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bFGF Upregulates the Expression of NGFR in PC12 Cells

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Abstract: The reciprocal and highly regulated processes of cellular proliferation, cellular differentiation, and progression to a postmitotic state during embryogenesis generate the cellular diversity in the developing nervous system. Growth factors, in part, can regulate these proliferative or differentiation processes by analogous mechanisms. Basic fibroblast growth factor (bFGF), a member of FGF family, has broad biological functions involving the regulation of cell growth, differentiation, and proliferation. As extension and remodeling of neurites play essential roles in development and neuronal plasticity, we investigated a role for nerve growth factor receptor (NGFR) on bFGF-induced neurite outgrowth in rat pheochromocytoma cell line, PC12. Our goal in the present study was to determine if there is a causal link between bFGF and NGFR. Results of these studies indicate that bFGF is required for NGFR-induced changes in morphology and transcriptional induction of the gene. We have provided convincing evidence that inhibitor of bFGF, PD173074, completely inhibited NGFR protein expression, whereas it partially blocked the NGFR protein expression in response to bFGF in PC12 cells. Another important finding of our study provides the data on the involvement of bFGF in MAPK-dependent signaling pathways and neurite outgrowth in PC12 cells, which suggests a central role of MAPK in the neuronal induction by bFGF. Taken together, these results raise the possibility that bFGF activates a MAPK-mediated pathway related to NGFR expression.

Key words: bFGF, NGFR, PC12

Introduction

Within the nervous system, individual neurons are exposed to many environmental signals, which influence their form and function. Prominent among these signals are growth factors and neurotransmitters1). These molecules exert their effects on a large spectrum of cells, comprising fibroblasts and endothelial cells and on nerve tissue including neurons and glial cells2).

Growth factors alter the expression of intrinsic neuronal genes involved in cell survival and regeneration3-4). Basic fibroblast growth factor (bFGF) is a member of the FGF family that has been shown to protect neurons against injury and degeneration5). bFGF increases long-term cell survival in frog retinal ganglion cells after optic nerve axotomy6). In vitro actions of bFGF on mesenchymal and neuroectodermal cells include induction of or increase in proliferation, mitogenesis in glial cells, the support of neuronal survival, the promotion of neurite outgrowth7,8), morphological changes, anchorage-independent growth, differentiation, delayed senescence, angiogenesis, neovascularization, tissue differentiation, regulation of tissue specific functions8), wound healing9), other regenerative processes, abnormal cell differentiation and growth9). These observations suggest that bFGF may be of importance for the development and maintenance of nervous system.

Given the complexity and extreme cellular diversity of the nervous system, the study of transformed neural crest-derived cell lines which can recapitulate many of the growth factor signaling events has been instrumental in defining the pathways resulting in neuronal differentiation and proliferation. The rat pheochromocytoma cell line, PC1211), which expresses receptors important for mitogenic signaling, such as the epidermal growth factor and insulin-like growth factor 1 receptors12,13), and those mediating cell differentiation and cessation of cell division, the nerve growth factor (NGF) and FGF receptors11,14), has uncovered many common pathways resulting in opposing biological effects15,16). In particular, NGF induction of PC12 cell differentiation involves activation of numerous enzymes that have been shown to be important for mitogenesis and transformation in other cell types. NGF promotes the survival and differentiation of sensory and sympathetic neurons17). NGF binds two receptors, TrkA and p75NTR, and induces neurite outgrowth in PC12 cells18). NGF promotes the
activation of p38 MAP kinase (MAPK) that is essential to neurite outgrowth in PC12 cells.\(^9,20\)

MAPK has been shown to be involved in bFGF-induced neuronal differentiation and neurite growth in embryonic rat hippocampal neurons\(^21-22\), with embryonic chick retinal neurons, have provided evidence for the role of this enzyme as a point of convergence of different pathways controlling neurite outgrowth. In addition, MAPK activation downstream of the FGFR has been shown to be involved in survival of different types of neurons in culture\(^23-26\).

