Brief Report

NF-κB Regulates B-Cell-Derived Nerve Growth Factor Expression

Klaus Heese[1, 2, 3], Noriko Inoue[2] and Tohru Sawada[2]

In the mammalian brain, four neurotrophins have been identified: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). NGF exerts an important role in the development and functions of the central and peripheral nervous system. However, it has recently been documented that several types of immune cells, such as mast cells, lymphocytes, basophils and eosinophils, produce, store and release NGF. Accumulating preclinical and clinical data indicate that dysfunctions of NGF and the other neurotrophins may contribute to impaired immune responses and concentration of NGF frequently correlates with disease severity. Thus, the aim of this study was to elucidate the potential signaling mechanisms of cytokine-neurotrophins interactions contributing to increased NGF levels. Our data show that the transcription factor NF-κB plays a pivotal role in regulating B-cell-derived NGF expression. *Cellular & Molecular Immunology.* 2006;3(1):63-66.

Key Words: immune system, inflammation, interleukin, NGF, neurotrophin

Introduction

Neurotrophins are a family of growth factors critical for the development and function of the nervous system. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) are included in this family. Although originally identified as neuronal survival factor, NGF is capable of exerting a far broader spectrum of biological activities (1). It has been documented that NGF is released by several types of immune cells such as mast cells, lymphocytes, basophils and eosinophils. NGF receptors are widely expressed in the immune system, indicating the possibility that the immune system responds to this neurotrophin through an autocrine mechanism. Heightened levels of NGF have been reported in patients with allergies, systemic lupus erythematosus, systemic sclerosis and chronic autoimmune arthritis (2, 3). Elevated levels of NGF found in sera and fluids of patients affected by autoimmune diseases could be explained by an increased production of NGF by immuno-competent B and T cells. Activation of these cells in response to antigens or cytokine stimulation could be the cause of this enhanced production. Data have suggested that NGF might be viewed as a Th2-like cytokine with a regulative role in inflammation and tissue remodeling and NGF might represent a clinical marker of inflammation (3).

Expression of neurotrophins in T cells, however, remains a controversial issue. A recent report indicates that B cells rather than T cells are the predominant if not the only source of leukocyte-derived NGF and BDNF and as such may provide "protective autoimmunity" in repair and regeneration of the injured nervous system (4-6). BDNF plays a pivotal role in B cell development. In $BDNF^{-/-}$ mice, maturation of B lymphocytes is specifically blocked at the pre-BII stage (7). Activated B cells release BDNF (5-7) and express the functional truncated form of the BDNF-receptor. B cells also respond to NGF by increasing their proliferation rate and NGF is essential for the survival of memory B cells (1, 3, 8). Thus, there might be a cross-talk between NGF and BDNF during B cell activation and BDNF may have autocrine functions under certain conditions.

It is known that B cells are able to produce cytokines such as IL-4 and IL-6 in an autocrine fashion. The activation of human B lymphocytes has been worked out in some detail

[1]Department of Molecular and Cell Biology, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore;

[2]BF Research Institute Inc., Itoyoshi Building 201, 2-5-9 Doshomachi, Chuo-ku, Osaka 541-0045, Japan;

[3]Corresponding to: Dr. Klaus Heese, Department of Molecular and Cell Biology, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore. Tel: +65-6316-2848, Fax: +65-6791-3856, E-mail: kheese@ntu.edu.sg.

Received Oct 19, 2005. Accepted Jan 25, 2006.

Copyright © 2006 by The Chinese Society of Immunology

Abbreviations: BDNF, brain-derived neurotrophic factor; CNS, central nervous system; IL-6, interleukin-6; NAC, N-acetyl-L-cystein; NGF, nerve growth factor; PDTC, pyrrolidine dithiocarbamate; PNS, peripheral nervous system; RT-PCR, reverse transcription-polymerase chain reaction; TNF, tumor necrosis factor.

and has been shown to be dependent on the synergistic effect of IL-4 and CD40 cross-linking (9).

