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Effects of the neuropeptide secretoneurin on natural killer cell migration and cytokine release

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Abstract

Secretoneurin has a widespread occurrence in airway mucosal innervation of patients with allergic diseases and may play an important role in the local traffic of immune cells in human airway mucosa. Whether secretoneurin affects natural killer cell migration and cytokine release in vitro was tested. Natural killer cells were obtained from venous blood of healthy donors. Cell migration was studied by micropore filter assays. Signalling mechanisms required for secretoneurin-dependent migration were tested using signalling enzyme blockers. Cytokine release was measured in natural killer cell supernatants by ELISA. Secretoneurin significantly stimulated natural killer cell chemotaxis via activation of phosphatidylinositol 3′ -kinase and protein kinase C. IL-2 stimulated natural killer cells showed a stronger response toward secretoneurin than unstimulated cells. Moreover, secretoneurin increased the release of interleukin-5 in a dose-dependent manner but did not affect Th1 cytokine release by natural killer cells. Data suggest that secretoneurin stimulates directed migration of natural killer cells and may modulate Th1/Th2-response via affecting chemokine release. Thus, secretoneurin may play an important role in the early stages of allergic inflammation.

Keywords: Natural killer cells; Secretoneurin; Neuropeptide; Chemotaxis; Cytokine release

1. Introduction

Sensory nerves mediate the inflammatory reaction to irritating substances in several organs and might co-ordinate the healing process. Neuropeptides, such as secteoneurin have been identified as potent mediators of inflammatory and immunologic processes in several organs such as the lung [1]. In accordance, neurogenic inflammation is suggested to play a crucial role in several diseases, e.g. bronchial hyper-responsiveness and bronchial asthma [2,3]. Secretoneurin is a neuropeptide derived from secretogranin II, an abundant protein in neuroendocrine storage vesicles and a member of the chromogranin/secretogranin family [4,5]. It acts as an inflammatory peptide released from primary afferent C-fibers of the peripheral nervous system and has been shown to induce directed migration of various cells [6,7]. Moreover, secretoneurin exhibits potent angiogenic properties, reveals antiapoptotic effects and modulates proliferation of endothelial cells [8,9].

Natural killer cells are granular lymphocytes that constitute approximately 5–15% of the total lymphocyte population and can be found in high numbers in human lung interstitium [10]. Their presence at high numbers in the lung parenchyma has suggested their involvement in processes like pulmonary immunity [11]. Recent data indicate an important role of natural killer cells in the development of allergic airway inflammation [12]. Furthermore, an increased number of these cells was demonstrated to be present in asthma [10]. These observations suggest

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that this might reflect a predisposition of several individuals with high natural killer cell activity to develop exaggerated T-cell responses to antigens and hence to be at risk for developing sustained asthma [13]. It has been shown that these immuno-modulatory cells may produce a great variety of cytokines which are involved in the inflammatory response. Natural killer cells are often associated with secretion of cytokines that contribute to the creation of microenvironments that promote the differentiation of naive T cells into a Th1-like phenotype [14]. However, natural killer cells can be triggered to secrete IL-4, IL-5 and IL-13, thereby promoting Th2 responses, too [15].

The aim of the present study was to investigate the effect of the neuropeptide secretoneurin on natural killer cell functions in vitro. We demonstrate a chemotactic effect of secretoneurin on natural killer cells in a concentration-dependent manner involving phosphatidylinositol 3-kinase and protein kinase C. Secretoneurin was also able to affect natural killer cell cytokine release in vitro.