Studies on the requirement for stimulation in some biological processes have provided deeper insight into the mechanisms of regulation in certain cell types. Recent studies have demonstrated that mesenchymal stem cells have the ability to differentiate into neurons\(^27\). Based on recent insights from stem cell research, we decided to study the process of proliferation and differentiation in PC12 cells, aiming to deduce the environmental components responsible for the development of chromaffin cell characteristics. Though bFGF may well have positive effects on each of precursor proliferation, neurogenic differentiation and neuronal survival, its interactions with NGFR are not at all understood. Our current experiments demonstrate that the expression of NGFR was increased in response to bFGF stimulation of PC12 cells. Furthermore, bFGF inhibitor suppressed the bFGF-induced NGFR expression, with obvious correlation with MAPK activation. These data suggest that NGFR transmits bFGF-dependent differentiation signals in PC12 cells.

**Materials and Methods**

**Reagents**

bFGF was purchased from Peprotech (Rocky Hill, NJ, USA). A specific inhibitor of bFGF, PD173034, was purchased from Calbiochem (San Diego, CA, USA). PC12 cell line was purchased from DS Pharma Biomedical Co., Ltd., Tokyo, Japan.

**Cell culture, induction of neurite outgrowth and proliferation assay**

PC12 cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated horse serum (HS) (Gibco), 5% heat-inactivated fetal bovine serum (FBS) (Gibco), and antibiotics (100 units/ml streptomycin and 100 units/ml penicillin) (Gibco) at 37°C under 5% CO\(_2\). To assess their differentiation, the cells were plated to a density of 5 x 10\(^4\) cells/well on collagen type IV-coated, 6 well culture plates (BD Biosciences, Bedford, MA, USA), maintained for 24 h, and incubated for 18 h in RPMI 1640 medium (Gibco) containing 1% HS, 0.5% FBS, and antibiotics (serum-starved media). Cells were treated with 5 ng/ml\(^19\) or 10 ng/ml bFGF in serum-starved media containing antibodies and 2 mg/ml BSA for 24 hours. The cells harboring above two-cell body lengths were evaluated to be positive for neurite outgrowth. For proliferation assay, the cell numbers were counted in triplicate assays with Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA). The absorbance was measured using a Microplate reader (iMark™, Bio-rad, Hercules, CA, USA).

**Quantitative Real-Time RT-PCR (QRT-PCR)**

Total cellular RNA was isolated using an RNeasy Mini Kit (QIAGEN KK, Tokyo, Japan). First-strand cDNA was synthesized from 1μg of total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA). One-hundredth aliquot of the cDNA was subjected to real-time RT-PCR using TaqMan Gene Expression Assays (Applied Biosystems) for NGFR, and Pre-Developed TaqMan Assay Reagents (Applied Biosystems) for ACTB as an internal control. Three independent measurements were averaged and relative gene expression levels were calculated as a ratio to ACTB expression of each cell.

**Western Blotting**

PC12 cells were lysed in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein concentration was determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). SDS-polyacrylamide gels were calibrated with molecular weight markers (Bio-Rad). NGFR (1:1000; Epitomics, Inc., Burlingame, CA, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000; Cell Signaling Technology, Danvers, MA, USA) were used as primary antibodies. Anti-rabbit secondary antibody (Cell Signaling Technology) was used at a dilution of 1:2000. Bound antibodies were visualized by chemiluminescence using the ECL Plus Western Blotting Detection System (Amersham, Upptala, Sweden), and images were analyzed by a Luminescent Image Analyzer (LAS 4000 mini; Fuji Film Inc., Japan). The experiment was repeated two times. Quantitative analysis of relative protein expression was calculated using Image J software.

**Statistical analyses**

Significant differences were analyzed by Fisher’s exact test. \(P<0.05\) was considered to be statistically significant.

**Results**

**The effect of bFGF on PC12 cells**

A preliminary screening was done to obtain the optimal concentration of bFGF using MTS assay in PC12 cells (Fig. 1). In line with the cytokine functions of bFGF, the data demonstrates that bFGF increased the cellular proliferation after 24 h exposure.

