ORIGINAL RESEARCH

Neuropeptide Substance P induces mRNA expression and secretion of CXCL8 chemokine, and HDC in human umbilical cord blood mast cells

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Abstract

Purpose: Mast cells play an important role in innate and acquired immunity and are thought to be the cellular origin of most proteases and cytokines. Substance P (SP) and its receptor, NK-1R, play critical roles in immune regulation in human and animal models of inflammation.

Methods: We used mature human cord blood mast cells (HCBMC) differentiated from cord blood CD34⁺ precursor activated with SP in culture.

Results: Our data indicate that Substance P strongly activates mature HCBMC in releasing CXCL8 expression and secretion (Control: 1.200 ± 1.0 ; SP: 4.10 ± 0.90 ; $P < 0.01$). Moreover, in a RT-PCR, HCBMC expressed CXCL8 mRNA after Substance P activation. Since calcium ionophore A23187 is a pharmacological activator

that raises cytosolic free calcium ion concentration and stimulates mast cells in the production and secretion of proinflammatory compounds, it was used as positive control. In addition, we found that HCBMCs generate the transcription of histidine decarboxylase (HDC), the enzyme responsible for the generation of histamine from histidine, after SP treatment. Since CXCL8 is a member of the CXC chemokine subfamily with potent chemotactic activity and is a primary inflammatory cytokine we conclude that our results, obtained from HCBMC cultures, a good and valid model in vitro, support the concept that the neurogenic system modulates inflammatory events by Substance P-mediated HCBMC chemokine CXCL8 release.

Conclusion: The expression, synthesis and release of CXCL8 suggest an increase of inflammatory process in vivo mediated by the recruitment and infiltration of inflammatory cells in inflamed tissues.

When nerve fibers come in contact with Substance P, they react by swelling—an effect that yields headaches and sinus symptoms. Sensory neuropeptides may activate brain mast cells to release inflammatory mediators such as histamine, serotonin, arachidonic acid compound and *de novo* synthesized cytokines/chemokines.¹⁻⁵

Human mast cells (MCs) develop from committed precursors in the bone marrow expressing the differentiation marker CD34+ and distinct from the three other myeloid cells. MCs have long been known to participate in the inflammatory process and there is evidence that they orchestrate inflammatory reactions.⁶⁻⁸ They are normally present in the brain, peritoneum, synovium, hair follicles, skin, gastrointestinal tract, airways, and many other organs, where they are in close contact with the outside environment. MCs are prominent in inflammatory diseases and have been implicated in the pathophysiology of the brain.⁹⁻¹² Recent evidence indicates that, in addition to direct effect of these mast cell products, some mast cell mediators themselves modulate inflammatory mediator production.¹³⁻¹⁵ MCs are thought to be the cell origin of most protease and cytokine production. These products are generated in response to anti-IgE, and several other compounds, and are governed by a number of transcriptional and translational processes.¹⁶⁻¹⁷ Moreover, MCs are involved in inflammation by secreting histamine, proteases and different cytokines, including inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor- α (TNF- α), and also express numerous chemokine receptors. MC disorders are defined by an abnormal accumulation of tissue mast cells in one or more organ systems.¹⁸⁻²²

Activation of cytokine receptors and alterations in cytokines are thought to play important roles in neuronal dysfunction and in the pathogenesis of nervous system diseases.²³⁻²⁷ The chemokine CXCL8 is a neutrophil-specific CXC subfamily with potent chemotactic and inflammatory properties activated and regulated by NF- κ B. CXCL8 binds to G protein-

coupled receptors CXCR1 and CXCR2 and is produced by many cells such as macrophages, T lymphocytes and astrocytes, in response to proinflammatory stimuli including IL-1, TNF α and LPS.²⁸ Since human mast cells are difficult to collect in large numbers we used human umbilical cord-blood mast cells for their abundance. It is pertinent therefore, to study the effect of the neuropeptide Substance P in the secretion of CXCL8, and HDC in human umbilical cord blood mast cells cultured *in vitro*.

Materials and methods

Materials

The drugs used in these studies were obtained from the following source: Substance P acetate salt hydrate, product # S6883, 98% purity, Lot No.016k51041, was obtained from Sigma Saint Louis, Missouri 63103, USA. Human CXCL8 (IL-8) Quantikine immunoassay (R&D Systems Inc., Minneapolis, MN, USA, cat. No.D8000C) activity in the culture supernatant was determined.

