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Cell type-specific regulation of brain-derived neurotrophic factor in states of allergic inflammation

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Summary

Background Brain-derived neurotrophic factor (BDNF) is a molecule influencing neuronal proliferation and differentiation. In states of allergy, it may orchestrate inflammatory changes by linking the immune system with the nervous system. Because the precise regulation of gene transcription in mast cells MCs is not clear, the present studies assessed the gene regulation of BDNF in this inflammatory cell type.

Methods Transcriptional expression of BDNF in human skin was studied in isolated cells using RT-PCR. *In situ* lesional MC BDNF protein expression was analysed by immunohistochemistry and related to the differential staining of MCs and functional effects of BDNF on HaCaT keratinocytes.

Results BDNF mRNA expression was found in isolated human skin MCs, keratinocytes, and fibroblasts. Also, low levels were found in endothelial cells and melanocytes. BDNF protein expression was found *in situ* in lesional and non-lesional MCs. A significantly decreased expression of BDNF protein was found in atopic dermatitis lesional MCs when compared with control MC expression. Functional *in vitro* experiments demonstrated that a decrease in BDNF stimulation led to increased secretion rates for stem cell factor and IL-8 in HaCaT keratinocytes.

Conclusion The demonstration of a decreased level of BDNF gene transcription in lesional MCs points to a differential regulation of MC-released neurotrophins in cutaneous allergic inflammation. Topically administered neurotrophin receptor-modulating compounds should be receptor target specific and not universally acting in diseases such as atopic dermatitis or allergic asthma.

Keywords allergy, atopic dermatitis, atopy, cytokines, neurotrophins

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Introduction

Atopic dermatitis is a common inflammatory skin disease that usually starts in early infancy. The disease is characterized by pruritus and eczema. It is also often associated with allergic airway diseases such as bronchial asthma or rhinitis [1]. Like other atopic diseases, its inheritance has recently been shown to be associated with several genes determining, in particular, immunological aspects of the disease [2]. Chronic lesions are character-

ized by an infiltration of mast cell (MCs), eosinophils, lymphocytes, and macrophages [1].

Elevated levels of total IgE are found in about 80% of patients with atopic dermatitis, and MCs are hypothesized to play a major role in IgE-mediated immediate hypersensitivity reactions. After allergen challenge, IgE-sensitized MCs liberate a large variety of mediator including histamine, prostaglandins, or leukotrienes [3]. Next to interactions with other inflammatory cells such as eosinophils, a major mechanism of MC action has been proposed for bi-directional MC–nerve interactions [4]. Yet, the scale and significance of these mechanisms have not been

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investigated in detail for cutaneous inflammatory diseases such as atopic dermatitis.

Probably the best-known example of elementary MC–nerve interactions is represented by the *in vitro* effects of the neurotrophin (NT) nerve growth factor (NGF), which lead to MC proliferation, differentiation, and histamine [5–8]. Neurotrophins are a family of structurally and functionally related polypeptides with up to 50% of amino acid homology. Their biological effects include an induction of neuronal proliferation and differentiation, modulation of the synaptic function, and regulation of apoptotic mechanisms [9]. Next to the most prominent member of the NTs family, NGF, the neurotrophic brain-derived neurotrophic factor (BDNF) is an important mediator of many organ functions [10].

As it was recently shown that the interaction between MC and nerves in allergic diseases is mediated by neuropeptides including substance P [4], calcitonin gene-related peptide (CGRP) [11, 12], or vasoactive intestinal peptide (VIP) [13–15] and there is increasing evidence that NTs such as BDNF can influence numerous cellular functions of the skin and the lungs in health and disease [16, 17], the present study was carried out to assess the expression and gene regulation of BDNF and functional effects as part of the cutaneous cytokine network in normal and atopic dermatitis skin.

Materials and methods

Tissues

Biopsies from 45 patients with atopic dermatitis (>16 years, mean age 38.5 years, 24 females, 21 males) and 23 normal controls (>16 years, mean age 42.8 years, 13 females, 10 males) were examined. Atopic dermatitis diagnosis was based on the criteria of Hanifin [18], and routinely performed histopathological examination revealed characteristic inflamed eczematous lesions. The SCORAD of the atopic dermatitis patients was >25 (moderate or severe).