Because bFGF is essential for the neurite outgrowth of neuronal cells, we examined the effect of bFGF on the neurite outgrowth of PC12 cells. We have confirmed neurite extension upon bFGF stimulation. PC12 cells did not show a clear extension...
Figure 1. Effect of bFGF on PC12 cellular proliferation in a concentration dependent assay. PC12 cells were exposed to different concentrations of bFGF for 24 h and MTS assay was performed. Each bar indicates the mean ± SD. The experiments were repeated twice in triplicate samples; *P < 0.05, bFGF treatment vs. control.

Figure 2. Neutrite growth in bFGF-treated cells. Cells were cultured in the absence of serum for 18 h and then stimulated with bFGF for 24 h. bFGF induced morphological changes, including flattening of cell bodies and promotion of process outgrowth. Neutrite growth was analyzed by an inverted microscope. Representative images for control and bFGF-treated cells are shown. Scale bar, 100 μm.

Figure 3. bFGF is primarily responsible for the up-regulation of NGFR mRNA in PC12 cells. PC12 cells were exposed to bFGF (5ng/ml) for 24 h, and bFGF increased NGFR mRNA expression by real-time PCR. The experiments were repeated twice in triplicate samples. Each bar indicates the mean ± SD. The experiments were repeated twice in triplicate samples; *P < 0.05, bFGF treatment vs. control.

Discussion

The mechanism of cell protection by bFGF appears to involve direct receptor-mediated induction of signaling transduction pathways leading to the expression of neuroprotective genes and their products. bFGF plays an important role as a mitogen and neurotrophic factor for the progenitor cells of both the central and peripheral nervous system. bFGF was found to induce growth of neuritic processes in an immortalized cell line of rat sympathoadrenal progenitor cells. In rat sympathoadrenal progenitor cells, which have the ability to differentiate into adrenal chromaffin cells or sympathetic neurons, bFGF induces differentiation into sympathetic neurons, with a concomitant development of a requirement for NGF for survival. To understand interactions between the neurotrophin and bFGF, implicated in aspects of proliferation of neural precursors/stem cells, neurogenic differentiation and neuronal survival, we investigated the effect of bFGF on the up-regulation of NGFR involved in signaling transduction pathways.

PC12 is a clonal cell line of rat pheochromocytoma cells that respond to NGF by extending neurites and acquiring the appearance of neurons. The differentiation of PC12 cells by NGF involves...
striking morphological and biochemical changes including the induction of numerous proteins required for the acquisition of a differentiated phenotype similar to that of a sympathetic neuron\textsuperscript{35}. Therefore, PC12 cells are frequently used as a model system for investigating nerve terminal events. In the present study, we investigated the regulatory mechanism of NGFR expression by bFGF in PC12 cells to clarify its physiological relevance. Our data showed that the changes in levels of NGFR alters neurite outgrowth following treatment with bFGF in PC12 cells. Photomicrographs clearly indicate major differences in the extent of neurite outgrowth in PC12 cells treated with bFGF. Similar changes in total free neurite length were seen in PC12 cells with altered NGFR levels following treatment with bFGF, which is known to activate a similar signaling pathway to NGF\textsuperscript{36-38}. These data are consistent with prior work showing that bFGF promotes branching neuritogenesis\textsuperscript{39} and survival in dissociated, cultured, rat hippocampal neurons\textsuperscript{40}. We extended these results to look for a correlation between levels of NGFR and the extent of neurite outgrowth. In our initial screen of NGFR expression levels in PC12 cells, bFGF increased NGFR protein expression and the outgrowth. In our initial screen of NGFR expression levels in PC12 cells, bFGF increased NGFR protein expression and the...
as playing a key role in regulating neurite outgrowth in PC12 cells. It will be important to determine whether a similar role can be attributed to bFGF in vivo. Further study of downstream signaling pathways activated by bFGF may give deeper insights into the mechanisms behind biological outcomes that are induced by differentiation factors.

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