Reagents for cell cultures were purchased from Sigma-Aldrich. HCBMCs were cultured in Iscove's Modified Dulbecco's medium supplemented with 10% bovine calf serum, 1.2 mM monothioglycerol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin either in 25 cm² tissue culture plates or in six-well tissue culture plates (Costar). Cells were plated at a density of 0.2x10⁶ per ml taken from the 3-day-old culture grown under the same conditions. Cells were grown in an incubator in a 5% CO₂ and air environment at 37°C.

Cultured HCBMC

In these studies, to investigate whether Substance P was indeed capable of inducing formation of new protein CXCL8 we used mature human mast cells developed from committed precursors in the bone marrow expressing the differentiation marker CD34+ and distinct from the three other myeloid cells. HCBMC

treated with Substance P at different concentrations (0.1, 1.0, 10, 100 and 200 μ M) and the pharmacological agonist calcium ionophore A23187 (5 μ M) is a good and valid model for the study of cytokine/chemokine release.

Informed patient consent to obtain cells was given and the study was approved by the University of Chieti Ethics Committee. Human umbilical cord blood was collected in tubes containing 10 U/ml heparin and diluted 1:2 with Dulbecco's phosphate buffered saline (DPBS) from GIBCO BRL (Life Technologies, Grand Island, NY) containing 2 mM ethylenediamine-tetraacetic acid (Sigma). Non-phagocytic mononuclear cells were separated by density-gradient centrifugation using Lymphocyte Separation Medium (LSM) from Organon Teknika Corp (Durham, NC). 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 (Miltenyi Biotec, Auburn, CA). 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 100 ng/ml rhSCF, 50 ng/ml IL-6, 10% fetal bovine serum (FBS; Bio Whittaker, Walkersville, MD), 5×10^{-5} M 2-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. Cells were stained with Toluidine blue (0.3%) to show metachromatic staining of mast cells and observed under light microscope (Fig.1D). HCBMCs were washed with DPBS and plain culture medium once in each and resuspended in serum-free complete culture medium. Cells were cultured at different periods of time (2h; 6h; 12h, 24h).

Stimulation with Substance P

Substance P (Sigma) at different dilutions was made directly in HCBMC culture medium. In separate tubes, in each experiment, cells were exposed to the vehicle alone, to determine non-specific release. HCBMC (1×10^6) in a six well tissue culture dish were washed with culture medium containing 1 mg/ml bovine serum albumin (BSA)

SP acetate salt hydrate was diluted in medium (RPMI 1640) and used at different concentrations. Calcium Ionophore (A23187) (Sigma) was dissolved in dimethyl sulfoxide (DMSO) and diluted to various concentrations in medium. Control cells were exposed to the medium alone at the identical concentrations of vehicle to determine baseline levels of CXCL8 expressed in pg/ml.

RT-PCR for CXCL8

HCBMCs (5×10^5 cells/sample) were incubated with SP (10 μ M) for 2 days in mast cell culture medium. After washing twice, HCBMCs were incubated with 1 μ g/mL CRA-1 for 2 hours before harvesting. Total cellular RNA was prepared with RNase mini Kits (Qiagen) according to the manufacturer's instructions. Cellular RNA was reverse-transcribed to complementary cDNA by using first-strand cDNA synthesis kits (Pharmacia Biotech). The RNA extract was denatured at 65°C for 10 minutes and mixed with RT solution containing a reverse transcriptase, deoxyribonucleoside triphosphate (dNTP). The mixture was incubated at 37°C for 60 minutes and then at 65°C for 5 minutes to inactivate reverse transcriptase.

Levels of cDNA were quantified by means of a competitive PCR method. PCR mimics were constructed by using the PCR MIMIC construction kit (Clontech). Cytokine mimics were prepared for CXCL8 by using PCR for 16 or 21 cycles. To construct the PCR mimics, two rounds of PCR amplification were performed. In the first PCR reaction two composite primers were used. Each composite primer had the target cytokine gene primer sequence attached

to a short nucleoside stretch of sequence attached to a short nucleotide stretch of sequence designed to hybridize to opposite strands of a fragment of v-erbB gene DNA. The first PCR was cycled at 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 90 seconds (*sense* 5'-ATGACTTCCAAGCTGGCCGTGGC T- 3'*antisense* 5'-ATGACTTCCAAGCTGGCCGTG GCT-3').