CD40 is a member of the tumor necrosis factor (TNF) receptor family and the development of the acquired immune response is dependent on bi-directional signaling mediated by CD40 and its ligand CD154 (CD40L), which is primarily expressed on activated $CD4^+$ T cells. Specifically, CD40 signaling is essential for activation of B cells by T cell-dependent antigens, germinal center formation, immunoglobulin (Ig) class switching, affinity maturation of B lymphocytes, and the development of plasma and memory B cells (10). Various analyses have shown that CD40L induces all NF-κB/Rel proteins, whereas LPS activates predominantly p50 and c-Rel (11-13).

In view of the important role played by the CD40-, IL-4-, NGF- and BDNF systems in B cell function and based on our previous reports about NGF activation in other cellular systems in the CNS (14-16), the present study aimed at getting insight into the potential role of BDNF and the transcription factor NF-κB on various B cell activators in regulating B-cell-derived NGF expression.

Materials and Methods

Animals

All experiments were carried out according to the guidelines of the Japanese Community's Council for Animal Experiments and conformed to international guidelines on the ethical use of animals. All animal protocols were approved by the Institutional Animal Care and Use Committee in compliance with the Public Health Service policy on human care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Isolation of B cells

Twelve-week-old 129SW or three-week-old $BDNF^{-/-}$ mice (kindly provided by Dr. Hatanaka (Osaka, Japan)) and wildtype littermates were sacrificed, genotyped (17) and spleens were collected. Resting splenic B cells were isolated by depletion of $CD43^+$ cells with magnetic beads by magnetic cell sorting on a MidiMACS cell separator (MACS; Miltenyi Biotec, Auburn, CA) and were further purified with specific magnetic anti-B220 microbeads and positively selected according to the manufacturer's directions. Resting B cells isolated by all methods were at least 98% B220-positive as verified by flow cytometry. Flow cytometric analysis was performed using a FACScan or a FACSCalibur and CellQuest software (BD Biosciences, Tokyo, Japan) as described previously (6).

Cell culture

B cells were grown in RPMI 1640 tissue culture media (Invitrogen Life Technologies, Tokyo, Japan) supplemented with 5% Fetal Bovine Serum (FBS), 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich, Tokyo, Japan), 10 mM HEPES (Invitrogen), 2 mM L-glutamine (Invitrogen). Resting B cells were cultured (10^6/ml) at 10^5/well in triplicate in 96-well plates for 24 h in the presence of complete RPMI medium alone, or stimulated with recombinant mouse IL-4 (5 ng/ml), IL-6 (5 ng/ml; BD Biosciences), lipopolysaccharide (LPS) (1 ng/ml; Sigma-Aldrich), or anti-mouse-CD40 monoclonal antibody (5 μg/ml; BD Biosciences) and compounds as indicated. Cell proliferation was assayed by determining cell viability using a trypan blue solution. In addition, cell survival was measured after 24 h by Cell Titer 96® AQueous One solution assay according to the manufacturers' protocol (Promega, Madison, WI) (7, 8, 18).

Enzyme immunoassay for NGF

To measure immunoreactive NGF released into the culture medium, a two-site ELISA for the determination of NGF as described by the manufacturer was used (Roche) (16). Briefly, polystyrene 96-well microtiter immunoplates (Nunc, Tokyo, Japan) were coated with either the monoclonal antibody against the β-subunit of NGF from mouse-hybrid cells (Roche) or a goat preimmune serum to determine nonspecific binding (Zymed Laboratories, San Francisco, CA, USA). Each plate included a complete standard curve ranging from 0 pg/ml to 1,000 pg/ml. Fifty microliters of the cell culture medium were added to each well over night at 4˚C. The plate was incubated with the β-galactosidase-coupled coating antibody. Specific signals were quantified using a chlorophenolred-β-D-galactopyranoside substrate solution (Roche). After incubation for 3 h at 37˚C, the optical density was measured at 595 nm using an ELISA-reader (Shimadzu, Tokyo, Japan). The NGF content in the samples was determined by comparison with the standard curve. Statistical analysis was performed by applying analysis of variance (ANOVA) with SPSS 10.0 software and the statistical error was indicated as the SEM (mean ± SD). Student's t test was also used for the parametric data analysis between two groups. Probability value $p < 0.05$ was considered statistically significant. All data are representative of at least three separate experiments.