Cutaneous keratinocytes, endothelial cells, fibroblasts, melanocytes, and MC were obtained from human foreskin or breast skin of non-atopic patients undergoing cosmetic surgery and isolated as described previously [19]. The skin MCs were enriched (95 ± 4% purity) using immunobeads (Dynal, Hamburg, Germany) coated with a c-Kit antibody YB5.B8 and magnetic cell sorting [20]. The human HaCaT keratinocyte cell line [21] was kindly provided by N. Fusenig (Heidelberg, Germany). All studies were performed according to the declaration of Helsinki, after patients had given their informed consent.

RNA-isolation

Isolation of RNA was performed as described previously [22]. In brief, 1×10^6 cells were lysed and total RNA was

Table 1. Primers used for PCR

Primer name	Primer sequence	PCR product (bp)
BDNF		
Forward	5' GAC CCT GCC GCG CGA GGG GAG	316
Reverse	5' AGT GTC TAT CCT TAT GAA TCG CC	
GAPDH		
Forward	5' GAT GAC ATC AAG AAG GTG GTG	197
Reverse	5' GCT GTA GCC AAA TTC GTT GTC	

BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

prepared using the RNeasy total RNA kit (Qiagen, Hilden, Germany). After digestion of genomic DNA by treatment with DNase, cDNA was synthesized by reverse transcription of 5 µg total RNA, using a cDNA synthesis kit (InVitrogen, Stade, Germany).

Reverse-transcription polymerase chain reaction

RT-PCR was carried out as described previously [23]. In brief, amplification with the BDNF-specific primer pairs (Table 1) was performed using taq polymerase (Gibco, Karlsruhe, Germany) with an automated thermal cycler (Perkin Elmer, Boston, MA). Each cycle consisted of the following steps: denaturation at 94 °C, annealing at 58 °C, and extension at 72 °C for 1 min each. PCR products were analysed by agarose gel electrophoresis and enzymatic digestion, using standard techniques.

Immunohistochemical staining

Immunohistochemistry was performed as described previously using paraffin-embedded sections [24]. The following antibodies were used: anti-MC tryptase (AA1) (donated by Dr A. Walls, Southampton, UK), anti-MC chymase (CC1) (Dako, Hamburg, Germany), and BDNF (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Toluidine blue staining was performed at pH 0.5 for 24 h.

To quantify MC numbers and assess MC BDNF staining intensity, previously described and validated protocols were used [25–27]. In brief, sections were evaluated by two independent investigators as described previously and counting nucleated-stained cells was performed using a raster covering 1/16 mm² at 1 : 400 magnification in at least five microscopic fields. Counts were expressed as stained cells per mm², as described previously [19, 25]. Quantification of MC staining intensity for NTs and their receptors was performed using an intensity ranging from 0 to 3.5, as described previously and validated [22, 26, 27]. Measurement of intensity was performed for at least four slides of each patient and control subject in a blinded fashion.

Cell culture and stimulation

Human HaCaT keratinocyte cells were kept routinely in Dulbeccos Eagle's medium (Gibco), supplemented with 5% fetal bovine serum (Biochrom, Berlin, Germany), 4 mM glutamine, and 100 U penicillin and streptomycin per mL (both from Gibco) [21]. Cells were seeded at 2×10^6 cells/cm² in culture plates, and the medium was routinely changed every 3 or 4 days. As described, in some experiments, the medium was removed after 3 day of culture and, following another 24-h culture with different concentrations of BDNF, the supernatants were collected to compare the secreted stem cell factor (SCF) and IL-8 quantities.

Cytokine measurements. Samples were analysed for SCF and IL-8 contents with a commercially available enzyme-linked immunoabsorbent assay Kit (Quantikine, R&D systems, Bad Nauheim, Germany). Values of duplicate measurements fluctuated within a very narrow margin (< 5%). The results were adjusted to viable cell counts and expressed as means $\pm$ SD of four different experiments.

Statistics

The results of the different parameters and groups are expressed as mean $\pm$ SEM unless stated differently. Statistical significance was examined using the unpaired two-tailed Student's *t*-test.