CXCL8 release assay

We used the Quantikine CXCL8 solid phase ELISA designed to measure CXCL8 in HCBMC supernatants. The assay was performed in accordance with the manufacturer. Secreted CXCL8 cytokine levels in culture supernatants of HCBMC (10⁵ cells/200 µl) were measure by a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Absorption of the avadin-horseradish peroxidase color reaction was measured at 405 nm and compared with serial dilutions of human recombinant as a standard. The minimum detectable dose of CXCL8 ranged from 1.5 – 7.5 pg/ml.

Preparation of the HDC probe

We used a probe made from a reverse transcribed polyA1 RNA and total cellular RNA was extracted. PolyA1 mRNA was purified by one-step chromatography on an oligo-dT column. A sample of 2 mg polyA1 mRNA was reverse transcribed at 42°C for 40 min in a 20 ml mixture containing 4 ml of 53RT buffer (250 mM Tris-HCl, pH 8.3, at 42 °C, 50 mM MgCl₂, 250 mM KCl, 15 mM dithiothreitol, 10 U placental RNase inhibitor, 0.5 mM each dNTP, 50 pmol oligo-dT primer and 20 U avian myeloblastosis virus RT). After RT the HDC cDNA was amplified by PCR using two specific primers synthesized on a gene assembler plus (Pharmacia LKB, Bromma, Sweden): 59 primer, 59-ATGATGGAGCCCCAGTGAATACC and 39 primer, 59-CCAGAATTCGCATGTCTGAGG TAG. Then a 4 ml single-stranded cDNA mixture was

supplemented with 50 pmol of each 59 (sense) and 39 (antisense) primers in a volume of 50 ml denatured for 2 min in a boiling bath and added to a 50 ml mix pre-warmed at 72 °C containing 0.25 mM each dNTP, 10 ml 103Taq polymerase buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, at 25 °C, 15 mM MgCl₂ and 0.1% gelatin) and 1.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). The PCR program consisted of one cycle of 1 min at 94 °C and 15 min at 72°C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 4 min at 72 °C, and was completed by an additional annealing at 55 °C for 30 s and a final elongation at 72 °C for 15 min. PCR was performed in a Techne PHC-2 programmable heating block. Amplified products were purified by glass-milk procedure (GeneClean Kit; BIO 101, Vista, CA), blunt-ended with the Klenow fragment and cut by *EcoRI*. The resulting blunt-end *EcoRI* fragments were cloned in the p-MAL vector (Biolabs) cut by both *StuI* and *EcoRI* restriction enzymes. The resulting recombinant plasmid was amplified in the TB1 *Escherichia coli* strain. Plasmid DNA was sequenced by the double-stranded protocol of the Sequenase kit (USB, Cleveland, OH). Plasmid containing amplified HDC cDNA was prepared according to the alkaline lysis method and purified on a CL4B column.

Statistical analyses

Data from different experiments were combined and reported as the mean ± SD. Student's *t*-test for independent means was used to provide a statistical analysis (*P* < 0.05 was considered significant).

Results

Microscopic studies

To analyze the purity of human cord blood mast cells, microscopic studies were performed. In Fig.1, we show (A) a transmission electron micrograph of a 2 weeks old immature cord blood mast cells, magnified 15,600 x; B) a transmission electron micrograph

FIGURE 1. (A) a transmission electron micrograph of a 2 weeks old immature cord blood mast cells, magnified 15,600 x; B) a transmission electron micrograph of a 9 weeks old immature cord blood mast cells magnified 15,600 x; C) a transmission electron micrograph of a mature cord blood mast cells, 15-weeks old, magnified 15,600 x. Samples were examined using a JEOL JEN-100s transmission electron microscope; D) Light microscopy cord blood mast cells in culture, stained with Toluidine blue 40 x; (Nikon light microscope, Japan).

of a 9 week old immature cord blood mast cells magnified 15,600 x; C) a transmission electron micrograph of a mature cord blood mast cells, 15-weeks old, magnified 15,600 x. In this last picture the mast cell has well-developed granules in which are present chemical mediators, such as proteases involved in the inflammatory response. Samples were examined using a JEOL JEN-100s transmission electron microscope; in panel D we depict a light micrograph cord blood mast cells in culture 40 x; (Nikon light microscope, Japan).

Production of CXCL8 by HCBMC activated with Substance P

Here we evaluated the generation of CXCL8 from isolated HCBM using ELISA method. In this study we show the amount of CXCL8 $10^6/\text{ml}$ cells released after treatment with Substance P ($10 \mu\text{M}$) and calcium ionophore A23187 ($5 \mu\text{M}$) (positive control). The results are derived from cells cultured (overnight incubation) of three different experiment in triplicate (Table 1).

