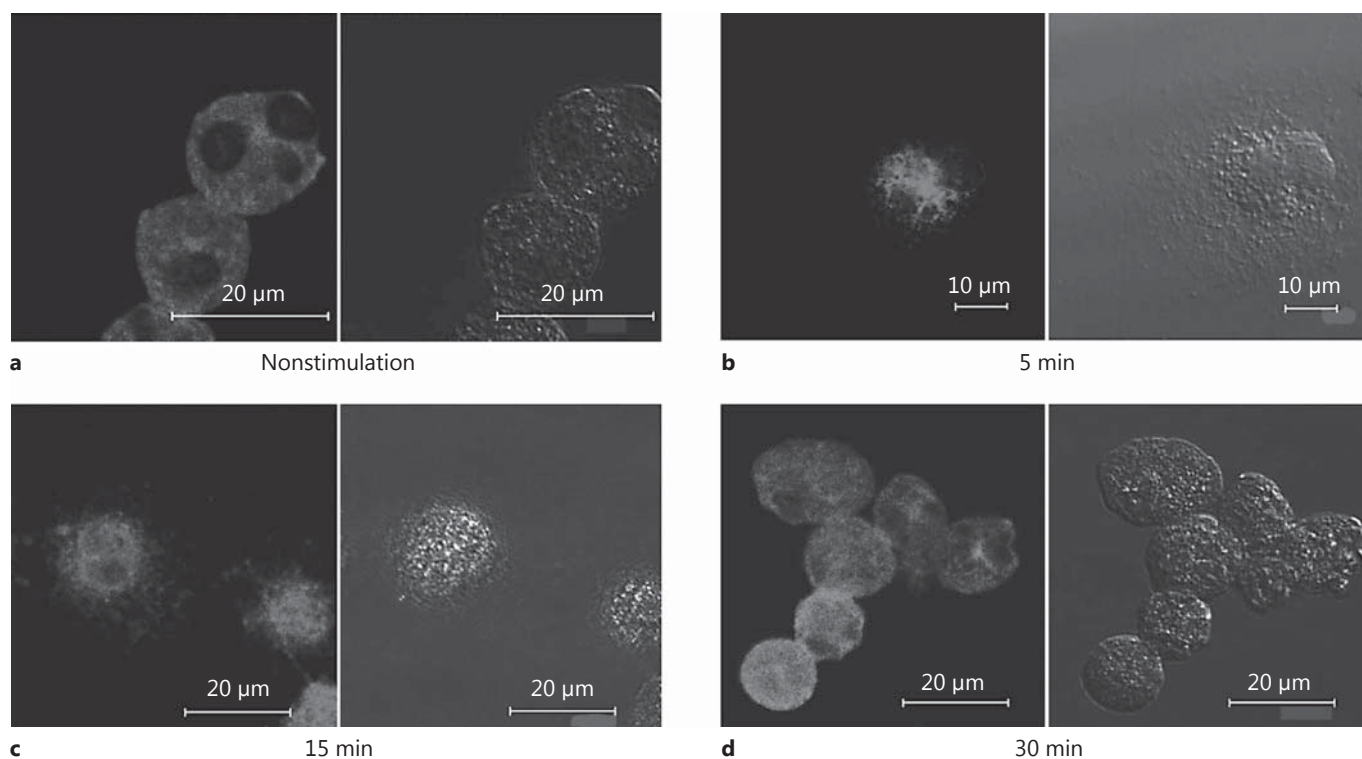


Fig. 1. Changes in Zn distribution in human MCs following FcεRI aggregation. CB-derived cultured MCs were preincubated with IgE and IL-4 and then stimulated with anti-IgE for 0 (**a**), 5 (**b**), 15 (**c**) and 30 min (**d**). Zn was detected by staining with DAEC and observed by laser confocal microscopy. A differential interference image is also shown. Scale bars are shown.

$$\frac{[\beta\text{-hex or histamine release} - \text{spontaneous release}]}{[\text{total } \beta\text{-hex and histamine release}]} \times 100.$$

Human ZnT1 Knockdown by RNA Interference

siRNA was transfected into cells using Gene Silencer transfection reagent according to the manufacturer's protocol (GenLantis, San Diego, Calif., USA). Human ZnT1 siRNA sequences were designed by Qiagen. The sequences of siRNA duplexes were as follows: sense, 5'-(GCA UUU GUA GAA AUA AUU A) dTdT-3'; antisense, 5'-(UAA UUA UUU CUA CAA AUG C) dTdT-3'. The effect of siRNA was evaluated using a SYBR Green real-time PCR system.