Results

Expression of brain-derived neurotrophic factor mRNA in different cutaneous cell types

To assess the cellular expression of BDNF mRNA in human skin, different cell populations were freshly isolated and subjected to RT-PCR. Using BDNF-specific primer pairs, repeated ($n = 4$) RT-PCR experiments were performed and BDNF-specific amplification products with a length of 316 bp were detected in different cell types. BDNF mRNA signals were present in MCs, keratinocytes, and fibroblasts. Only weak signals were found in endothelial cells and melanocytes (Fig. 1). The PCR product identities were confirmed by direct sequencing, which revealed identity with the published sequences.

Mast cell-specific expression of brain-derived neurotrophic factor in normal and lesional atopic dermatitis skin

To assess the protein expression of BDNF in human skin *in situ*, BDNF immunohistochemistry was performed, leading to the identification of BDNF in MCs of both normal and atopic dermatitis MCs.

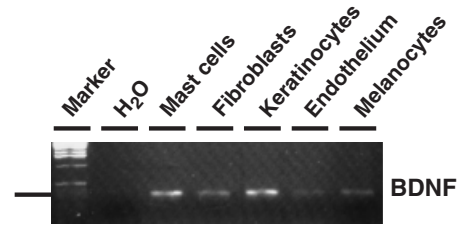


Fig. 1. Brain-derived neurotrophic factor (BDNF) mRNA expression in isolated cutaneous cells. Expression of mRNA specific for BDNF (RT-PCR) in isolated skin keratinocytes, fibroblasts, endothelial cells, melanocytes, and mast cells (representative results from four different experiments).

The transcriptional regulation of BDNF in lesional MCs of atopic dermatitis patients was assessed using quantitative immunohistochemistry, and significant changes in protein expression were found between normal and atopic dermatitis MCs. A signal intensity of 2.5 ± 0.2 in control MCs was present whereas an intensity of 1.0 ± 0.1 was found in lesional MCs ($P < 0.0001$) (Fig. 2).

Incubation of HaCaT cells with decreasing concentrations of brain-derived neurotrophic factor

To assess the functional effects of a decreased MC BDNF secretion, *in vitro* studies were performed that assessed the secretory inflammatory activity of HaCaT keratinocytes, and significant differences were found. The secretion of SCF in HaCaT cells after stimulation with different levels of BDNF changed in a dose-dependent manner and decreases in BDNF stimulation from 10 to 1, 0.1, and 0 ng/mL led to an increase in SCF secretion that was significant ($P < 0.01$) (Fig. 3). Also, the secretion of IL-8 in this keratinocyte cell culture changed after incubation with different levels of BDNF: parallel to the increase in SCF secretion, decreases in BDNF stimulation from 10 to 1, 0.1 and 0 ng/mL led to an increase in IL-8 secretion that was significant ($P < 0.05$) (Fig. 3).

Discussion

In the present study, we have provided evidence that in atopic dermatitis, BDNF is down-regulated in lesional MCs, suggesting that for one, there is a decreased production of BDNF in the pathophysiological important cellular compartment of MCs that are increased in numbers in atopic dermatitis lesions.

Until now, numerous inflammatory diseases have been shown to be associated with increased serum concentrations of BDNF, including allergic diseases [28]. In contrast to bronchial asthma, for which detailed knowledge on BDNF-related pathomechanisms has been gained recently [29–31], only little is known about the precise function of BDNF in atopic dermatitis. The decreased MC expression of BDNF in patients with atopic dermatitis, as shown here,