Results and Discussion

Increasing evidence identifies NGF as a signaling molecule modulating inflammatory processes associated with tissue repair (1, 2). Stimulation of B cells with LPS, CD40, IL-4, or IL-6 resulted in a significant increase in NGF protein release. In $BDNF^{-/-}$ mice, LPS-/cytokine-induced secretion of NGF protein by splenic B cells was markedly reduced (Figure 1). Under such experimental conditions, the cell number did not change significantly with respect to time or in response to cytokine stimulation. RT-PCR analysis revealed that the same B cell activators (LPS, IL-4, CD40 and IL-6) also stimulated NGF and trkA mRNA expression. On the contrary, the $p75^{NTR}$ mRNA level was not significantly altered by any one of these activators (data not shown).

Accumulating evidence has convincingly demonstrated the pivotal role of CD40 in B cell activation and no compensatory mechanism appears to replace CD40's

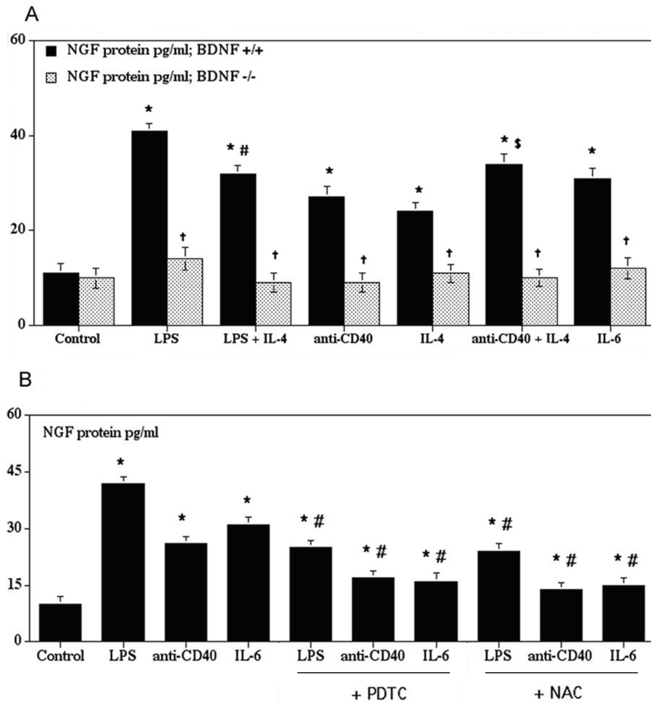

Figure 1. Induction of NGF protein release from purified splenic B cells by various B cell activators or NF-κB inhibitors. (A) LPS (1 ng/ml), anti-CD40 (5 μg/ml), IL-4 (5 ng/ml) and IL-6 (5 ng/ml) were used (* $p < 0.05$ compared to unstimulated controls, # $p < 0.05$ compared to LPS-stimulated cells, \$ $p < 0.05$ compared to anti-CD40 or IL-4 alone, † $p < 0.05$ compared to BDNF$^{+/+}$ mice). (B) B cell activators used were: LPS (1 ng/ml), anti-CD40 (5 μg/ml), IL-4 (5 ng/ml), IL-6 (5 ng/ml); inhibitors: PDTC (30 μM) and NAC (2 mM) (* $p < 0.05$ compared to unstimulated controls, # $p < 0.05$ compared to stimulated cells in the absence of NF-κB inhibitors).

function (10). Though the NGF receptor p75^{NTR} (also a member of the TNFR superfamily) and CD40 are expressed in a co-ordinate fashion on B cells, they may differ in their activities at different stages of B cell differentiation as a result of their interactions with other cytokine- or growth factor-systems (19). It remains unclear whether p75^{NTR} expression is essential for B cell functioning. However, it is of special interest as to whether p75^{NTR} mediates apoptosis upon activation by proNGF (20). Recently, important differences in cytokine production and expression of surface-bound growth factors by CD40L-activated B cells, compared to LPS-activated B cells, have been described. The findings indicate that the means by which B cells are activated and not simply the enhanced expression of co-stimulatory molecules underpin the ability of B cells to respond to the stimuli and to activate, for instance, naïve CD8^{+} T cells (10-13). Although continuous studies have revealed CD40 signaling pathways that ultimately result in B cell activation, it still remains to be explored how these signaling events are initiated after receptor ligation and how the CD40- and NGF-receptor systems interact with each other.

