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Short communication

Characterization of an early signaling defect following FcεRI activation in the canine mastocytoma cell line, C2

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ABSTRACT

A comparison of IgE recognition by cognate receptors expressed on the C2 canine mastocytoma cell line with analogous events in a rat basophilic leukemia cell line transfected with the α-chain subunit of the canine high-affinity IgE receptor using flow cytometry show that canine IgE recognizes the α-chain of its cognate receptor on both cell lines. Our study confirms the expression of functional IgE receptors in both cell lines, but receptor-mediated signaling in the C2 line only supports the early stages of downstream signaling as shown by the phosphorylation of the γ-chain and the failure to effect the phosphorylation of Syk. In contrast RBL-2H3 cells respond to sensitization with IgE and challenge with cognate antigen with tyrosine phosphorylation of the γ-subunits of the receptor complex followed by downstream phosphorylation of Syk and Ca^{2+} mobilization, culminating in β-hexosaminidase release. We propose that the identification of the precise signaling defect in C2 cells will yield useful information regarding the pathway leading to mast cell exocytosis and facilitate the restoration of the complete signaling cascade through complementation of the missing/defective signal transducer since signaling events downstream of Ca^{2+} mobilization are intact as demonstrated by β-hexosaminidase release following non-immunologic stimulation with the calcium ionophore, A23187.

1. Introduction

The difficulty of obtaining sufficient quantities of primary mast cells has limited our ability to study IgE receptor-mediated mast cell activation. This process culminates in the release of preformed and *de novo* synthesized mediators responsible for the initiation of allergic responses which affect a number of mammalian species including dogs, horses and humans. The generation of the rat basophilic leukemia cell line (Eccleston et al., 1973) has greatly increased our general understanding of IgE-mediated signaling pathways, although the commonality of downstream signaling events in mast cells from different species is by no means established. Dogs, in particular purebreds, are prone to allergic diseases and mast cell malignancies (AVMA, 2002; Hunter et al., 2008), and a study of signaling events in FcεRI activated mast cells of canine origin could yield information that may translate into therapeutic intervention strategies to control canine mast cell disorders.

The establishment of two mastocytoma cell lines by Gold and collaborators (Lazarus et al., 1986) which led to the cloning of the C2 cell line (DeVinney and Gold, 1990) showed that this cell line, following repeated passage and propagation under defined culture conditions, could be activated via an IgE-mediated antigenic stimulus to release histamine, proteases and tumour necrosis factor-α. More recently, following extended maintenance in continuous culture, Puigdemont and collaborators (Brazis et al., 2002) re-evaluated IgE receptor-mediated signaling in this cell line. The outcome of their studies showed cell surface expression

Abbreviations: BSA, bovine serum albumin; C2, canine mastocytoma cell line; cFcεRIα, canine IgE high-affinity receptor alpha subunit; DMEM, Dulbecco's modified Eagle's medium; DNP-HSA, dinitrophenol coupled to human serum albumin; FBS, fetal bovine serum; FcR-γ, Fc receptor gamma subunit; HRP, horseradish peroxidase; IMDM, Iscove's modified Dulbecco's medium; IP, immunoprecipitation; NIP-HSA, 4-hydroxy-3-iodo-5-nitrophenylacetyl coupled to human serum albumin; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RBL-2H3, rat basophilic leukemia cell line; Syk, spleen tyrosine kinase; T-PBS, 0.05% Tween-20 in PBS; WB, Western blot.

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of IgE receptors. However, an IgE-mediated antigenic stimulus failed to induce the release of mast cell mediators including β-hexosaminidase and tumour necrosis factor-α. In contrast, bypassing the receptor-mediated events with a non-immunologic stimulus (Ca^{2+} ionophore and phorbol myristate acetate) supported degranulation of these mediators. The authors concluded that the failure to initiate IgE-mediated downstream signaling events is attributable to an impaired function of cell surface IgE receptors (Brazis et al., 2002).

Although it is established that FcεRI crosslinking initiates the signaling cascade culminating in the release of mediators causing the symptoms of allergy, the identification of the pathway leading to exocytosis is far from complete and awaits the identification of further signaling events. In the past identification of defects in signaling pathways (Zhang et al., 1996; Siraganian, 2003) has frequently assisted our understanding of normal events and our present study aimed at a more detailed characterization of the signaling defect in C2 cells. Receptor aggregation following mast cell sensitization is thought to bring the transmembrane and intracellular domains of the α-subunit into close proximity to the γ-subunit. This association leads to the recruitment of the cytoplasmic kinase Lyn which results in phosphorylation of tyrosine residues in the ITAMs of the β- and γ-subunits of the receptor complex (Reischl et al., 1999; Kawakami and Galli, 2002; Rivera, 2002; Siraganian, 2003). The next major event in the signaling cascade proposed links the recruitment of spleen tyrosine kinase, Syk, to the γ-subunits. It has been suggested that Syk becomes tyrosine phosphorylated through interaction with the phosphorylated γ-subunits and this in turn initiates downstream signaling events by activating several other proteins culminating in the release of inflammatory mediators and the phosphorylation of Syk has been shown to be essential for the propagation of downstream signaling events (Zhang et al., 1996).