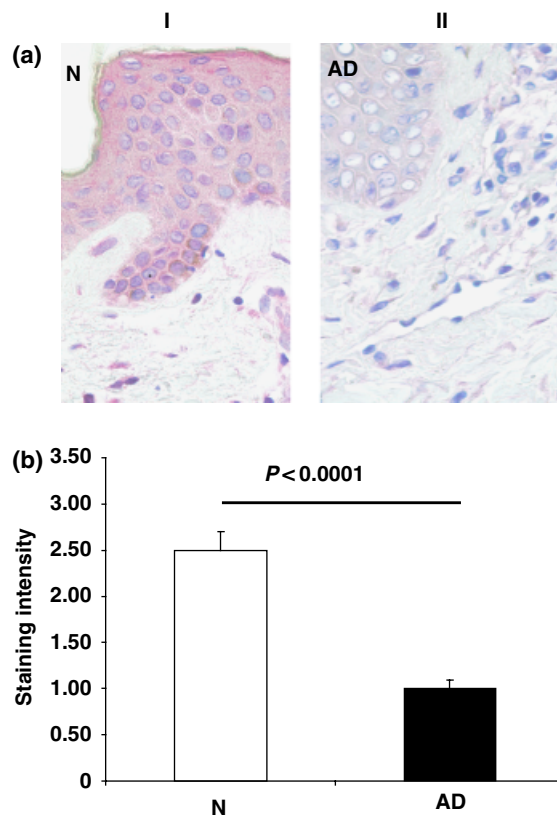


Fig. 2. Brain-derived neurotrophic factor (BDNF) expression in atopic dermatitis lesional mast cells. (a) Immunohistochemical staining (APAAP) of normal skin sections (I) and from patients with atopic dermatitis (II) using specific antibodies against BDNF. N, normal skin; AD, atopic dermatitis ($\times 40$ magnification). (b) Quantitative analysis of BDNF immunohistochemical staining (APAAP) of normal skin sections and from patients with atopic dermatitis. Black bars, atopic dermatitis; open bars, controls.

together with an increase in MC expression of NGF as shown previously [22], indicate a subtle cellular regulation of different NT molecules in states of allergic cutaneous inflammation. The differential regulation may also reflect changes in the cellular microenvironment and an interaction of the different cytokines and cells in the disease.

A differential regulation for NTs with a reduction vs. induction of different molecules has so far only been demonstrated for a limited number of pathological states [32]. By combining the data of the present and previous studies, it can be concluded that the NT system does not act uniformly with an up-regulation of gene expression towards any inflammatory stimulus as shown, i.e. for allergic asthma. The changes observed are therefore not an epiphenomenon of the ongoing inflammation but should be considered to be disease specific and related to the microenvironment.

The present demonstration of a cell type-specific regulation of the NTs BDNF in atopic dermatitis also stands in line with differential regulations observed for other med-

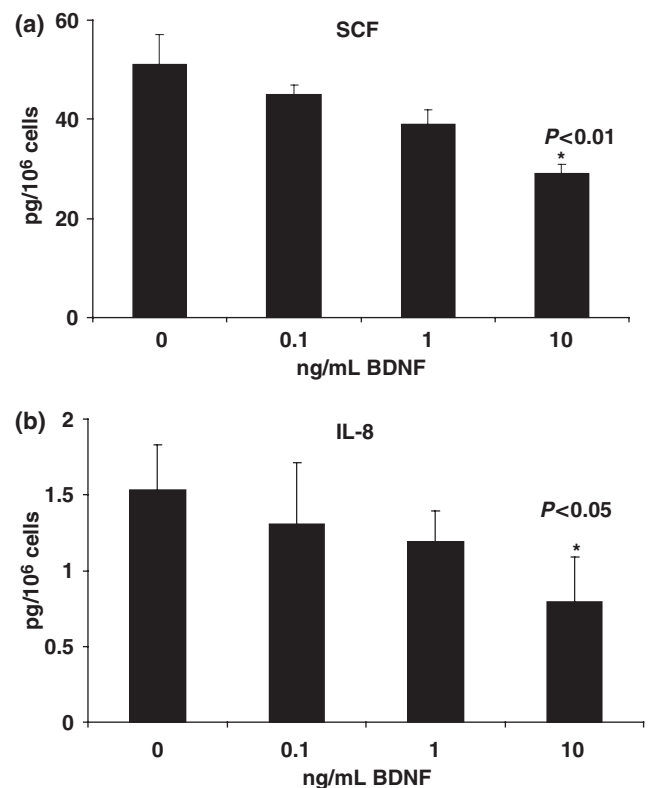


Fig. 3. Functional effects of increasing brain-derived neurotrophic factor (BDNF) levels on HaCaT keratinocyte secretion. (a) Secretion of stem cell factor (SCF) in HaCaT cells after stimulation with different levels of BDNF. (b) Secretion of IL-8 in HaCaT cells after stimulation with different levels of BDNF.

iators: VIP, one of the most abundant biologically active polypeptides, and its receptors have been shown to be up-regulated in many diseases. However, a down-regulation of this system was only found recently for atopic dermatitis when it was shown that the VIP receptor VPAC2 is down-regulated in MCs of atopic dermatitis skin lesions [26]. Interestingly, the mediator itself has been shown to be up-regulated in many inflammatory diseases including aspirin-sensitive upper airway inflammation [14], toxic rhinitis, environmentally triggered hyperreflective rhinitis [13], perennial persistent allergic rhinitis (AR) [33], and seasonal intermittent AR [15].

