

IgE receptor-mediated release of nerve growth factor by mast cells

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Summary

Background It has previously been demonstrated that the level of nerve growth factor (NGF) is increased in serum from humans with allergic diseases and asthma.

Aim A question raised by these observations is whether NGF could be released from degranulating mast cells during an allergic reaction. The aim of this study was to investigate if NGF is released from mast cells after activation through cross-linkage of the high-affinity IgE receptor.

Methods Mouse and human *in vitro* cultured mast cells were activated by IgE and specific antigen, stem cell factor or lipopolysaccharide. Release of NGF was measured by ELISA and mRNA expression was detected by RT PCR.

Results We found that mast cells not only express NGF transcripts, but also release NGF polypeptide in response to IgE and specific antigen. Activation of mouse mast cells for 30 min induced significant release of NGF ($32.9 \pm 1.3 \text{ pg}/2 \times 10^6 \text{ cells}$) compared to spontaneous release ($13.9 \pm 2.7 \text{ pg}/2 \times 10^6 \text{ cells}$) ($P < 0.01$). Similarly, activation of human cultured mast cells also resulted in a significant increase of NGF release ($733 \pm 310 \text{ pg}/3 \times 10^5 \text{ cells}$) compared to spontaneous release ($9.2 \pm 4.0 \text{ pg}/3 \times 10^5 \text{ cells}$). Two other mast cell secretagogues studied, stem cell factor and lipopolysaccharide, were not able to induce release of NGF.

Conclusion This study provides evidence that NGF could be specifically released by stimuli causing an allergic reaction, and mast cells can thereby be the source of NGF in IgE-mediated allergic diseases. Our findings add further support for a close correlation between NGF and mast cells that could be of importance for the allergic inflammation.

Keywords: allergy, asthma, FcεRI, IgE, inflammation, mast cells, nerve growth factor

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Introduction

Mast cells are widely distributed throughout vascularized tissues and certain epithelia where they play a fundamental role in the pathogenesis of immediate hypersensitivity reactions such as allergy and anaphylaxis [1,2]. Recent evidence suggests that the pathophysiological role of mast cells is much more global in nature. In addition to being effector cells in immediate hypersensitivity reactions, mast cells are involved in other pathophysiological processes including delayed-type hypersensitivity, wound healing,

connective tissue homeostasis, fibrosis, neuroimmunological disorders and active immunology [3–6], suggesting a profound role of mast cells in health and disease. The exertion of mast cell function in the pathophysiological conditions is thought to in part reflect the actions of the mediators released by those cells in response to their activation by secretagogues. The most extensively studied stimulus is the aggregation of their high-affinity IgE-receptor (FcεRI) by IgE and antigen that results in release of preformed mediators stored in granules (e.g. histamine, tryptase, heparin) and newly synthesized lipid mediators and cytokines [7]. Apart from classical IgE-mediated mechanisms, mast cells can also be activated by other physiological stimuli such as bacterial lipopolysaccharide

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(LPS), stem cell factor (SCF), substance P and immunocomplexes as well as calcium ionophores and phorbol esters [8–12].

Nerve growth factor (NGF) was discovered in the 1950s and represents the best characterized member of the neurotrophins [13]. NGF regulates the survival, differentiation, development and functional maintenance of both peripheral and central neurones in the nervous system. Biological actions of NGF are mediated by low and high-affinity cell surface receptors which, upon binding and internalization of the ligand, trigger a cascade of biological and morphological events in NGF-target cells [14]. In recent years, numerous studies have provided evidence that the effects of NGF are not limited to the peripheral nervous system but also affect non-neuronal cells, the first evidence of which was that injection of NGF into newborn rats caused an increase in the number and size of mast cells [15]. The effects of NGF on cellular biology have been extended to other cell populations of the haemopoietic lineage, including lymphocytes, granulocytes and monocytes (reviewed in [16,17]).

Associations between inflammatory responses, allergy and NGF levels have been observed [18,19]. Both plasma and inflamed tissues of patients affected by vernal keratoconjunctivitis contain elevated levels of NGF, which is associated with the number and distribution of mast cells [20]. It has also been observed that NGF levels are increased in humans with allergic diseases and asthma [21,22]. These findings, together with the fact that NGF is synthesized by mast cells [23,24] prompted us to investigate whether mast cells release NGF after aggregation of the FcεRI and thereby could be the source of NGF in IgE-mediated disease.