Results

Zn Distributions Changed Dramatically in Human MCs following FcεRI Aggregation

We first investigated the changes in Zn distribution in human CB-derived cultured MCs after FcεRI cross-linking using DAEC staining and confocal microscopy. In resting MCs, Zn was diffusely distributed in the cytoplasm (fig. 1a). At 5 min after FcεRI aggregation, human

MCs extruded membrane-free granules to the external environment (fig. 1b, differential interference image). Zn appeared to move to the extracellular region (fig. 1b). Degranulation was obvious 15 min after FcεRI aggregation (fig. 1c). Unexpectedly, 30 min after stimulation, Zn returned to the intracellular region of MCs (fig. 1d).

Release and Uptake of Zn by Human MCs following FcεRI Aggregation and Ionomycin Stimulation

To confirm Zn release from MCs after FcεRI cross-linking, we measured extracellular Zn levels by ICP-AES. Figure 2a shows the kinetics of Zn release from MCs following FcεRI aggregation or ionomycin stimulation. Zn was not detectable in the supernatants of MCs that had not been stimulated with anti-IgE or ionomycin. Zn release peaked 5 min after FcεRI cross-linking and was no longer detectable 30 min after stimulation. After stimulating MCs with ionomycin, Zn release was rapid and peaked at 1 min. At 5 min after ionomycin stimulation, Zn levels in supernatants returned to their basal levels.

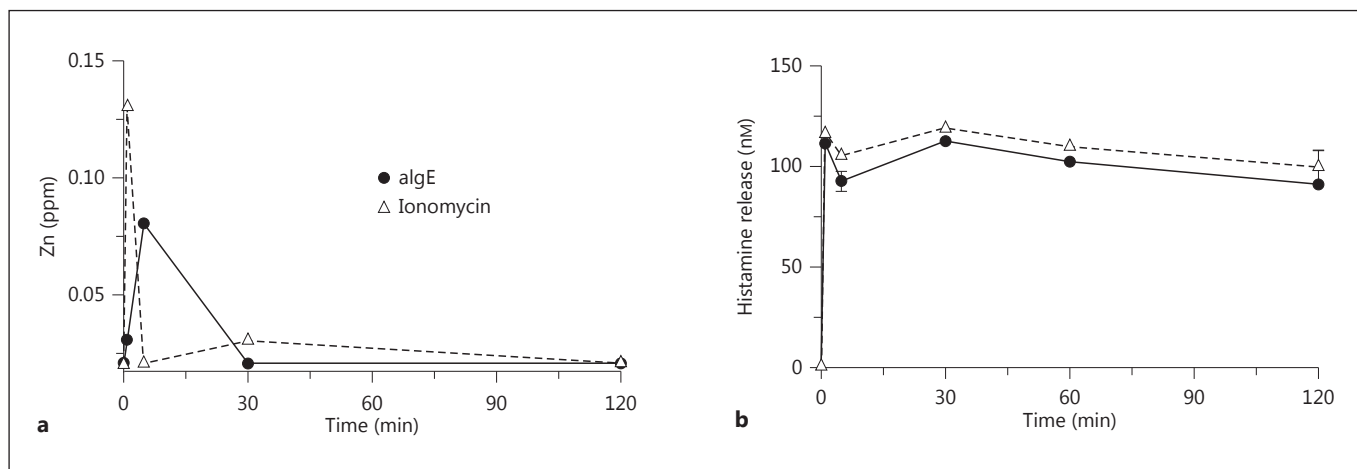


Fig. 2. Kinetics of histamine and Zn release from human MCs following FcεRI aggregation or ionomycin stimulation. CB-derived cultured MCs were preincubated with IgE and IL-4 and then stimulated with anti-IgE or ionomycin over 120 min. Released Zn (**a**) and histamine (**b**) were measured by ICP-AES and EIA, respectively.