TABLE 1. Stimulation of CXCL8 (ng/ml) in HCBMCs by substance P (SP) for 12 h, and inhibition of dexametasone 10^{-5} M

Treatment	CXCL8 (ng/ml)	<i>P</i> <
Control	1.200 ± 1.0	*
A23187 (5 µM)	5.30 ± 1.10	0.001
SP (10µM)	4.10 ± 0.90	0.01
DEX (10^{-5} M)+ A23187 (5 µM)	2.300 ± 1.1	N.S.
DEX (10^{-5} M)+ SP (10µM)	2.40 ± 1.00	N.S.

Dose-response curve for Substance P on CXCL8

In order to study the full influence of Substance P on CXCL8 production we used Substance P at different concentrations (0.1, 1.0, 10, 100, 200 µM). Calcium ionophore A23187 (5µM) was used as a positive control. In this study (Fig.2) we show a dose-response of Substance P on CXCL8 released by mature human mast cell cord blood. Substance P at 0.1 µM did not

give any appreciable increase in CXCL8 compared to the control. The maximum stimulation was seen at 100 and 200 µM but, since 10 µM produced not statistically different results than 100 and 200 µM, here in all experiments we used it at 10 µM.

RT-PCR for IL-8 mRNA

Semi-quantitative RT-PCR for IL-8 mRNA expression was performed and studied. In Fig.3 we show CXCL8 mRNA after treatment of human cord blood mast cell with Substance P. In lane 2 it is evident that CXCL8 mRNA is increased compared with the control (lane 1). Lane 3 is the positive control A23187. In order to determine whether SP (10µM) can activate CXCL8 mRNA expression the mRNA was isolated from HCBMC and reverse-transcribed into cDNA. The cDNA was amplified by PCR with primer specific for

FIGURE 2. Dose-response graph of Substance P on CXCL8 released by mature human cord blood mast cells after 12 h incubations. This is a representative experiment of 4.

FIGURE 3. Semi-quantitative RT-PCR for CXCL8 mRNA. CXCL8 mRNA generation after treatment of human mast cell cord blood with Substance P. Lane 1-Control; lane 2- Substance P; lane 3- A23187. The top line represents the treatments, the bottom line GAPDH. This is a representative experiment of 4.

CXCL8. After incubation in SP (10 μ M) for 6 h, CXCL8 expression was strongly increased compared with that in the absence of SP (PBS, control).

We determined HDC activation and mRNA levels, important mast cell functional marker responsible for the production of histamine from histidine (Fig. 4). A probe was prepared to detect mRNA encoding the HDC gene oligonucleotide primers specific for HDC sequences were used to amplify a product from rat brain cDNA and this yielded a 1.019-bp fragment of DNA following digestion with EcoRI restriction enzyme. After cloning into the p-Mal plasmid, this was used to detect HDC mRNA by RT-PCR. Since SP has been presented as an activator for mast cells, in this study we analyzed the generation of HDC mRNA was strongly induced on HCBMC by SP, compared with control (A23187 was used as a positive control).

Discussion

In this study we show that Substance P, a neuropeptide involved in neurogenic inflammation, stimulates HCBMC to generate CXCL8. We also examined the possible stimulation of Substance P on HCBMC in

FIGURE 4. RT-PCR HDC mRNA. 1) Control; 2) Substance P (10 μ M); 3) A23184 5 (μ M) Effects of SP on HDC mRNA in HUCBMC: the cells were treated with SP and A23184 (5 μ M) (positive control) and not treated (control, PBS) for 6 h. The difference between control and A23187 treatment is evident. The top line represents the treatments, the bottom line GAPDH. This is a representative experiment of 4.

generating HDC mRNA. Results in this study demonstrate that Substance P is a potent stimulus in HCBMC in *in vitro* assay. The enhanced response of Substance P treated HCBMC followed time- and dose-dependent curves for CXCL8. The effect of substance P was significant at 12 and 24 h, compared to the control groups; while at 2 and 6 h the effect was negligible.

HDC mRNA was indeed stimulated by Substance P. Therefore, we can speculate that the effect of SP may influence levels of histamine synthesis *in vitro* and *in vivo*. The activation of histamine acts by binding to target cell receptors and initiates intracellular events that causes different changes in different cell types; for example the binding of histamine to the endothelium causes cell contraction leading to leakage of plasma into tissues and stimulates synthesis of cytokines and arachidonic acid products. As a positive control we used calcium ionophore A23187, a pharmacological activator which raises cytosolic free calcium ion concentration and stimulates mast cells in the production and secretion of proinflammatory compounds such as, cytokines/chemokines, proteases and arachidonic acid products. This pharmacologic agent mimics the effect of antigen and induces many biological and functional responses in the absence of antigen.