Materials and methods

Mast cell cultures

Bone marrow-derived cultured mouse mast cells (BMCMC) were obtained by culturing mouse (C57BL/6, 3- to 4-month-old, Bommice, Ry, Denmark) bone marrow cells for 5–6 weeks in 10% WEHI-3 enriched conditioned RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL, Eggenstein, Germany), 4 mM L-glutamine, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM Hepes, and 100 μg/mL penicillin/streptomycin (Sigma Chemical Co., St. Louis, MO, USA). The mast cell differentiation was confirmed by toluidine blue staining and flow cytometry. The growth factor-dependent mouse mast cell line MCP5/L [25] was maintained in the same medium described above. The mouse mast cell line MTC and *in vivo*-derived mouse MTC tumour cells [26] were cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum, 4 mM

L-glutamine, and 100 μg/mL penicillin/streptomycin. Cord blood cultured human mast cells (CBCMC) were differentiated from cord blood cells cultured for 8–10 weeks in 50 ng/mL SCF and 10 ng/mL IL-6 (Amgen, Thousand Oaks, CA, USA) as described by Saito *et al.* [27]. The purity of human mast cells were >95% by tryptase staining.

Analysis of NGF transcripts by RT PCR

Total RNA was extracted using the TriPure isolation reagent (Boehringer Mannheim, Mannheim, Germany). Since mast cell secretory granules contain heparin which may inhibit the RT reaction, RNA isolated from MCP5/L cells either at resting state or activated was treated with heparinase I (Sigma) for 2 h at 25°C [28] and purified again after the enzymatic treatment. For RT PCR, 1 μg of RNA was reverse-transcribed in a 20-μL reaction by oligo-p(dT)₁₅ priming using the 1st strand cDNA synthesis kit for RT PCR (Boehringer Mannheim) according to the manufacturer's instructions.

The PCR primers used for fragment amplification of mouse NGF were 5'-GGCAGACCCGCAACATCACT-3' (upper) and 5'-GTCTTATCTCCAACCCACAC-3' (lower) spanning a region of 247 bp starting from position 466 (GenBank accession number k01759). For glyceraldehyde-3-phosphate dehydrogenase amplification (GAPDH), as a control for the integrity of each cDNA, a 452-bp fragment starting from position 312 was generated with primers as described [28]. Amplifications were performed using the PCR core kit (Boehringer Mannheim) as follows: 94°C, 5 min; followed by 25 amplification cycles for GAPDH and 38 for NGF, respectively (94°C, 30 s; 56°C, 45 s; and 72°C, 1 min); a 7-min elongation at 72°C was maintained after the final cycle. PCR products were separated by electrophoresis through 2% agarose gels, stained with ethidium bromide, and photographed.

Activation of mast cells in vitro

MCP5/L cells were resuspended at 2×10^6 cells/mL and sensitized using a monoclonal murine IgE anti-TNP antibody (IgE1-b4, ATCC, Manassas, VA, USA) (15% hybridoma supernatant) for 90 min. The cells were washed twice, followed by challenge with 1 μg/mL TNP-BSA (coupling ratio: 4.8, kindly provided by Dr Birgitta Heyman, Uppsala, Sweden) for various time periods. Both the antibody sensitization and the antigen challenge were carried out in RPMI medium supplemented with 0.5% BSA (Sigma) at 37°C on a rocker platform. After stimulation, supernatants were collected after centrifugation. Where indicated, cell lysates were obtained by sonication. Both supernatants and lysates were stored at –20°C until further analysis of NGF and β-hexosaminidase. Human myeloma IgE (ND)

and mouse antihuman IgE (clone 346) (kindly provided by Dr Torbjorn Karlsson, Pharmacia & Upjohn Diagnostics, Uppsala) were used to activate CBCMC. Human mast cells at 1×10^6 cells/mL were incubated with $1 \mu\text{g/mL}$ IgE overnight, washed twice with PBS, plated at 3×10^5 cells/mL, and challenged with $2.0 \mu\text{g/mL}$ anti-IgE for 30 min at 37°C . Supernatants were collected as described above. Alternatively, mast cells were activated by LPS or SCF treatment. MCP5/L cells were washed, resuspended at 2×10^6 cells/mL in the same medium as mentioned above, and stimulated for 2 h with various concentrations of SCF (Pepro Tech, London, England) or LPS (Sigma). CBCMC at 3×10^5 cells/mL were also incubated with LPS in a similar way. Supernatants were collected as described above.