In contrast to the present finding, an up-regulation of NGF was previously reported in atopic dermatitis [22]. NGF expression was analysed *in situ* in lesional MLs and immunohistochemistry revealed increased protein expression of NGF [22]. By contrast, here, we demonstrate that the BDNF is highly significantly down-regulated in lesional MCs. This imbalance may cause the disproportional neuro-immune interactions underlying the development of atopic dermatitis lesions.

In order to assess the functional significance of decreased lesional MC BDNF production, we incubated the HaCaT keratinocyte cell line with decreasing

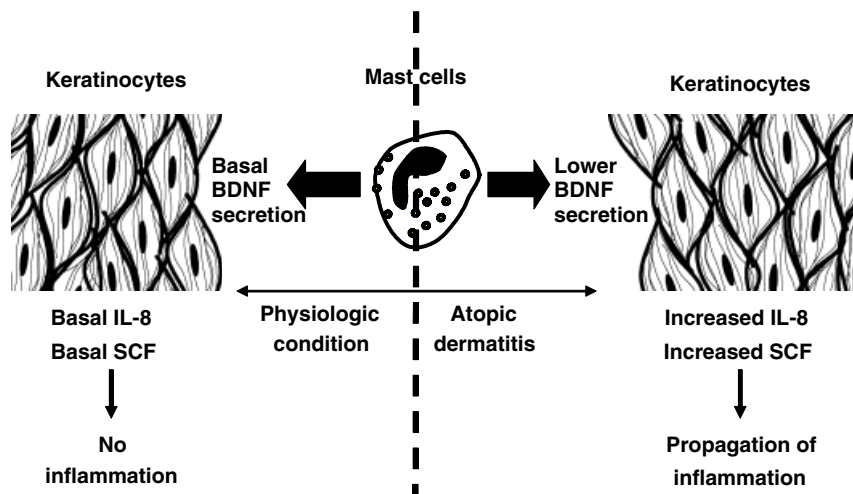


Fig. 4. Schematic illustration of effects of altered lesional mast cell brain-derived neurotrophic factor (BDNF) levels in atopic dermatitis. In atopic dermatitis, decreased levels of BDNF are found in lesional mast cells. Decreased BDNF levels from mast cells in the lesions may lead to increased IL-8 and stem cell factor (SCF) production by keratinocytes as shown by the HaCaT *in vitro* studies. Thus, it can be hypothesized that these mechanisms lead, via an increase in proinflammatory IL-8, to a propagation of inflammation.

concentrations of BDNF and measured the secretion of SCF and IL-8. Interestingly, decreased levels of BDNF led to an increased secretion of SCF and IL-8 by HaCaT cells. Because previous studies demonstrated a close interaction between MCs and keratinocytes in atopic dermatitis [25], it can be suggested that the presently observed decrease in MC BDNF production may also have functional effects on keratinocyte IL-8 and SCF secretion in states of atopic dermatitis. In this respect, the decreased BDNF levels may lead to increased IL-8 and SCF production by keratinocytes as shown by the HaCaT *in vitro* studies and thus to a propagation of inflammation (Fig. 4).

In summary, the present study presents novel data on the gene regulation of BDNF in lesional cutaneous MCs in atopic dermatitis and relates these findings to functional *in vitro* data. By revealing a disease- and cell type-specific differential regulation of BDNF, it can be assumed that the NT system undergoes a microenvironmental switch in its phenotype in states of allergic inflammation. These changes are not an epiphenomenon of general inflammatory conditions such as atopic dermatitis, bronchial asthma, or COPD [32,34]. It is tempting to speculate that molecules modifying the NTs balance may represent promising future therapeutic targets to treat numerous diseases with a neuro-immunological background.

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Potential conflict of interest: none declared.

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