TABLE 2. Time-course experiment

HCBMCs treatment	Incubation time (h)	CXCL8 (ng/ml)	<i>P</i> <
Control	2	N.D.	-
A23187 (5 μ M)	2	N.D.	-
SP (10 μ M)	2	N.D.	-
DEX (10 ⁻⁵ M)+ SP (10 μ M)	2	N.D.	-
Control	6	1.30 $\pm$ 1.25	*
A23187 (5 μ M)	6	3.4 $\pm$ 1.2	N.S.
SP (10 μ M)	6	1.6 $\pm$ 1.0	N.S.
DEX (10 ⁻⁵ M)+ SP (10 μ M)	6	2.2 $\pm$ 1.1	N.S.
Control	12	1.40 $\pm$ 1.19	*
A23187 (5 μ M)	12	6.55 $\pm$ 1.00	0.001
SP (10 μ M)	12	4.10 $\pm$ 1.20	0.005
DEX (10 ⁻⁵ M)+ SP (10 μ M)	12	1.9 $\pm$ 1.20	N.S.
Control	24	1.24 $\pm$ 1.0	*
A23187 (5 μ M)	24	6.40 $\pm$ 1.5	0.001
SP (10 μ M)	24	4.00 $\pm$ 1.1	0.05
DEX (10 ⁻⁵ M)+ SP (10 μ M)	24	2.7 $\pm$ 1.0	N.S.

There is evidence that mast cells orchestrate the inflammatory reactions and play a pivotal role in the hypersensitivity reaction by releasing cytokines and chemical mediators such as proteases. Considerable attention has recently been devoted to the study of cytokines/chemokines mechanisms having the ability to induce biological activities. One of the hypotheses is that chemokines activate through its receptor several cells, including monocytes, lymphocytes, polymorphonuclear leukocytes and mast cells.²⁹⁻³³ One of the most important aspects of Substance P action is the induction and modulation of the secretion of cytokines by immune cells.³⁴⁻³⁷ Here, we hypothesize that Substance P stimulates CXCL8 chemokine transcription and translation protein in HCBMC. It has been reported that mast cells express a number of immunological important cytokines and chemokines.³⁹⁻⁴² Many immune cells have been reported that express specific receptors for neuropeptides and respond to neuropeptide stimulation by releasing cytokines.⁴³⁻⁴⁵

These studies demonstrate that the steady state levels of HCBMC CXCL8 mRNA and secreted CXCL8 are increased by Substance P in a dose-

dependent manner. The concentration of Substance P (10 μ M) used to produce an effect on CXCL8 expression may mimic the effect in *in vivo* microenvironments, although this concentration is far too high from the physiological concentrations. However, it is difficult to directly extrapolate the *in vitro* Substance P concentrations used in these studies to *in vivo* physiologic conditions. These data suggest that chemokine production in mast cells may be differentially influenced by Substance P. It has previously been shown that mast cells *in vitro* release significant amounts of preformed C-C subfamily chemokines such as RANTES and MCP-1 after stimulation with biological proinflammatory stimuli and anti-IgE.^{38, 46-50} Our experiments cannot distinguish whether the induction of CXCL8 by Substance P in HCBMC is due to new synthesis of CXCL8, or release of preformed molecules. CXCL8, like many chemokines, displays a broad range of biological activities, such as chemoattraction, direct inflammation, that influences hematopoiesis, angiogenesis, sepsis, and adaptive and innate immunity. Since CXCL8 is found to be highly expressed in polymorphonuclear leukocytes, these results suggest that CXCL8 may play an important role in neurogenic inflammatory diseases characterized by elevation of neutrophils and mast cell infiltration. In this article we show that HCBMC can synthesize significant quantities of CXCL8, after Substance P activation, which may be important in anaphylactic reactions.

In conclusion, these results indicate that Substance P may be critically involved in the regulation of the CXCL8 chemokine production *in vitro* and probably *in vivo*, providing a potential therapeutic target for inflammatory disorders. Understanding the cellular and molecular mechanisms of cross-talk among neurogenic inflammatory mediators and immune cells, such as mast cells, is central for the prevention or treatment of inflammatory neurological diseases.

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