Measurements of mediators

NGF was assayed by ELISA (NGF ImmunoAssay System, Promega, Madison, WI, USA) according to the manufacturer's instructions. Before ELISA analysis supernatants representing the spontaneous and activated releases of NGF from MCP5/L cells were concentrated by ultrafiltration through Amicon's Centricon-10 concentrators (Amicon, Inc., Beverly, MA, USA). For detection of the granular enzyme β -hexosaminidase an enzymatic colourimetric assay was used. Briefly, $60 \mu\text{L}$ of supernatant or lysate was mixed in a 96 well plate with identical volume of substrate solution (7.5 mM p-nitrophenyl N-acetyl- β -D-glucosaminide (Sigma) in 80 mM citric acid, $\text{pH } 4.5$), and incubated at 37°C for 2 h on a rocker platform. After the incubation, $120 \mu\text{L}$ of glycine (0.2 M , $\text{pH } 10.7$) was added to each well and the absorbance was read using an ELISA reader (Molecular Devices Corporation, Menlo Park, CA, USA).

Statistical analysis

Statistics were calculated using an analysis of variance (ANOVA), followed by multiple comparison using Fisher's method. *denotes $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Values presented are the means $\pm$ SEM ($n = 3-9$).

Results

Constitutive NGF gene expression by mouse mast cells

We have previously reported that a human mast cell line and primary human mast cells express NGF mRNA and spontaneously secrete biologically functional NGF [24]. To determine if mouse mast cells also constitutively express NGF mRNA, we examined NGF transcript expressed in

BMC MC, mouse mast cell lines MCP5/L and MTC, and *in vivo*-derived mouse mast cell tumour (MTC *in vivo*). As shown in Fig. 1(a), mRNA for NGF was detected in all the mouse mast cells analysed. Cross-linking Fc ϵ RI did not increase the density of the band amplified from MCP5/L (Fig. 1b). However, owing to the qualitative rather than quantitative feature of RT PCR assay, the regulation of NGF at transcription level needs to be further confirmed by other quantitative measurements.

Fc ϵ RI-mediated NGF release

Activation through Fc ϵ RI aggregation is a primary stimulus capable of degranulating mast cells and is the immunologically

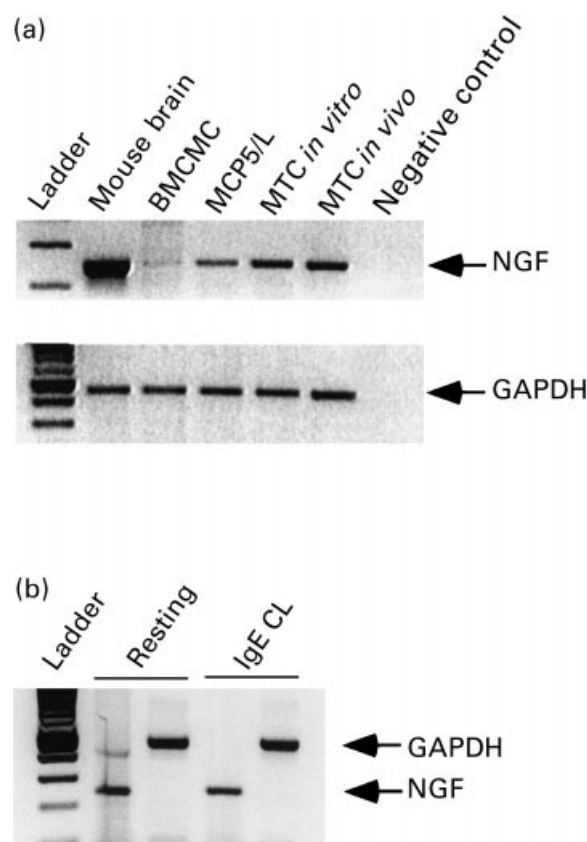


Fig. 1. RT PCR analysis of mouse mast cells for NGF mRNA expression. RNAs from mouse mast cells were extracted and cDNAs were synthesized for PCR screening for both NGF and GAPDH amplification which is served as a control for cDNA integrity. In (a), mouse brain was used as a positive control for NGF; BMC MC, bone marrow-cultured mouse mast cells; MCP5/L, heparinase-treated RNA from mouse mast cell line MCP5/L; MTC *in vitro*, MTC cell line; MTC *in vivo*, mouse MTC tumour. In (b), heparinase-treated RNAs from either resting MCP5/L cells or cells activated for 2 h via IgE cross-linking were examined by RT PCR for NGF and GAPDH transcript expression.