Figure 2b shows the kinetics of histamine release from MCs following FcεRI aggregation or ionomycin stimulation. Histamine release peaked 1 min after anti-IgE or ionomycin stimulation. Subsequently, the histamine levels in supernatants remained the same over time.

Comparison of Zn Release and Degranulation Mechanisms

To further investigate the signaling pathway(s) involved in IgE-mediated Zn release, we compared the effects of inhibitors of PLC (U73122), MAPK (tamoxifen), PI3K (wortmannin, Ly294002) and a β-adrenergic agonist (formoterol) on IgE-mediated β-hex and Zn release from LAD2 cells. Wortmannin and U73122 significantly inhibited β-hex release but not Zn release. However, tamoxifen inhibited both Zn release and degranulation. In agreement with previous reports, formoterol significantly inhibited β-hex release from anti-IgE-stimulated MCs ($p < 0.05$). Interestingly, Zn release was almost completely inhibited by formoterol ($p < 0.05$). Thus, these findings suggested that both common and distinct signaling cascades are involved in β-hex and Zn release from human MCs induced by anti-IgE.

Suppressed ZnT1 Expression in MCs by siRNA Reduces FcεRI-Mediated Degranulation and Zn Release

We hypothesized that signaling cascades for Zn release were distinct from those for β-hex release induced by anti-IgE from human MCs and included Zn transporters.

Our preliminary microarray analysis of human tissue and progenitor-derived MCs revealed that at least three Zn efflux transporters and eight Zn uptake transporters were expressed in human MCs (data not shown). Among the three Zn efflux transporters, the expression level of ZnT1 (SLC30A1) was the highest in skin MCs, lung MCs, CB-derived cultured MCs and PB-derived cultured MCs. Thus, we selected ZnT1 for knockdown and investigated its effects on Zn and β-hex release. We suppressed ZnT1 transcripts in LAD2 cells using siRNA. ZnT1 mRNA expression decreased by approximately 98% (fig. 3a, $p < 0.01$). Suppression of ZnT1 mRNA expression resulted in a partial, but significant, downregulation of Zn release from LAD2 cells following FcεRI aggregation (fig. 3b, $p < 0.05$). Thus, endogenous ZnT1 may be involved in FcεRI-mediated Zn release from human MCs. Interestingly, suppression of ZnT1 mRNA expression also resulted in downregulation of β-hex release from LAD2 cells following FcεRI aggregation (fig. 3c).

Discussion

This is the first report to show that Zn-containing human MCs release Zn into the extracellular microenvironment immediately after FcεRI aggregation or ionomycin stimulation. We also provide the first characterization of Zn release from these cells. Unlike the release of some mediators such as histamine, Zn release is not completely

