Monocytes of allergics and non-allergics produce, store and release the neurotrophins NGF, BDNF and NT-3

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Abstract

Introduction: Recent studies have shown that neurotrophins (NTs) are involved in inflammatory processes. Elevated plasma levels of NTs were found allergic diseases with the highest levels in allergic asthma. However, the exact cellular sources involved in the regulation and release of neurotrophins in allergic inflammation are still not well defined.

Objective: The aim of this study was to assess whether monocytes of allergic and non-allergic subjects produce, store and release the neurotrophins NGF, BDNF and NT-3.

Methods: Monocytes of allergic and non-allergic donors were purified by immunomagnetic selection. APAAP-staining for the presence of NTs and their receptors was performed. RT-PCR and Western blot evaluated the production and storage of NTs. Monocytes were incubated and supernatants were collected for measurement of neurotrophic factors after stimulation with lipopolysaccharide (LPS) as inflammatory stimulus. The neurotrophin content in lysates and cell culture supernatants was determined by ELISA.

Results: Human monocytes express the neurotrophins NGF, BDNF and NT-3 but also their specific receptors TrkA, TrkB and TrkC. RT-PCR amplification of isolated mRNA demonstrated expression of the examined neurotrophins. Proteins were detectable by Western blot. NTs were found in the monocyte lysates and supernatants at different levels in allergic and non-allergic donors. Cell stimulation with LPS leads to release of NGF and NT3.

Conclusions: Monocytes, produce, store and release NGF, BDNF and NT-3. They are a possible source of elevated neurotrophin levels found in allergy and asthma.

Keywords: NGF; BDNF; NT-3; Monocytes; Asthma; Allergy; Inflammation

1. Introduction

The neurotrophins (NTs) Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF) and Neurotrophin 3 (NT-3) belong to a family of related polypeptides sharing a high homology and a remarkable range of biological activities modulated through activation of their high affinity specific receptors trkA, trkB, trkC and their low affinity unspecific receptor p75 [1]. NTs play an important and well described role in the development, function and survival of neuronal cells [2].

Recently, several studies have described the effects of NTs in inflammatory and autoimmune diseases [3,4]. In relation to allergic diseases, NTs plasma levels were found to be increased in vernale keratoconjunctivitis [5], allergic rhinitis and at highest levels in allergic asthma [6]. In a previous study, we were able to prove elevated NGF levels

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in allergic asthmatics and also found elevated levels of BDNF and NT-3 in untreated allergic asthmatics. After treatment with inhaled corticosteroids, a decrease in circulating neurotrophin levels was observed [7]. The mechanism of increased NT levels in inflammatory conditions is poorly understood. After segmental allergen provocation, an increase of NTs in the bronchoalveolar lavage (BAL) fluid has been described in the late allergic response [8]. In animal models also the immediate response was influenced while treatment with NGF antibodies reduced airway inflammation [9,10]. In different animal models NGF can influence the asthmatic phenotype by augmenting bronchial hyperresponsiveness (BHR) and allergic inflammation [11]. This effect might be modulated by inducing up-regulation of neuropeptide production and release in sensory neurons [12].

Neurotrophins not only affect nerves but can also activate immune cells. Neurotrophin receptors have been found on various immunological active cells involved in allergic sensitisation and inflammation, e.g., lymphocytes and mast cells [13,14]. Recently also the production of neurotrophins by various immune cells has been proved [15,16]. Documented neurotrophin effects on immune cells include proliferation and activation [17–20] and enhancement of neutrophils and macrophages survival [21–23]. Besides these cells, NGF is produced by residential cells such as epithelial cells [24]. Recently, we were able to demonstrate the production of NGF, BDNF and NT-3 by human peripheral eosinophils [25]. This cell type plays a major role in allergic inflammatory processes of the airways and is found accumulated in the bronchial tissue of asthmatics. While the viability of peripheral eosinophils is not influenced by neurotrophins, an influence of the survival after migration into the lung was lately observed. NGF stimulation leads to an increased production of IL-4 [26,27].

Monocytes are known as main effector cells of the immune system and play a crucial role in host defence mechanisms. They internalize, process and present antigens, tumour cells and parasites, and secrete various cytokines and chemokines.