specific stimuli that can activate mast cells in an allergic reaction. Therefore we investigated whether mast cells activated by this means can release NGF. We also measured the release of β -hexosaminidase, an enzyme stored in mast cell [29]. Monoclonal IgE anti-TNP supernatant was used to sensitize MCP5/L cells for 90 min followed by the antigen TNP challenge. Release of β -hexosaminidase from the activated cells was approximately 2.5-fourfold as compared to control cells at all time points tested. NGF was also released by this immunological specific activation with approximately twofold induction as compared to spontaneous controls when measured at 0.5, 2 and 6 h after IgE-receptor cross-linking (Fig. 2).

The increase in NGF polypeptide released by mast cells after IgE-dependent activation raised the question of whether this increase in NGF-release is due to an increase in membrane permeability or if it is due to enhanced NGF synthesis after activation. We therefore examined the total content of NGF in resting and activated mast cells (NGF in cell lysates + released NGF). No significant differences were found between the two groups, indicating that stimulation through the IgE-receptor results in enhancing the release of presynthesized NGF molecules but not in up-regulation of NGF synthesis (Fig. 3a). Similarly, the release levels of β -hexosaminidase were not affected by activation (Fig. 3b).

We also determined if human primary mast cells release NGF after an allergic activation. CBCMC were activated through aggregation of Fc ϵ RI by myeloma IgE (ND) and antihuman IgE antibody treatment. Release of NGF increased approximately 80-fold compared to the spontaneous release by control cells (Fig. 4a). The release level of β -hexosaminidase was also found to be substantially increased following IgE-dependent activation of CBCMC (Fig. 4b).

LPS or SCF do not induce release of NGF

Besides aggregation of Fc ϵ RI, mast cells can be activated to release mediators by other physiological stimuli such as LPS or SCF. We therefore examined if these two factors, which do not represent specific allergic activation of mast cells, could induce release of NGF by mast cells. Mouse mast cells were treated for 2 h with LPS at 5, 50 and 500 μ g/mL concentrations or with SCF at concentrations of 1, 10, and 100 ng/mL. The results revealed that neither LPS nor SCF could significantly influence the release of NGF (Fig. 5). As for β -hexosaminidase release, SCF was found to be capable of enhancing the release whereas LPS was not (Fig. 5). The above finding suggests that the release of NGF and β -hexosaminidase might be differentially regulated by MCP5/L cells.