2. Materials and methods

2.1. Peptides and reagents

RPMI 1640 with phenol red was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Bovine serum albumin (BSA) was from Dade Behring (Marburg, Germany). Secretoneurin, staurosporine, isobutylmethylxanthine (IBMX), wortmannin, rolipram, tyrphostin-23, EDTA and Interleukin-2 were obtained from Sigma (St. Louis, MO). RANTES was from PeProTech (Rocky Hill, NJ). Bisindolylmaleimide I GF 109203X (GFX) was from Boehringer Ingelheim (Ingelheim am Rhein, Germany). RANTES was from PeProTech. Lymphoprep® was purchased by Nycomed Pharma (Oslo, Norway). Dulbeccos' phosphate buffered saline (PBS), Mycoplex™ foetal calf serum (FCS) were from PAA Laboratories (Linz, Austria). Hanks' balanced salt solution (HBSS) without phenol red was obtained from Invitrogen (Carlsbad, CA). MACS separation columns and microbeads were from Miltenyi Biotech (Auburn, CA). The microchemotaxis chambers were from Neuroprobe (Bethesda, MD) and cellulose nitrate filters obtained by Sartorius (Goettingen, Germany). Interleukin-4, interleukin-5, interleukin-8, interleukin-10 and interferon-γ-ELISAs were from Biosource (Camarillo, CA). Anti-secretoneurin antibodies were a gift from Reiner Fischer-Colbrie (Department of Pharmacology and Toxicology, Faculty of Pharmacology, Innsbruck Medical University). All stock solutions were stored at −20 °C before use.

2.2. Isolation of human peripheral blood natural killer cells

Human natural killer cells were isolated from freshly prepared mononuclear cell fractions (MNC) from peripheral blood of healthy volunteers without history of asthma, atopic rhinitis or atopic dermatitis. After Lymphoprep® density gradient centrifugation, MNC were collected and washed three times with normal saline solution. After washing, 10^7 cells were re-suspended in 50 μl of ice cold MACS buffer (0.1 M phosphate-buffered saline [PBS] containing 2 mM EDTA and 0.5% human albumin). For depletion of $CD3^+$ T-lymphocytes, MACS colloidal super-paramagnetic microbeads conjugated with monoclonal anti-human CD3-mAb were added and the mixture was then incubated at 6 °C for 30 min. After washing with MACS buffer, the cell suspension was loaded onto MACS LD separation columns. The depletion procedure was repeated to increase the purity of $CD3^-$ cells. After this, natural killer cells ($CD3^-/CD56^+$) were isolated from the remaining $CD3^-$ cells using CD56 microbeads, as previously described [16]. Separation was again repeated at this step to increase the purity of the selected cell population. Purity of sorted cells was always >98%, as determined by fluorescence-activated cell sorting (FACS) analysis.

2.3. Natural killer cell chemotaxis assay

Migration assays were performed using a modified 48-well Boyden microchemotaxis chamber in which a 8-μm-pore-size cellulose nitrate filter separated the upper and the lower chamber. Purified natural killer cells were re-suspended in RPMI 1640/ 0.5% BSA (1×10^6 cells/ml). Fifty microliters of the cell suspension were placed into the upper chamber and allowed to migrate either towards various concentrations of secretoneurin (1 μM to 10 fM) or RANTES (10 ng/ml) placed in the lower chamber for 120 min at 37 °C in a humidified atmosphere (5% CO_2). After the migration period, nitrocellulose filters were dehydrated, fixed, and stained with haematoxylin. Migration depth of the cells into the filters was quantified microscopically, measuring the distance (μm) from the surface of the filter to the leading front of cells. Data are expressed as chemotaxis index (CI), which is the ratio between the distance of directed and undirected migration of cells into the nitrocellulose filters.

In further experiments, secretoneurin (10 nM) was pre-treated with different concentrations of a specific secretoneurin antibody for 20 min before testing effects on natural killer cell migration as described previously. Intracellular signalling of secretoneurin on natural killer cells was tested by pre-incubation of the cells with the intracellular enzyme blockers staurosporine (10 ng/ml), bisindolylmaleimide I GF 109203X (GFX) [500 nmol/l], wortmannin [10 nmol/l] (from penicillium fumiculosum), rolipram [10 μmol/l], isobutylmethylxanthine (IBMX) [10 ng/ml] or tyrphostin 23 [10 ng/ml]. The cells were then washed twice, resuspended in RPMI/ 0.5% BSA, and tested for migration towards secretoneurin.

2.4. Natural killer cell culture

Freshly isolated human natural killer cells (5×10^5 cells/ml) were transferred in a 24-well-plate (Falcon®, Franklin