NGF induces monocytes to generate reactive oxygen metabolites, promotes macrophages chemotaxis and stimulates macrophage phagocytosis and cytokine production [22,23,28]. A low production of NGF in human monocytes/macrophages has been described, increasing after stimulation [29]. Kerschensteiner et al. [30] demonstrated the expression of BDNF only on stimulated human monocytes. NT-3 was not detected on human alveolar macrophages by immunocytochemistry [31]. NT-3 mRNA expression has been shown on mice macrophages after activation by lipopolysaccharide (LPS) [32]. The aim of the presented study was to evaluate if human monocytes are a possible source for the production, storage, and release upon an inflammatory stimulus of the neurotrophins NGF, BDNF and NT-3, particularly in allergic and non-allergic subjects.

2. Materials and methods

2.1. Subjects

Nineteen voluntary blood donors (18 to 56 years), including 10 patients with allergic asthma and nine healthy controls, were examined. Detailed history, skin prick test to common inhalative allergens, with at least one reaction equal to the histamine control, and specific IgE were made to differentiate between atopics and non-atopics. All subjects gave informed consent.

2.2. Isolation of monocytes

Monocytes were isolated from human peripheral blood as previously described [33]. Heparinized venous blood (130 ml) was collected and allowed to sediment in 6% dextran (Sigma, USA) at room temperature for 40 min. The leukocyte rich fraction was aspirated gently, washed and centrifuged on Ficoll-Paque (density 1.077) (Biochrome, Berlin, Germany) for 45 min at 900 × g. The washed pellet of PBMC was added to micromagnetic beads (Monocyte Isolation Kit; Miltenyi Biotec, Germany) for negative selection. The tagged cells were then passed through a magnetic field (MACS, Miltenyi) and the negative selection was collected. Monocytes were concentrated at high purity of >95% assessed by flow cytometry using anti-CD14 IgG and FITC conjugated antibodies (Jackson Immuno Research, USA). Isolated monocytes demonstrated a viability of more than 95% as evaluated with propidium iodide and trypan blue staining (Sigma).

2.3. Culture and stimulation of isolated monocytes

To assess NTs storage and release after stimulation, isolated cells were cultured in RPMI 1640 medium, containing 10% FCS and 5% penicillin/streptomycin. Approximately 10^5 cells/well were supplemented with lipopolysaccharide (LPS), (Sigma) of 10, 1 or 0.1 μg/ml, and cultured at 37 °C in humidified atmosphere, 5% CO₂. After 24 and 48 h, the stimulated cells were detached, centrifuged and cell culture supernatant collected.

2.4. Immunocytochemistry

The alkaline phosphatase anti-alkaline phosphatase procedure (APAAP) (Dako, Denmark) was used as previously described. Cytospins of isolated CD14+ cells were air dried, fixed for 20 min in methanol and stained by specific antibodies for NGF, BDNF and NT-3 and their receptors trkA, trkB and trkC (dilution 1:100). For visualisation, a hexazotized fuchsine was used and counterstaining was performed with Mayer’s haematoxylin. For negative controls, the primary antibody was omitted.
2.5. RNA isolation and reverse transcription PCR amplification

To demonstrate expression of RNA encoding for NTs in monocytes, total RNA was extracted from 5×10⁶ cells by using the commercial RNeasy kit (Qiagen, USA). The first-strand cDNA synthesis reaction was catalysed by AMV Reverse Transcriptase and oligo (dT) primer according to the manufacturer’s instructions (Boehringer, USA). The generated complementary DNA was amplified in a total volume of 25 µl with a 1.25 U of Taq DNA polymerase, 0.2 mmol/l dNTP mixture and 0.5 µmol/l of neurotrophin primers (Table 1). Neurotrophin mRNA has been shown to be present in HMC-1 cells [34]. cDNA of these cells was used as a positive control. Amplification steps in the thermocycler (MWG Biotech, Germany) for NTs consisted of 94 °C for 5 min and 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Amplified products were electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed under UV light. The specificity of the used primers was demonstrated previously [24].

2.6. Protein electrophoresis and Western immunoblotting

To confirm the storage of neurotrophins, 10⁶ human peripheral M/M was homogenized on ice with 1.5% SDS, 62.5 mM Tris–HCl buffer (pH 6.8) supplemented with 1% nonidet P-40 (NP-40), 5% mercaptoethanol and 2 mM PMSF. Fifty microliters of proteinase inhibitors (Sigma) was added. Lysates were centrifuged at 15 000 rpm for 15 min and supernatants were collected as total lysate. Protein content was measured at 280 nm by using a modified Lowry protein assay (BCA, Pierce, USA). Aliquots were stored at −80 °C until used. Samples of protein 50 µg/lane in parallel were loaded on a 10–20% polyacrylamide gel and electrophoresed with a molecular weight marker (Bio Rad, USA). Recombinant NGF, BDNF and NT-3 were used as standard. Proteins were blotted on supported nitrocellulose membrane (Schleicher and Schuell, USA). Western immunoblotting was performed using 1:500 rabbit polyclonal anti-human NGF, BDNF or NT-3 antibody (Santa Cruz Biotechnology, USA) and 1:10000 horse-