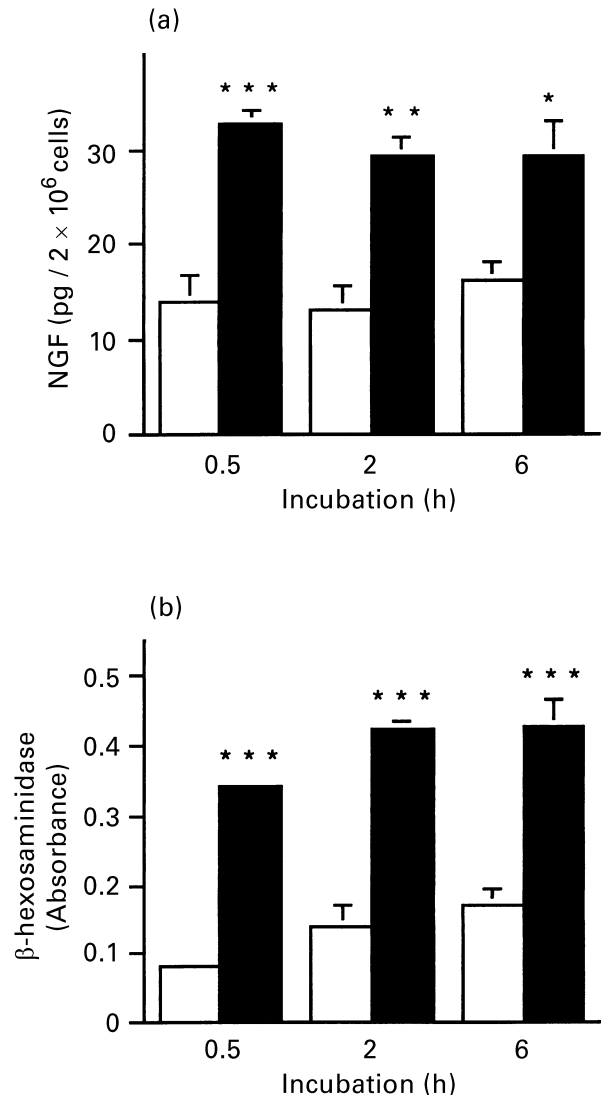


Fig. 2. Detection of released NGF protein (a) and β -hexosaminidase (b) in MCP5/L mouse mast cell culture. Supernatants from either resting control cells (open bars) or cells stimulated through IgE receptor cross-linking (solid bars) were assayed at the time points indicated. Released NGF was measured by ELISA and β -hexosaminidase detection was carried out by an enzymatic colourimetric assay.

Since CBCMC were cultured in the presence of SCF, we therefore only determined whether LPS could induce release of NGF and β -hexosaminidase.

Similar to the finding with mouse mast cells, LPS did not cause release of either NGF (Fig. 4a) or β -hexosaminidase (Fig. 4b) from CBCMC. LPS incubation was carried out for 2 h whereas the IgE aggregation was kept for only 30 min, which explains the increase of β -hexosaminidase level ($21.6 \pm 2.2\%$) at LPS concentration 0 (curve point which corresponds to 0 μ g/mL of LPS), compared

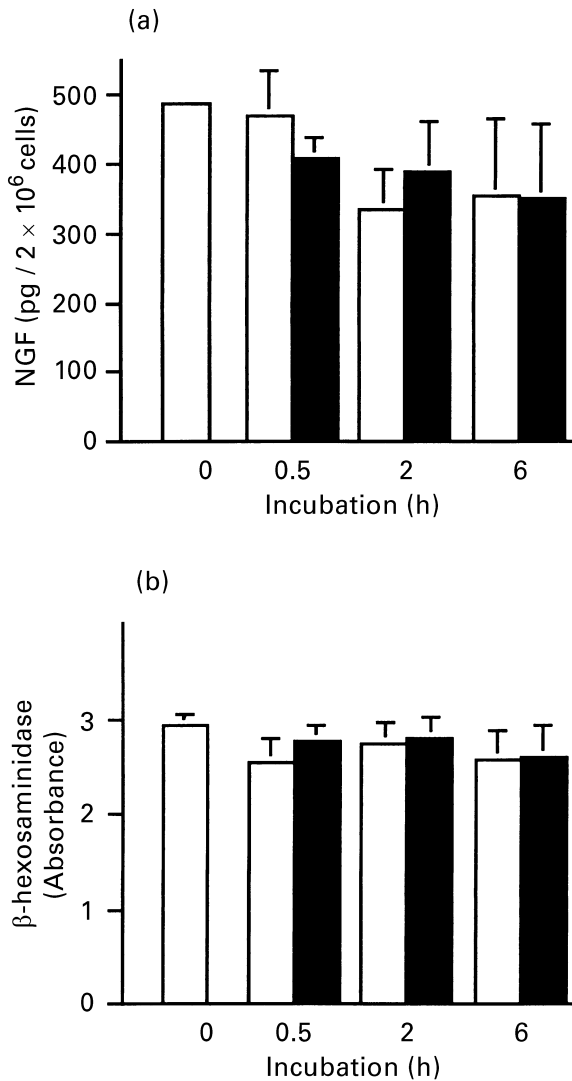


Fig. 3. Detection of total NGF protein (a) and β -hexosaminidase (b) in MCP5/L mouse mast cell culture. Cell suspensions were sonicated, and the lysates from resting control cells (open bars) or cells activated through Fc ϵ RI aggregation (solid bars) were assayed as explained in Fig. 2.

with the value ($5.0 \pm 1.0\%$) representing resting cells (open bar), which serve as control for IgE crosslinking (Fig. 4b). However, this prolongation of incubation time didn't increase the release of NGF (Fig. 4a), where NGF spontaneous release levels were 7 ± 3.5 pg/mL for 2 h and 9.1 ± 4.0 pg/mL for 30 min. Taken together, these data indicate that the spontaneous release of NGF is lower compared to that of β -hexosaminidase in these cells.