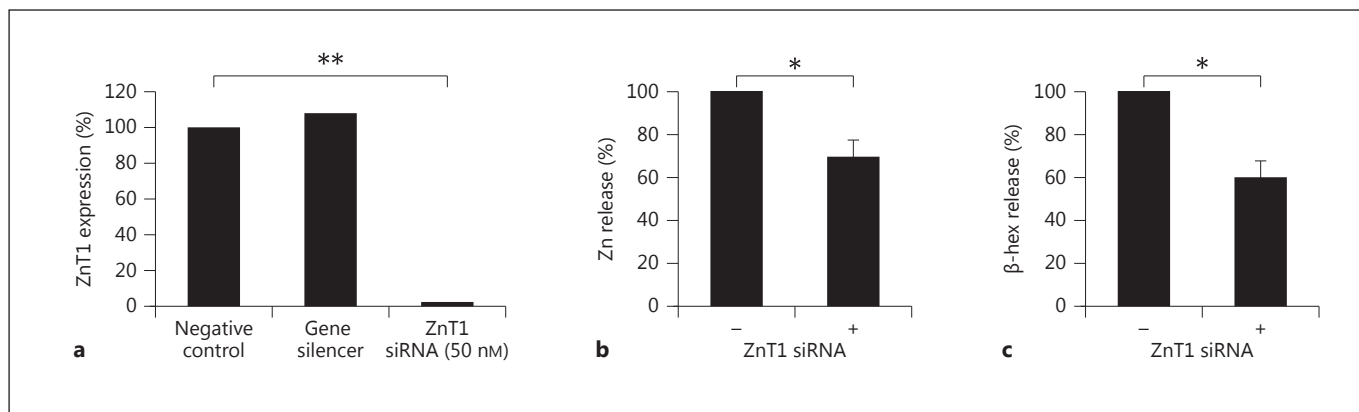


Fig. 3. Suppressed ZnT1 expression in MCs by siRNA reduces FcεRI-mediated Zn and β-hex release. LAD2 cells were incubated with ZnT1 siRNA (50 nM) or appropriate controls for 48 h. The cells were then processed for quantitative-PCR analysis (**a**), Zn release experiments (**b**), and degranulation experiments (**c**). **a** The ZnT1 mRNA expression level is shown as the ratio to the level in cells treated only with the reagent. As a negative control, LAD2 cells were incubated with a gene silencer other than ZnT1 siRNA. Results are the means ± SEMs from 3 independent experiments. **b**,

c After ZnT1 siRNA treatment, LAD2 cells were preincubated with 1 μg/ml of anti-NIP-IgE for 24 h and then stimulated with NIP-BSA (100 ng/ml) for 20 min to induce Zn release (**b**) and degranulation (**c**), which was assessed by β-hex release. IgE-dependent release of Zn and β-hex from LAD2 cells without ZnT1 siRNA treatment was set as 100% release. Release results are the means ± SEMs of 3 independent experiments each performed in duplicate. * $p < 0.05$; ** $p < 0.01$.

dependent on extracellular Ca^{2+} . It requires a lower concentration of allergen than β-hex release. Once released, Zn levels are restored in MCs presumably via transporters. Our results suggest that Zn may also have a unique function as a mediator in allergic inflammation.

Using ICP-AES, we estimated that approximately 0.8 ppm ($= 0.36 \mu\text{M}$) of Zn was released from CB-derived cultured MCs (2×10^5 cells/ml) following FcεRI aggregation. LAD2 cells (2×10^5 cells/ml) released approximately 9 μM Zn after FcεRI cross-linking. These differences in Zn release could have been due to the different methods used to measure Zn or the different cell types used. ICP-AES is an emission spectrophotometric technique based on the fact that excited electrons emit energy at a given wavelength as they return to their ground state. In contrast, a fluorescent reagent detects its target via a chemical reaction. Thus, it is possible that Zn atoms detected by these techniques are not in the same state because Zn can exist in different physical or chemical states, such as those observed when it is ionized or bound to protein.