In the present study we demonstrate cell surface expression of FcεRIα, in C2 cells by ligating canine IgE to its cognate receptor. An assessment of β-hexosaminidase release in response to IgE sensitization and antigenic challenge demonstrated the failure of these cells to support cell exocytosis of cellular mediators. A further investigation into the nature of this defect compared the phosphorylation status of γ-subunits of FcεRI and Syk in the C2 line with corresponding events in RBL-2H3 cells and showed that while C2 cells express a functional FcεRI receptor complex, the signaling defect occurs downstream of FcεRI-γ phosphorylation and upstream of the phosphorylation of Syk.

2. Experimental procedures

2.1. Reagents and immunoglobulins

Cell culture reagents were purchased from Sigma–Aldrich and fetal bovine serum from Autogen Bioclear. Anti-phosphotyrosine, clone 4G10 and anti-FcR-γ-subunit antibodies were purchased from Upstate Biotechnology. Anti-Syk monoclonal and polyclonal antibodies were purchased from Abcam Ltd. and Santa Cruz Biotechnology, respectively. Goat anti-mouse and anti-human IgE were both purchased as FITC conjugates from Bethyl Laboratories. All other reagents and antibodies were obtained from Sigma–Aldrich, unless stated otherwise.

2.2. Flow cytometry

Cell surface expression of FcεRIα receptors was assessed using flow cytometry. 1×10^7 cells/ml were washed in PBS, harvested with cell dissociation solution (Sigma–Aldrich) and re-suspended in wash buffer (PBS containing 1% FBS). Incubation with 1 μg of canine Fc IgE was followed by addition of 1/400 dilution of goat anti-canine IgE (Bethyl Laboratories) and anti-goat FITC-labeled antibody (Sigma–Aldrich, 1/2000 dilution). All of the incubation steps were performed for 30 min on ice. Data were collected using Cyan™ ADP Flow Cytometer using Summit software (Dako).

2.3. Cell culture and cell degranulation

C2 cells were maintained in IMDM supplemented with 10% FBS, 20 ml 100 mM L-glutamine, and 5 ml 100 U/ml penicillin and 100 μg/ml streptomycin solution at 37 °C with 5% CO_2. RBL-2H3 cell lines were maintained in DMEM supplemented with 10% FBS and 5 ml 100 U/ml penicillin and 100 μg/ml streptomycin solution at 37 °C with 5% CO_2. Receptor activation experiments required sensitization of C2 and RBL-2H3 cells with 1 μg/ml of either NIP-specific canine Fc IgE or DNP-specific mouse IgE for 16 h. Cells were harvested with cell dissociation solution and re-suspended at 5×10^6 cells/ml in a buffered solution (120 mM NaCl, 5 mM KCl, 25 mM PIPES, 0.04 mM $MgCl_2$, 1 mM $CaCl_2$, final pH 7.4). IgE sensitized cells were activated with NIP-HSA or DNP-HSA (100 ng/ml) as appropriate for 120 s and the reaction was stopped with ice-cold PBS.

2.4. β-Hexosaminidase release assays

IgE receptor-mediated degranulation of preformed mediators was assessed in the C2 cell line and RBL-2H3 cells assessing β-hexosaminidase release as described previously (Carroll et al., 2001). Data are presented as the mean ± S.D. representative of at least three experiments performed in triplicate.

2.5. Calcium mobilization

C2 or RBL-2H3 cells were harvested in cell dissociation solution and washed in PBS. 1×10^7 cells were re-suspended in the appropriate media and incubated at 37 °C for 1 h with 10 μg/ml of canine Fc IgE for C2 cells or mouse IgE for RBL-2H3 cells. Following incubation, the cells were centrifuged and re-suspended at a density of 5×10^6 ml^{-1} in balanced salt solution (BSS) (148 mM NaCl, 49 mM KCl, 63 mM D-sorbitol, 2.63 mM $K_2HPO_4 \cdot 3H_2O$, 1 mM KH_2PO_4, 10 mM HEPES). 1×10^6 unlabeled cells were removed for the negative control, and the calcium probe Fluo-3 AM was added to the remaining sample at a final concentration of 5 μM. Cells were incubated for 15 min at 37 °C in the dark, after which aliquots of 1×10^6 cells were analyzed on a Beckman Coulter FACS analyzer. A base reading was taken for 30 s and 100 ng/ml DNP-HSA or NIP-HSA was added as appropriate. Ionomycin (10 μM) was used as a positive control to demonstrate that the cells had been adequately loaded with Fluo-3 AM.

2.6. Immunoprecipitation and immunodetection

Cells were solubilized with RIPA lysis buffer (1% NP-40, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris, pH 7.4, supplemented with 1 mM Na_3VO_4 and Complete protease inhibitor cocktail). Post-nuclear supernatants (PNS) were obtained by centrifugation at $10{,}000 \times g$ for 15 min at 4 °C. A Bradford assay (Bio-Rad) was performed on PNS samples to determine protein concentration for normalization between C2 and RBL-2H3 samples. Normalized samples were incubated overnight with 1 μg/mg of the appropriate antibodies at 4 °C. Samples were precipitated with Protein A-agarose (Upstate Biotechnology) for 4 h. Immunoprecipitates were washed in ice-cold RIPA lysis buffer and eluted in SDS loading buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol and 100 mM DTT) and resolved by 12.5% SDS-PAGE followed