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CALCIUM IONOPHORE A23187 AND COMPOUND 48/80 INDUCE PGD2 AND TRYPTASE IN HUMAN CORD BLOOD-DERIVED MAST CELLS: LACK OF EFFECT OF IL-18

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Immunological and biochemical reactions associated with inflammation are elicited in response to a physical or immunological challenge. Early in inflammation there is mobilization and infiltration of neutrophils, mast cells and macrophages to the site of inflammation. These cells release pro-inflammatory compounds icluding cytokines, vasoactive peptides (eg., histamine), and eicosanoids. The release of prostaglandin D2 (PGD2) and tryptase induced by anti-IgE, A23187 and compound 48/80 were studied using *in vitro* a good and valid model of human cord blood-derived mast cells (HCBDMC). Tryptase is a mast cell product and enhances vasopermeability with anticoagulant activities. In this study we measure the release of PGD2 and tryptase on mast cells activate by anti-IgE, calcium ionophore A23187, polybasic compound 48/80 (an agent containing a cationic region adjacent to a hydrophobic moiety, which works by activating G proteins) and IL-18. The generation of PGD2 was measured by radioimmunoassay. Release of PGD2 was detectable (after 12 h) following challenge with anti-IgE, A23187 and compound 48/80. Our data show that mature HCBDMC produce proinflammatory PGD2 following triggering with anti-IgE and with IgE-independent agonists, such as calcium ionophore A23187 and polybasic compound 48/80, while IL-18 was unable to stimulate the release of PGD2 or tryptase on HCBDMC. Although a great deal has been learned about the mediators produced by mast cells, the ultimate biologic function(s) of mast cells remains a mystery.

Eicosanoids are synthesized by oxidative metabolism of arachidonic acid, which is generally

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esterified at the *sn*-2 position of the major classes of glycerophospholipids. The first step of eicosanoid generation involves the liberation of arachidonic acid from the phospholipid pools. The second step, leading to PG generation, involves cyclo-oxygenyses (COXs), which catalase the conversion of arachidonic acid to PGH2. There are at least two COX isoforms, COX-1 and COX-2. The two forms differ widely in their locations, activities and roles. COX-1 is expressed constitutively in almost all the tissue and cells. COX-2 is induced markedly in response to a wide variety of stimuli, such as cytokines. Cytokines play a central role in the pathogenesis of allergic diseases and allergic inflammation. Several pro-inflammatory cytokines, including interleukin-1 beta (IL-1 beta) and tumor necrosis factor-alpha (TNF alpha), are known to stimulate a number of cells to produce inflammatory mediators such as eicosanoids (1-8). IL-1 consistently induces COX-2 gene expression and prostaglandin synthesis in several cell lines (9-13). Several proinflammatory cytokines and cross-linking of bound IgE by antigen, in mast cells is thought to activate protein tyrosine kinases (Syk and Lyn) which in turn cause activation of a mitogen-activated protein (MAP) kinase cascade and phosphatidylinositol-specific phospholipase C (PI-PLC-gamma) (14-17). Calcium and MAP kinases combine to activate the enzyme cytosolic phospholipase A2 (cPLA2), which initiates in these cells the synthesis of lipid mediators such as PGD2, leukotriene C4, D4 and E4 (18-23).

The ability of mast cells to generate or release the vasoactive/spasmogenic mediators histamine, PGD2, sulfidopeptide leukotrienes, platelet-activating factor, cytokines/chemokines and other factors is thought relevant to immediate bronchospastic responses in association with mucus secretion and airway inflammation (24-27). Cytokines of the interleukin-1 (IL-1) family, such as IL-1 alpha/ beta, IL-18 and IL-33, have important functions in host defense, immune regulation, allergy and inflammation (28-32).

IL-33 or IL-1beta also induce IL-8 and IL-13 production in naïve human cord blood derived mast cells (HUCBMCs), and enhance production of these cytokines in IgE/anti-IgE-stimulated HUCBDMCs, without enhancing secretion of either PGD_2 or histamine (33-36). Mast cells could be an important source of PGD_2 and may mediate or regulate vascular inflammation by releasing PGD2 in response to various stimuli, contributing to the innate and/or acquired immune responses (37-40).