To determine the mechanism involved in the enhanced

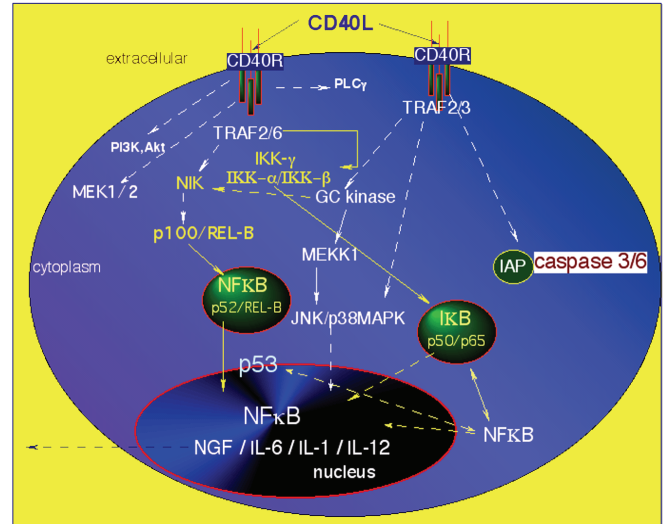

Figure 2. Schematic illustration of the signaling mechanisms underlying the coupling of CD40 to NGF expression in B cells.

release of NGF protein upon cytokine stimulation of B cells, we studied different NF-κB inhibitors. Using ELISA and RT-PCR techniques, the effects of the antioxidants PDTC (known to prevent cellular reactive oxygen intermediate (ROI) production and cytokine-induced NF-κB activation) and N-acetyl-L-cystein (NAC) were investigated (12, 16).

B cells incubated with PDTC (30 μM), which alone does not influence basal NGF mRNA/protein expression levels (data not shown) (16), revealed the inhibitory effect of PDTC on LPS-/cytokine-induced NGF protein release 24 h after stimulation (Figure 1). Moreover, the LPS-/cytokine-induced NGF protein release was abolished by NAC (2 mM) (Figure 1) suggesting that reactive radical intermediates are indeed involved in LPS-/cytokine-induced B cell NGF synthesis. It is interesting to note that both inhibitors, PDTC and NAC, blocked the effect on cytokine-induced NGF release (Figure 1). PDTC and NAC also reduced the LPS-/cytokine-activated NGF mRNA expression at maximal inhibitory concentrations (data not shown). Thus, our present study suggests that LPS-, IL-4- and CD40-stimulated NF-κB activation is a key regulatory event for B-cell-derived NGF production. The investigation on BDNF-deficient mice points to a crucial cross-talk between these two neurotrophins during B cell activation (12, 13) (Figures 1, 2).

However, the biological significance of the interaction between the CD40- and the neurotrophin-systems and its pivotal impact on specific B cell functions remain to be elucidated.

An inflammation-induced increase of NGF might cause this factor to act like a cytokine that modifies functions of mast cells, macrophages and B cells. Instead of working singly, neurotrophins and cytokines appear to follow a concerted path to achieve an array of responses necessary to preserve homeostatic integrity. Acute or chronic dysfunction of this cross-talk can upset the delicate equillibrium that

regulates intersystemic communication, hence may potentially provoke or exacerbate immune-mediated pathological processes, such as multiple sclerosis. A clearer understanding of the specific roles played by these neurotrophins in this context will lead to new opportunities for pharmacological modulation of neuro-immune dysfunctions.

Acknowledgements

This study was supported by grants from Nanyang Technological University (SBS/SUG/22/04) and The Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan.