Table 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Upstream (5′–3′)</th>
<th>Downstream (5′–3′)</th>
<th>Size (bp)</th>
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<td>GGC ACT CCG TT</td>
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<td>TGC TCT TTC</td>
<td>CCC CTG CAG CCT T</td>
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</tr>
<tr>
<td>NT-3</td>
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<td>GGC ATC C</td>
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Fig. 1. APAAP-staining of CD14+ cells for NGF, BDNF, NT-3 and high affinity receptors TrkA, TrkB and TrkC (1:100). Negative control with omitting of primary Ab. Magnification ×500.

radish peroxidase-linked donkey anti-rabbit secondary antibody (Amersham Pharmacia Bioscience, USA). The blot was analysed using a chemiluminescence system (ECL, Amersham Life Sciences, Sweden). For absorption control experiments, the membrane was stripped of bound antibody, and the first antibody was incubated with the

Fig. 2. RT-PCR amplificates of the neurotrophins NGF, BDNF and NT-3. Lane 1: 100-bp ladder; 1–3: samples of allergies; 4 and 5: non-allergics; +: positive control (cDNA from HMC-1 cells); −: negative control.
2.7. Determination of neurotrophin content in cells and supernatants

Both primary and stimulated cells and their supernatants were investigated for their content of neurotrophins NGF, BDNF and NT-3. Therefore, cells were lysated by sonification. NTs were detected in sandwich ELISAs according the manufacturer’s instructions for NGF, BDNF and NT-3 (R&D Systems, USA). All assays were performed on F-bottom 96-well plates (Nunc, Wiesbaden, Germany). Tertiary antibodies were conjugated to horseradish peroxidase. Wells were developed blocking peptide overnight before reprobing as previously described.

Fig. 3. Western blot analysis of NGF, BDNF and NT-3. Lanes 1, 6 and 7 represent samples of allergics. Lanes 2–5 are cell lysates of non-allergics. Lane 8: positive control (recombinant protein).

Fig. 4. Neurotrophin content of monocytes lysates (left part of graph) and cell culture supernatants (right part of graph) after 48 h determined by ELISA.

Fig. 3. Western blot analysis of NGF, BDNF and NT-3. Lanes 1, 6 and 7 represent samples of allergics. Lanes 2–5 are cell lysates of non-allergics. Lane 8: positive control (recombinant protein).
with tetramethylbenzidine and measured at 450 nm using a microplate ELISA reader (Molecular Devices, USA). Neurotrophin content was quantified against a standard curve calibrated with known amounts of protein. The detection limits were <4 pg/ml for NGF and BDNF and <8 pg/ml for NT-3. Measurements were performed in duplicate and are expressed as means. Cross-reactivity to related neurotrophic factors was less than 3% for all three analysed NTs.

2.8. Statistical analysis

Results are presented as mean values±S.E.M. Values were compared by using the nonparametric test for independent (Whitney–Mann U-test) or dependent (Wilcoxon signed rank test) values with SPSS Version 10 (SPSS, USA). P values <0.05 were regarded as significant.

3. Results

3.1. Immunohistchemistry

The content of NT and their corresponding receptors in cells was demonstrated by APAAP. Positive immunoreactivity was found for NGF, BDNF and NT-3. Also staining for neurotrophin receptors TrkA, TrkB and TrkC was positive for CD14+ cells, while negative controls did not demonstrate immunoreactivity (Fig. 1).

3.2. RNA isolation and reverse transcription PCR amplification

Each cDNA sample from isolated monocytes demonstrated gluceraldehyde 3-phosphate dehydrogenase (GAPDH) amplification products of the expected size and comparable intensity. There was no evidence of a genomic amplification product. During PCR amplification, control reactions were negative for amplification products, demonstrating that the PCR method and reagents used yielded specific amplified products only when a cDNA source was included. Specific bands of RNA encoding for NGF, BDNF as well as NT-3 were detectable in all samples after reverse transcription (Fig. 2).