IL-18 is a pleiotropic proinflammatory cytokine produced by monocyte/macrophage lineage as well as epithelial cells, such as human keratinocytes. IL-18 leads to increased immunoglobulin E production from B cells and enhanced production of IL-4 and IL-13 by basophils, mast cells, and CD4(+) T cells. Inflammation is thought to be initiated by overelease of IL-18 and accelerated by IL-1 (41-45).

Recent studies also demonstrate a convincing role for IL-18 in atopic responses, including atopic inflammatory diseases (46-50). Moreover, IL-18 directly stimulates basophils and mast cells to produce Th2 cytokines and histamine independently of IgE. IL-18 also induces IL-13 and/or IL-4 production by NK cells, mast cells and basophils (51-54). Human mast cells are known to be a rich source of prostaglandin D_2, however, the regulation of cyclooxygenase (COX) isoform and prostanoid production by proinflammatory IL-18 cytokine has not been clearly elucidated in HCBDMC (55-57). Here, we studied the effects of IL-18, anti-IgE, A23187 and the compound 48/80 (an agent containing a cationic region adjacent to a hydrophobic moiety, which works by activating G proteins), on the generation of prostaglandin D_2 (PGD_2) and tryptase by HCBDMC.

MATERIALS AND METHODS

Cells and materials

Reagents for cell culture were purchased from Sigma-Aldrich. HCBDMC were cultured in Iscove's Modified Dulbecco's medium supplemented with 10% bovine calf serum, 1.2 mM monothioglycerol, 2 mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin either in 25 cm^2 tissue culture plates or in six-well tissue culture plates (Costar). Cells were plated at a density of $0.2x10^6$ per ml taken from the 3-day-old culture grown under the same conditions. Cells were grown in an incubator in a 5% CO_2 and 95% O_2 at 37°C.

Human cord blood mast cell isolation, culture and treatment

Umbilical cord blood was collected in tubes containing

10U/ml heparin and diluted 1:2 with Dulbecco's phosphate buffered saline (DPBS) from GIBCO BRL (Life Technologies, Grand Island, NY, USA) containing 2 mM ethylenediamine-tetra acetic acid (Sigma). Non-phagocytic mononuclear cells were separated by density-gradient centrifugation using Lymphocyte Separation Medium (LSM) from Organon Teknika Corp (Durham, NC, USA). The isolation of hematopoietic stem cells (CD34+) was performed by positive selection of CD34+/AC133+ cells by magnetic associated cell sorting (MACS) using an AC133+ cell isolation kit (Milltenyi Biotec, Auburn, CA, USA). CD133 expression is restricted to a subset of CD34 bright positive stem cells in human cord blood. Mast cells had been obtained by culturing cord blood mononuclear cells in the presence of SCF, IL-6. CD34+ cells were suspended in Iscove's Modified Dulbecco's Medium (IMDM; GIBCO BRL), supplemented with 100ng/ml rhSCF, 50ng/ml IL-6, 10% fetal bovine serum (FBS; Bio whittaker, Walkesville, MD), $5x10^{-5}$ M2-Mercaptoethanol, and 1% penicillin-streptomycin (GIBCO BRL) for 12 to 16 weeks. During this culture period, the cells were washed with DPBS every week and resuspended using fresh complete culture medium. Mast cell viability was determined by trypan blue (0.3%) exclusion and observed under light and electron microscope. For sensitization, HCBMCs were washed with DPBS and plain culture medium (without any growth factors) once in each and resuspended in serum-free complete culture medium, but without IL-6 supplementation. Cells (1 x 10^5 or 1 x10^6 cells/ml) were then incubated with human myeloma-IgE (2µg/ml/10^6 cells) at 37°C for 48 h in 24-well Falcon cell culture plates (Becton Dickinson, Franklin Lakes, NJ, USA). These sensitized HCBMCs were used in all the experiments and treated for 12 h with Il-18, A23187 0r 48/80.