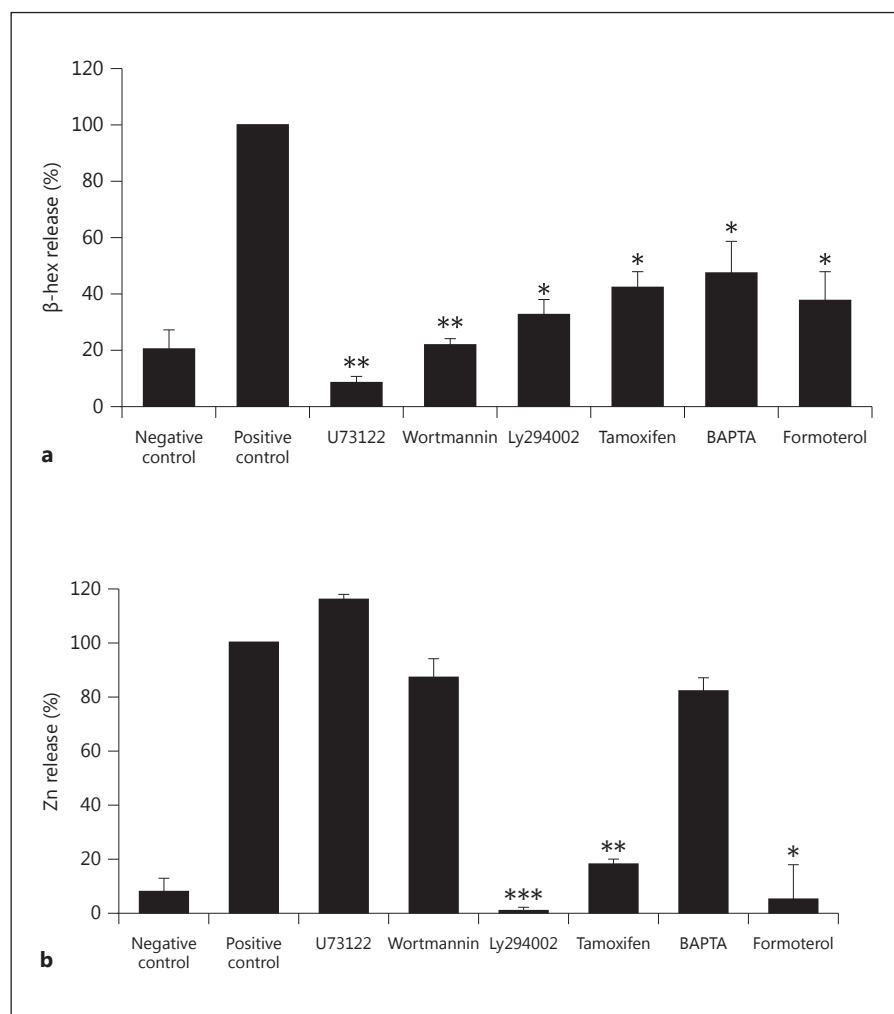
Microfluorescence imaging of murine hippocampal slices revealed that brief trains of electrical stimulation of mossy fibers caused immediate release of Zn from synaptic terminals into the extracellular microenvironment [17, 18]. Zn release was induced across a wide range of stimulus intensities and frequencies, including those likely to induce long-term potentiation [17]. The amount of

Zn released depended on the stimulation frequency (1–200 Hz) and intensity. Once released, Zn crossed the synaptic cleft and entered postsynaptic neurons, which resulted in increased intracellular Zn levels.

There are many differences in Zn release from synaptic terminals and human MCs. Zn release from synaptic terminals is dependent on extracellular Ca^{2+} [17]. Zn release is highly correlated with glutamate release from mossy fiber terminals induced by action potentials using a stimulation protocol similar to physiological synaptic activity [18]. Forskolin is a diterpene that activates adenylyl cyclases and enhances neurotransmitter and Zn release at the mossy fiber synapse. Induced Zn release is not observed in ZnT3-deficient mice [18]. These results suggest that the mechanism of Zn release from synaptic terminals induced by action potentials is different from Zn release from human MCs induced by FcεRI aggregation.

Although Zn and tryptase were colocalized in resting MCs, different distributions of Zn and tryptase were observed 30 min after FcεRI aggregation (data not shown). In a comparison of Zn and β-hex release from human MCs following FcεRI aggregation with regard to the requirement of extracellular Ca^{2+} and the optimal concentrations of NIP-BSA, we demonstrated that the signaling cascades for Zn and β-hex release were distinct (data not shown). We speculate that Zn efflux transporters are in-

Fig. 4. Correlation between IgE-dependent Zn (a) and β -hex release (b) from LAD2 cells. LAD2 cells were preincubated with 1 μ g/ml of anti-NIP-IgE for 24 h and treated with formoterol, BAPTA, U73122, wortmannin, Ly294002 or tamoxifen. Subsequently, the cells were washed and stimulated with NIP-BSA (100 ng/ml) for 20 min. IgE-dependent release of Zn and β -hex from LAD2 cells without treatment was set as 100% release. Results are the means $\pm$ SEMs of 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.



involved in the mechanism of Zn release, which is distinct from the mechanisms of degranulation.