References

1. Levi-Montalcini R, Skaper SD, Dal Toso R, Petrelli L, Leon A. Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci. 1996;19:514-520.
2. Bonini S, Rasi G, Bracci-Laudiero ML, Procoli A, Aloe L. Nerve growth factor: neurotrophin or cytokine? Int Arch Allergy Immunol. 2003;131:80-84.
3. Lambiase A, Micera A, Sgrulletta R, Bonini S, Bonini S. Nerve growth factor and the immune system: old and new concepts in the cross-talk between immune and resident cells during pathophysiological conditions. Curr Opin Allergy Clin Immunol. 2004;4:425-430.
4. Moalem G, Gdalyahu A, Shani Y, et al. Production of neurotrophins by activated T cells: implications for neuroprotective autoimmunity. J Autoimmun. 2000;15:331-345.
5. Kerschensteiner M, Gallmeier E, Behrens L, et al. Activated human T cells, B cells, and monocyte produce brainderived neurotrophic factor *in vitro* and in inflammatory brain lesions: a neuroprotective role of inflammation. J Exp Med. 1999;189: 865-870.
6. Edling AE, Nanavati T, Johnson JM, Tuohy VK. Human and murine lymphocyte neurotrophin expression is confined to B cells. J Neurosci Res. 2004;77:709-717.
7. Schuhmann B, Dietrich A, Sel S, et al. A role for brain-derived neurotrophic factor in B cell development. J Neuroimmunol. 2005;163:15-23.
8. Torcia M, Bracci-Laudiero L, Lucibello M, et al. Nerve growth factor is an autocrine survival factor for memory B lymphocytes. Cell. 1996;85:345-356.
9. Oettgen HC. Regulation of the IgE isotype switch: new insights on cytokine signals and the functions of epsilon germline transcripts. Curr Opin Immunol. 2000;12:618-623.
10. Quezada SA, Jarvinen LZ, Lind EF, Noelle RJ. CD40/CD154 interactions at the interface of tolerance and immunity. Annu Rev Immunol. 2004;22:307-328.
11. Lee JR. Reactive oxygen species play roles on B cell surface receptor CD40-mediated proximal and distal signaling events: effects of an antioxidant, N-acetyl-L-cysteine treatment. Mol Cell Biochem. 2003;252:1-7.
12. Dedeoglu F, Horwitz B, Chaudhuri J, Alt FW, Geha RS. Induction of activation-induced cytidine deaminase gene expression by IL-4 and CD40 ligation is dependent on STAT6 and NF-κB. Int Immunol. 2004;16:395-404.
13. Krappmann D, Wegener E, Sunami Y, et al. The IκB kinase complex and NF-κB act as master regulators of lipopolysaccharide-induced gene expression and control subordinate activation of AP-1. Mol Cell Biol. 2004;24:6488-6500.
14. Heese K, Fiebich BL, Bauer J, Otten U. NF-κB modulates lipopolysaccharide-induced microglial nerve growth factor expression. Glia. 1998;22:401-407.
15. Heese K, Hock C, Otten U. Inflammatory signals induce neurotrophin expression in human microglial cells. J Neurochem. 1998;70:699-707.
16. Heese K, Beck KF, Behrens MH, et al. Effects of high glucose on cytokine-induced nerve growth factor (NGF) expression in rat renal mesangial cells. Biochem Pharmacol. 2003;65:293-301.
17. Henneberger C, Grantyn R, Rothe T. Rapid genotyping of newborn gene mutant mice. J Neurosci Meth. 2000;100:123-126.
18. Heese K, Yamada T, Nagai Y, Sawada T. CGI-94 controls neuronal survival. Brain Aging. 2002;2:44-48.
19. Brodie C, Oshiba A, Renz H, Bradley K, Gelfand EW. Nerve growth-factor and anti-CD40 provide opposite signals for the production of IgE in interleukin-4-treated lymphocytes. Eur J Immunol. 1996;26:171-178.
20. Nykjaer A, Lee R, Teng KK, et al. Sortilin is essential for proNGF-induced neuronal cell death. Nature. 2004;427:843-848.