Discussion

This study shows that mast cells release NGF upon activation through the high affinity IgE receptor, suggesting that

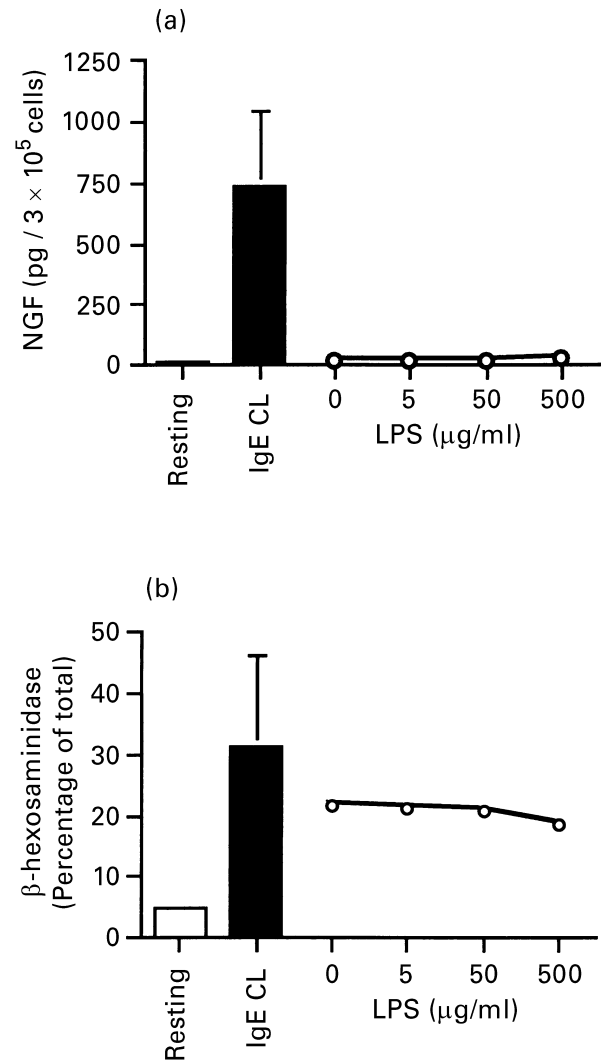


Fig. 4. Effects of IgE-dependent mast cell activation or LPS treatment on NGF and β -hexosaminidase release from CBCMC. Supernatants from either resting control cells (open bars) or cells activated through Fc ϵ RI aggregation for 30 min (solid bars), or cells treated with LPS for 2 h (curves), were measured for NGF (a) and β -hexosaminidase (b) as explained in Fig. 2. β -hexosaminidase release levels are expressed as percentage of total cellular content of the enzyme.

mast cells might be an important source of preformed NGF that is released upon allergic stimuli and can be detected in body fluids in allergic diseases [20–22]. Mast cells are important effector and regulatory cells secreting a variety of different cytokines important for the inflammatory process [30]. A number of cytokines such as TNF α are preformed and stored in granules whereas others such as IL-13 and lymphotactin are induced upon activation [10,31–33]. Furthermore, our data also indicate that NGF mRNA is constitutively transcribed (Fig. 1 and [24]) which

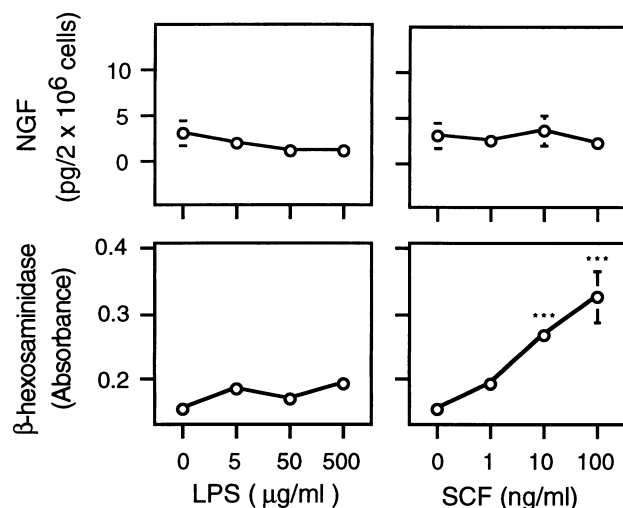


Fig. 5. Effects of LPS or SCF on release of NGF protein and β -hexosaminidase. MCP5/L mouse mast cells were stimulated by LPS or SCF for 2 h at concentrations indicated. NGF and β -hexosaminidase release was measured as in Fig. 2.

adds proof to the finding that NGF polypeptide is preformed and stored in mast cell granules [23,24]. After primary activation through IgE-receptor cross-linking, we observed no further increase in the amount of NGF released into the supernatant over time. A similar observation has been reported for IL-6 released by mast cells [12]. Our finding could be explained by a number of possibilities. Some of the released NGF may have been degraded by mast cell-derived proteases with prolonged incubation as suggested [12], or the release of NGF is actually a rapid response completed within the first 30 min.