Activation of HCBMC with Anti-IgE

Anti-Human IgE (DAKO Rabbit anti-Human IgE, Specific for Epsilon-chains. Code number A 0094; Lot no. 069. Edition 05.07.00) was used in this study at different concentrations: 1, 5 and 10 mg/ml. For anti-IgE stimulation experiments, HCBDMC (10^5 or 10^6 cells/ml) were washed with Iscove's modified Dulbecco's medium and Tyrode's buffer, once in each, and passively sensitized by incubation with human myeloma IgE (2mg/ml/10^6 cells; Chemicon Inc.) for 48 h in culture medium at 37°C. Cells were then washed two times and resuspended in fresh culture medium. Cells were stimulated with anti-IgE (Dako) at 15mg/ml in 96-well round bottom culture plates (1 x 10^6 cells/ml in 200 ml medium/sample) for 12 h at 37°C in 5% CO_2.

Immunoassays for tryptase

Tryptase was measured in the supernatants and cell pellets by UniCAP Tryptase Fluoroenzyme-immunoassay System (Pharmacia Diagnostics AB, Uppsala, Sweden) as reported previously (Schwartz LB, 1994). Briefly, anti-tryptase covalently coupled to ImmunoCAP® reacts with the tryptase in the specimen. After washing, enzyme-labeled antibodies against tryptase are added to form a complex. After incubation, unbound enzyme-anti-tryptase is washed away and the bound complex is then incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate is measured in the FluoroCount™ 96 microplate reader. The fluorescence is directly proportional to the concentration of tryptase in the sample. To evaluate the test results, the response for the samples is compared directly to the response of the standards. Tryptase was measured in the supernatants and cell pellets by fluoroenzyme-immunoassay (Pharmacia, Uppsala, Sweden). These peptides were measured in the supernatant and pellet to calculate percent release.

Tryptase release assay

For tryptase measurements, HCBDMC were sensitized with human myeloma IgE for 48 h in culture medium and were then stimulated for different periods of time in Tyrode's buffer (133 mM NaCl, 4 mM KCl, 0.64 mM KH_2PO_4, 10 mM HEPES, 1g/L glucose, 1 mM $CaCL_2$, 0.6 mM $MgCl_2$ and 0.03% human serum albumin, pH 7.2) Tryptase was measured in the supernatant by fluoroenzyme-immunoassay (Amersham-Pharmacia Biotech). Results were expressed in ng/10^6 cells/ml.

Determination of cell viability

HCBDMC were harvested, washed in PBS and centrifuged for 5 min at 400xg at room temperature. Cells were then resuspended in culture medium, 0.1% trypan blue solution was added for 5 min at room temperature and the cells were counted using a haemocytometer. Viability was 99%, expressed as percentage of cells which do not take up trypan blue.

Stimulation of HCBDMC

Calcium ionophore A23187, compound 48/80, or anti-IgE at different dilutions were made directly in HCBDMC

culture medium. In separate tubes, in each experiment, cells were exposed to the vehicle alone, to determine non-specific release. HDCBMC ($1x10^5$ or $1x10^6$) in a six-well tissue culture dish were washed with culture medium containing 1mg/ml bovine serum albumin (BSA) and without Ca++ to reduce spontaneous secretion. They were then sensitized in the same medium for 30 min at 37°C with 2 ml of mouse monoclonal anti-DNP IgE (500ng/ml). After sensitization, the cells were washed again and treated for 30 min at 37°C with 2 ml of DNP-BSA (1 ng/ml) in the same medium, but now supplemented with 0.5 mM calcium to permit secretion. Control samples without IgE were run simultaneously in the presence of 0.5 mM Ca^{++} and these values represented non-specific release. The cells were also activated with anti-IgE and with IgE-independent agonists such calcium ionophore A23187, polybasic compound 48/80 and IL-18.