Ten different Zn efflux transporters comprise the so-called solute-linked carrier 30 (SLC30 or ZnT) family [19, 20]. DNA chip analysis revealed that human MCs expressed three efflux transporters: ZnT1, ZnT5 and ZnT9. Among these, ZnT1 (SLC30A1) expression was the highest in skin MCs, lung MCs, CB-derived cultured MCs and PB-derived cultured MCs. To date, ZnT1 is the only Zn transporter protein that has been shown to be localized to the plasma membrane among mammalian ZnT members [21]. ZnT5 has been reported to be associated with insulin granules in β cells, suggesting that ZnT5 transports Zn into secretory granules [22]. ZnT9 exhibits translocation from the cytoplasm to the nucleus in a cell cycle-dependent manner [23], although its role and localization have not been well studied.

ZnT1 knockdown revealed that ZnT1 was significantly, but only partially, involved in Zn release following Fc ϵ RI aggregation (fig. 3). Because the expression levels of three different efflux transporters, ZnT1, ZnT5 and ZnT9, were almost the same in LAD2 cells, ZnT1 knockdown alone would not completely inhibit Zn release. Zn efflux via ZnT1 following Fc ϵ RI aggregation may only partially account for this effect. Exocytosis of Zn-containing granules may also be involved in Zn release.

After ZnT1 knockdown, MC degranulation was also significantly reduced. The reason for this is not certain, although it was recently reported that Zn is required for the Fc ϵ RI-induced translocation of granules to the plasma membrane. We speculate that disturbances in Zn influx and efflux may affect Fc ϵ RI-induced translocation of granules to the plasma membrane.

A β -adrenoceptor agonist, formoterol, significantly inhibited both Zn and β -hex release from MCs (fig. 4). It has been reported that there is a good correlation between the extent to which agonists inhibited histamine release and the degree to which agonists increased c-AMP levels in MCs, suggesting that the mechanism by which β -adrenoceptor agonists inhibit MC function is driven by c-AMP [24]. Zn release from MCs was also inhibited by β -adrenoceptor agonists. Thus, the pathway for Zn transfer to the extracellular region might be partly inhibited via c-AMP.

Our findings that Zn levels were restored in the cytoplasm and that Zn was no longer detectable in cell supernatants 30 min after Fc ϵ RI aggregation led us to speculate that Zn was taken up by MCs after Zn release. Fourteen different Zn uptake transporters have been reported, a so-called SLC39 or ZIP family. DNA chip analysis revealed that human MCs express seven different uptake transporters. The expression levels of these transporters varied among different tissue-derived MCs and different progenitor-derived MCs (data not shown). Thus, it was difficult to identify the transporter primarily involved in Zn uptake following aggregation using RNAi to knock down these transporters.

Zn affects many aspects of immune function, including thymic development and activities of T cells, B cells, NK cells and monocytes [25, 26]. Zn appears to regulate several signaling pathways that interact with the signals transduced by Toll-like receptors (TLR), and after activation, leads to the secretion of proinflammatory cytokines.

The interaction of Zn with protein tyrosine phosphatases and protein kinase C and the direct modulation of lipopolysaccharide binding to TLR-4 all result in enhanced cytokine production. In contrast, a complex interaction between Zn, NO and cyclic nucleotide signaling, inhibition of IL-1 receptor-associated kinase-1 and inhibitors of κ B kinase all counteract the production of proinflammatory cytokines.

In Jurkat cells, exogenously added Zn ions increased the intracellular Zn ion level and inhibited calcineurin activity, thereby suppressing mitogen-induced IL-2 production [27]. Thus, Zn released from human MCs might control the inflammatory status during allergic responses.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.

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