3.3. Protein electrophoresis and Western immunoblotting

To confirm the expression of the neurotrophins NGF, BDNF and NT-3 on the protein level, lysates and recombinant proteins as controls were immunoblotted using specific antibodies. As shown in Fig. 3, specific bands were detectable for the neurotrophins NGF, BDNF and NT-3. The specificity was confirmed by the fact that the staining was absorbed by preincubation of antibodies with an excess of blocking peptide.

3.4. Determination of neurotrophin content in monocytes and culture supernatant

The neurotrophins NGF, BDNF and NT-3 were detectable in cell lysates and cell culture supernatants in different amounts between different individuals. To avoid differences in cell counts in the lysates, neurotrophin content was divided by protein content. Comparing allergic versus healthy persons, cells of allergic asthmatics released a significant higher amount of NGF after 48 h (allergic asthmatics: 25.16±9.08 pg/ml, non-allergic: 4.68±1.79 pg/ml; p<0.05) and showed a significant higher amount of cellular neurotrophin (allergic asthmatics: 161.38±43.74 pg/μg protein, non-allergic: 91.75±23.10 pg/μg protein; p<0.05). BDNF and NT-3 demonstrated no significant difference (Fig. 4).

Cell stimulation with LPS for 24 h led to a dose-dependent release of the neurotrophins NGF and NT-3 (Fig. 4).

![Fig. 4. Bar graph showing the release of NGF, BDNF, and NT-3 in response to LPS stimulation.](image-url)
5), while BDNF release did not increase after LPS stimulation. The maximal NGF release was found on a concentration of 0.1 μg/ml LPS. Higher concentrations of LPS were not able to accumulate further NGF.

4. Discussion

In this study, we demonstrated that human monocytes are able to produce, store and release the neurotrophins NGF, BDNF and NT-3. The expression of the high affinity neurotrophin receptors trkA, trkB and trkC was confirmed by immunocytochemistry on human peripheral monocytes. We have been able to demonstrate the production of NTs on the transcriptional as well as on the translational level. NGF and NT-3 are released in response to an inflammatory stimulus such as LPS, which is a strong stimulus for monocytes due to the presence of LPS receptors. BDNF was released in constant amount, regardless of the presence of an added trigger. This contributes to a different mechanism of release or regulation of neurotrophic mediators.

The expression of trkA was described on human monocytes/macrophages [21], with an increase after LPS stimulation [27] and a down-regulation during in vitro differentiation to macrophages, suggesting that NGF effects are more important for monocytes than for mature macrophages [20,21]. There is evidence suggesting a self-limiting autocrine circuit between NGF and trkA. TrkB was identified on monocytes/macrophages and trkC was recently described on human peripheral blood mononuclear cells (PBMC) and T-cells [35] and on mouse peritoneal macrophages, up-regulated by LPS [36].

Increased neurotrophin serum levels were seen in allergic diseases, with the highest circulating levels in allergic asthma [6]. Elevated levels in BAL fluid after segmental allergen provocation have also been reported [8]. In the presently investigated patient group, the content of NGF in isolated monocytes of allergic asthmatic was significantly higher than in the non-allergic group. Allergics released higher quotes into the supernatants for all NTs. Consequently, monocytes may be a source of local and circulating NTs. The predominance of NGF as an inflammatory mediator and the apparent lack of BDNF suggest an imbalance, leading to local inflammation and airway hyperreactivity (AHR). Anti-NGF treatment prevented the development of increased AHR in mice [10], and an effective treatment with inhalative corticosteroids decreased circulating NTs in allergic asthmatic patients [7]. Furthermore, NGF is a potent inductor of neuropeptide expression, e.g., substance P (SP) [37], and vice versa, since SP also increases NGF production [38]. Substance P is known to be elevated in BAL of asthmatics [39], probably triggering inflammation in the airways, leading to vasodilatation, and increasing vascular permeability and smooth muscle contraction [40]. Therefore, a bidirectional interchange seems to exist. Additionally, NGF and NT-3, but not BDNF, promote receptor-mediated macrophage chemotaxis [22]. Triggering trkA with NGF protects human M/M from apoptosis [20,27].

Enhanced cell migration, prolonged cell survival and release of mediators on a paracrine pathway influence the activation as well as the differentiation of immune cells in inflamed tissue. NTs may act as autocrine or paracrine factors in the inflammatory response. Here, we have shown that human monocytes produce, store and release NTs and may be a possible source of elevated neurotrophin levels found in allergy and asthma. Future studies on the role of monocyte-derived NTs in allergic disease need to be conducted to identify the precise role of these cells as NT source.

References