ELISA for PGD_2

All the experiments were performed with HCBMC obtained in about 8 weeks. Briefly, the cells were cultured for 12 h with and without reagents to be tested (anti-IgE, 48/80, A23187, and IL-18). After incubation, the cells were centrifuged at 4,000 r.p.m. for 8 min. The supernatants were removed and stored at -20°C. The levels of the PGD_2 were determined by a PGD_2-specific ELISA kit composed of 96 determinations and the assays were performed exactly as recommended by the manufacturer. The ELISA reader was set at 405 nm absorbance and all the samples were read after 1 h after addition of stop solution. The wells were protected from light and incubated at room temperature. The antiserum anti-PGD_2 (rabbit) was reconstituted in phosphate buffer containing BSA and sodium azide, as recommended by the manufacturer. PGD_2 alkaline phosphatase conjugate was supplied ready to use in bicarbonate buffer containing 0.1% BSA, magnesium chloride and sodium azide at pH 9.8 and stored at 4°C. PGD2 was determined in cell-free supernatants with commercial ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions (sensitivity of the assay, 3 pg/ml).

Statistical analysis

All assays were performed in triplicate. The results were expressed as mean ± SD. Data from three different experiments were combined and reported as the mean ± SD. Student's *t* test for independent means was used to provide statistical analyses ($p>0.05$ was considered as not significant). Comparisons were made using ANOVA.

RESULTS

Tryptase release from HCBDMC

HCBDMC are cells which have tryptase

Table I. *Tryptase (ng/ml) release from HCBDMC (10^5 or 10^6 cells/ml) following the addition or not of IL-18 (50 ng/ml) and the corresponding samples treated with calcium ionophore (A23187) (0.5 μg/ml), C48/80 (10^{-5} M) or anti-IgE (10 μg/ml).*

	TRYPTASE RELEASE (pg/ml)			
	10^5 CELLS	p values	10^6 CELLS	p values
Spontaneous	13.6 ± 6.0	-	23.0 ± 9.0	-
Anti-IgE (10 μg/ml)	129.0 ± 11.5	-	210.0 ± 32.0	0.005
IL-18 (50 ng/ml)	15.5 ± 8.0	0.05	11.0 ± 5.0	N.S.
A23187 (0.5 μg/ml)	186.7 ± 12.1	0.05	240.0 ± 21.0	0.005
C 48/80 (10^{-5} M)	156.4 ± 24.8		170.5 ± 30.1	0.005

The cells were cultured for 30 min. at 37°C, 5% CO_2. P values (Student's t test) are calculated by comparing untreated HCBDMC with anti-IgE, calcium ionophore (A23187), C48/80 or IL-18-treated cells. The values ± S.D. are representative of three experiments in triplicate.

Table II. *PGD_2 production from HCBDMC (10^5 or 10^6 cells/ml) following the addition or not of IL-18 (50 ng/ml) and the corresponding samples treated with the calcium ionophore A23187 (0.5 μg/ml), anti-IgE or C48/80.*

TREATMENT FOR 12 H	PGD2 GENERATION (pg/ml) 10^6 CELLS	p values
Spontaneous (PBS)	37.4 ± 15.3	-
Anti-IgE (10 μg/ml)	1650.4 ± 170.4	0.001
A23187 (0.5 μg/ml)	1970.5 ± 111.2	0.001
C 48/80 (10^{-5} M)	1822.6 ± 150.7	0.001
IL-18 (50 ng/ml)	70.6 ± 100.4	0.01
Indomethacin (10^{-6} M) plus (Anti-IgE (10 μg/ml)	53.5 ± 17.2	N.S.
Indomethacin (10^{-6} M) plus A23187 (0.5 μg/ml)	70.3 ± 28.3	N.S.
Indomethacin (10^{-6} M) plus C 48/80 (10^{-5} M)	62.8 ± 25.5	N.S.
Indomethacin(10^{-6} M) plus IL-18 (50 ng/ml)	27.2 ± 15.4	N.S.

The cells were cultured for 12 h at 37°C, 5% CO_2. P values (Student's t test) are calculated by comparing untreated HCBDMC with -treated cells. The values ± S.D. are representative of three experiments in triplicate.

containing granules and receptors for IgE. In this report HCBDMC were cultured with IL-18 (50ng/ml), a classic specific secretagogue anti-IgE, or a non-specific compound calcium ionophore A23187 (0.5μg/ml) or compound 48/80 (10^{-5} M). Table I shows in three representative experiments the release of tryptase following HCBDMC treatment and incubated for 30 min with C48/80, anti-IgE, A23187 and with or without IL-18. Anti-IgE, calcium ionophore A23187 (0.5μg/ml) or compound 48/80 (10^{-5} M) strongly stimulated tryptase on HCBDMC; while IL-18 was unable.