The total levels of NGF synthesized by mast cells did not change before and after IgE-receptor aggregation (Fig. 3a). This indicates that the enhancement of release upon activation is not a result of increased synthesis of the protein, but rather due to increased release of the protein into the extracellular medium. Therefore it is likely that NGF is synthesized by mast cells at a constant rate and is stored in the granules as a preformed protein. Activation of mast cells appears to only increase the secretion of NGF polypeptide. In contrast, other cytokines released by mast cells such as TNF α [10], and IL-16 [11] are immunologically inducible, which means that the activation of mast cells results in an elevated release of the protein as well as an increased intracellular production of this specific cytokine.

The NGF secretion pattern we observed in this study is compatible with findings obtained *in vivo*. A rapid appearance (within 2 h) of NGF in rat skin blister fluid can be found [34], and in human allergic inflammation and asthma NGF serum levels could increase 2–40-fold, depending on the seriousness of the disease as compared

with healthy controls [21]. More interestingly, this increase in NGF levels correlates with the increase of total IgE serum levels as evidenced by the fact that the highest NGF values are found in patients with severe allergic asthma, which is manifested by high total IgE levels [21]. Furthermore, in allergen provoked asthma patients increased levels of NGF were detected after 18 h [22].

In contrast to mast cell IgE-dependent activation, neither SCF nor LPS were able to stimulate mast cells to release NGF (Fig. 4), though SCF could stimulate release of β -hexosaminidase. This differential release of cytokines and mediators by mast cells has been described previously. For example, bacterial LPS induces rat peritoneal mast cells to release IL-6 independent of histamine release while mast cells treated with the calcium ionophore A23187, which triggers high histamine release, do not produce IL-6 [8]. SCF induces mouse BMCMCs to release IL-6 but fails to release significant amounts of histamine [12]. We believe that the differential release of NGF seen in our study is important for the role mast cells play during the inflammatory process. Our data indicate that NGF secretion is only stimulated by mast cell Fc ϵ RI crosslinking, but not by other physiological stimuli such as SCF and LPS treatments. NGF released through Fc ϵ RI-cross-linking ensures that the intracellular NGF pool is reserved mainly for release during an allergic response.

Mast cells reside in tissues and are abundant in and around blood vessels, where they have close contact with other cell types. Therefore, mast cell released NGF could activate other inflammatory cells during an allergic response, and thereby increase and perpetuate the inflammatory response. It has been described that NGF significantly increases the differentiation of basophils [35], primes IgE-mediated mediator release from human circulating basophils [36] and promotes neutrophil chemotaxis and superoxide production [37,38]. NGF is also active in modulation of B-cell-mediated immune [39]. Furthermore, upon activation mast cells not only directly release NGF, but could also indirectly upregulate NGF production in surrounding cells by releasing other pro-inflammatory cytokines such as IL-1 or TNF α that is known to stimulate synthesis of NGF [40,41].

Another important effect of NGF is the stimulation of axon regeneration in injured nerve cells and to enhance the synthesis and release of neuropeptides such as substance P and calcitonin gene-related peptide in peripheral neurons [42,43]. Transgenic mice overexpressing NGF in the lung have hyperinnervated airways with increased numbers of substance P containing sensory nerve fibres [44]. Neuropeptides have potent pro-inflammatory roles in the airways and are believed to be involved in airway hyperreactivity [45]. Mast cells have been shown to be in close contact with nerve endings in different tissues including

the lung (reviewed in [46]). In asthma models, we and others have found an increased expression of neuropeptides in sensitized and challenged animals [47,48], suggesting that the cause of increased neuropeptide content in the airways in asthma could be due to release of NGF from allergen-activated mast cells.

In conclusion, we have shown that mast cells release NGF upon stimulation through FcεRI-aggregation. This may be an important step in unraveling the multifunctional role of mast cells in asthma and allergy.

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