Generation of PGD2 from HCBDMC after treatment with IL-18, anti-IgE, A23187 or C48/80

Stimulated HCBDMC were treated overnight (12 h) with C48/80, anti-IgE, A23187 or IL-18, with or without indomethacin 10^{-6} M (a classic

cyclooxygenase-inhibitor) (Table II).

The production of prostanoid PGD_2 was examined after 12 h mast cell activation. Prostaglandin D_2 (PGD_2) was strongly produced after stimulation of HCBDMC with calcium ionophore A 23187, compound 48/80 or anti-IgE compared to the control; while IL-18 was not affected. HCBDMC pretreated with indomethacin, completely suppressed PGD_2 synthesis.

DISCUSSION

Considerable attention has recently been devoted to the study of cytokines, and in particular IL-1 family mechanisms having the ability to induce biological activities on mast cells. One of the hypotheses is that IL-18 activates several cells through its receptor, including monocytes, lymphocytes basophils and mast cells (41, 58-62). Since IL-1 stimulates PGE2 in macrophages, we hypothesize that IL-18 stimulates arachidonic acid in human mast cells and then may affect the production of prostaglandins such as PGD_2.

This study demonstrates that human cultured cord blood mast cells release PGD_2 in response to calcium ionophore A23187, compound 48/80 or anti-rat IgE stimulation, but not to IL-18 and surely the response is mostly mediated by the induction of COX-2 rather than COX-1 isoenzyme, implying that mast cells may be an important source of prostaglandin D_2 in human tissue where mast cells are present and that COX-2 may play an important role in the regulation of the inflammatory diseases. In these studies the stimulatory effect of IL-18 on HCBDMC *in vitro* were not seen. HCBDMC pretreatment with the conventional non-steroidal anti-inflammatory drug (NSAID), indomethacin, completely blocked PGD2 release, induced by calcium ionophore A23187, compound 48/80 or anti-IgE. The effects obtained with calcium ionophore A23187, compound 48/80 or anti-IgE stimulation is associated with many pathophysiologic phenomena including thrombocytopenia, intravascular coagulation, endotoxic shock and acute inflammation (63-66). Our study demonstrates that treatment of HCBDMC by calcium ionophore A23187, compound 48/80 or anti-rat IgE strongly produces prostaglandin D_2 *in vitro* after 12 h incubation; while IL-18 was unable to. These effects may explain that the inflammatory action of IL-18, where mast cells are present, is not due to the activation of arachidonic acid cascade. Inflammatory cells, such as mast cells, play important roles not only in inflammation but also in innate and adaptive immunity (67-70). The migration of leukocyte inflammatory cells is the central event in the inflammatory response and it is presumably based on the existence of locally produced chemotactic factors such as members of the CXC and CC chemokine groups induced by certain cytokines (49). Moreover, the response of leukocytes of tissue injury and infection can be potentially harmful and contribute to the pathogenesis of many diseases and inflammatory disorders (71-74).

In the present study we used an experimental and valid model, using HCBDMC culture for the production of PGD_2 and tryptase after IL-18 treatment. It has been recently reported that peripheral inflammation results in enhanced PGD_2 biosynthesis by mast cells in the central nervous system (CNS) (75-79), therefore at first sight it may seem important that immunomodulators such as cytokines and chemokines not only have a physiological effect on cells, but also, at high concentrations, they have a pathological impact. The continual release of pro-inflammatory mediators by CNC following inflammatory proteins may contribute, in part, to the destruction of surrounding normal tissue, therefore attenuating the CNS cytokine-chemokine actions having therapeutic benefits. However, IL-18 even if it is a potent inflammatory cytokine, in our study it lacked stimulation of PGD_2 and trypsin release. It seems very likely that IL-18 and its antagonist(s) will be the topic of future biomedical research including immunotherapy of rheumatoid arthritis and other inflammatory diseases, even if many of its functions still remain to be elucidated.

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