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Nerve Growth Factor Down-regulates the Expression of Chemokine Receptors in Rat PC12 Cells

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ABSTRACT

Nerve growth factor (NGF) is the classical inducer of neuronal differentiation. PC12 cells provided a model system of neuronal differentiation by NGF. Genes associated with the acquisition of a neuronal phenotype in PC12 cells need to be further explored. There has been growing interest in the role of chemokine and chemokine receptors in central nervous system development and disease. In this study, we determined the changes in mRNA expression of chemokine receptors by NGF treatment in PC12 cells. Using a real-time reverse transcriptase polymerase chain reaction assay, we demonstrated that the amounts of CCR3, CCR5, CCR7, and CXCR4 mRNAs were reduced in response to NGF. Although it is unclear which aspects of PC12 cell differentiation they control, expression of the chemokine receptors may have a negative effect on development of the neuronal phenotype and their down-regulation is potentially important in neuronal differentiation. This study also suggests that broader neurophysiological activities of chemokine receptors deserve to be investigated.

Key words: Nerve growth factor, chemokine receptors, mRNA expression, real-time PCR, PC12 cells

INTRODUCTION

Studying the pathway of neural cell differentiation is a key to understanding normal brain development and advancing neural cell transplantation therapy as well. Neurotrophic factors play a pivotal role in neuronal survival and plasticity. Nerve growth factor (NGF) is well known for inducing neuronal differentiation in rat pheochromocytoma cell line PC12, which provides a model system. PC12 cells differentiate into sympathetic neuron-like cells in response to NGF, which is characterized by growth arrest, the elaboration of long, branching neurites, and

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electrical excitability (Green and Tischler, 1976). Several studies have described the global change of differentially expressed genes in PC12 cells before and after NGF treatment (Brown et al., 1999; Lee et al., 1995). However, the underlying mechanisms of neuronal differentiation in PC12 cells are still largely unidentified.

Chemokines were initially recognized to control leukocyte communication and migration, which is especially important in inflammatory responses. Chemokines play crucial roles in immune system functions, angiogenesis and tumor progression (Gerard and Rollins, 2001). Recent studies have revealed that some chemokines and chemokine receptors are also expressed in the central nervous system (CNS) and their expression is increased in neuroinflammatory and neurodegenerative conditions such as multiple sclerosis, brain tumors, Alzheimer's disease (AD), and human immunodeficiency virus (HIV)-related dementia (Bajetto et al., 2002). Moreover, these molecules are of great importance in directing the proliferation and migration of multipotent progenitor cells during the development of the nervous system (Zou et al., 1998; Ma et al., 1998; Lu et al., 2002; Tsai et al., 2002). Chemokine receptor signaling modulates synaptic activity in the brain (Bajetto et al., 2002; Tran and Miller, 2003). Besides these known functions, chemokines and their receptors are likely to be involved in other important neurophysiological functions. In this study, we investigated whether and which chemokine receptors are implicated in neuronal differentiation by performing real-time reverse transcriptase PCR analysis of mRNA expression profiles in PC12 cells cultured in the absence or presence of NGF.

MATERIALS AND METHODS

PC12 cells, rat pheochromocytoma cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) and 10% horse serum (Invitrogen, Gaithersberg, MD). Cells were plated on poly-D-lysine-coated plates and grown for 24 h. For neuronal differentiation, cells were treated with NGF (100 ng/ml; R&D Systems, Minneapolis, MN) for 5 d.

Total RNA was extracted by using Trizol reagent (Invitrogen) and treated with RNase-free DNase (Promega, Madison, WI). The purity of RNA was assessed by spectrophotometer, and the integrity of RNA was verified by agarose gel electrophoresis. DNase-treated RNA was subjected to PCR analysis, as described below, using primers to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) to ensure that each RNA preparation was free of DNA. Independent RNA preparations from different sets of experiments were used for replicate experiments.

RNA (1μg) was reverse-transcribed in a 20μl reaction mixture using Moloney murine leukemic virus

Table 1. Primers and expected sizes of PCR products with each primer pair

Gene		Primer	Size (bp)
GAPDH	Sense	5'-atcccatcaccatcttccag-3'	579
	Antisense	5'-cctgcttcaccaccttcttg-3'	
CCR3	Sense	5'-ggcatccaacgaagaggaactcaa-3'	372
	Antisense	5'-atctcgctgtacaaggccaggtaa-3'	
CCR5	Sense	5'-aacctggccatctctgacctg-3'	431
	Antisense	5'-gtagcagatgaccatgac-3'	
CCR7	Sense	5'-acagcggcctccagaagaacagcgg-3'	445
	Antisense	5'-tgacgtcataggcaatgttgagctg-3'	
CXCR4	Sense	5'-catgacagacaagtaccggct-3'	475
	Antisense	5'-caggataaggatgaccgtagt-3'	
Tβ4	Sense	5'-tgtacattccacgagcattgcctt-3'	246
	Antisense	5'-ctctcaattccaccatctcccacc-3'	
ATP1B1	Sense	5'-ggaagccctgtatcattatcaagctc-3'	194
	Antisense	5'-gaatccgcccatcccaaagtactc-3'	

Tβ4, thymosin β4; ATP1B1, Nα+/K+ATPase β1

reverse transcriptase (Invitrogen). The cDNA was amplified using Top-Taq Premix kit (CoreBio Lifescience & Biotech, Seoul, South Korea), gene specific primers, and SYBR Gold (Molecular Probes, Eugene, OR). The real-time PCR was performed in the Rotor-Gene 3000 (Corbett Research, Sydney, Australia) using 0.2-ml capped tubes. The cycle profile was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 10 sec, and 72°C for 20 sec. As a control of sample loading and normalization between samples, PCR amplification of the housekeeping gene GAPDH was included for each sample at each run. Fluorescence measurements were obtained online and analyzed with the version 6.0 software (Corbett Research). To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis. The relative quantification of gene expression was computed by using the comparative C_t (threshold cycle) method with a mathematical formula as previously described (Pfaffl et al., 2002). The primer sequences specific for the genes examined and predicted product sizes are shown in Table 1.

RESULTS AND DISCUSSION

One of the dramatic aspects of NGF-mediated PC12 differentiation is the neurite outgrowth (Green and Tischler, 1976). Consistently, PC12 cells treated with NGF for 5 d exihibited extensive neurite outgrowth (Fig. 1A, B). cDNAs were prepared from

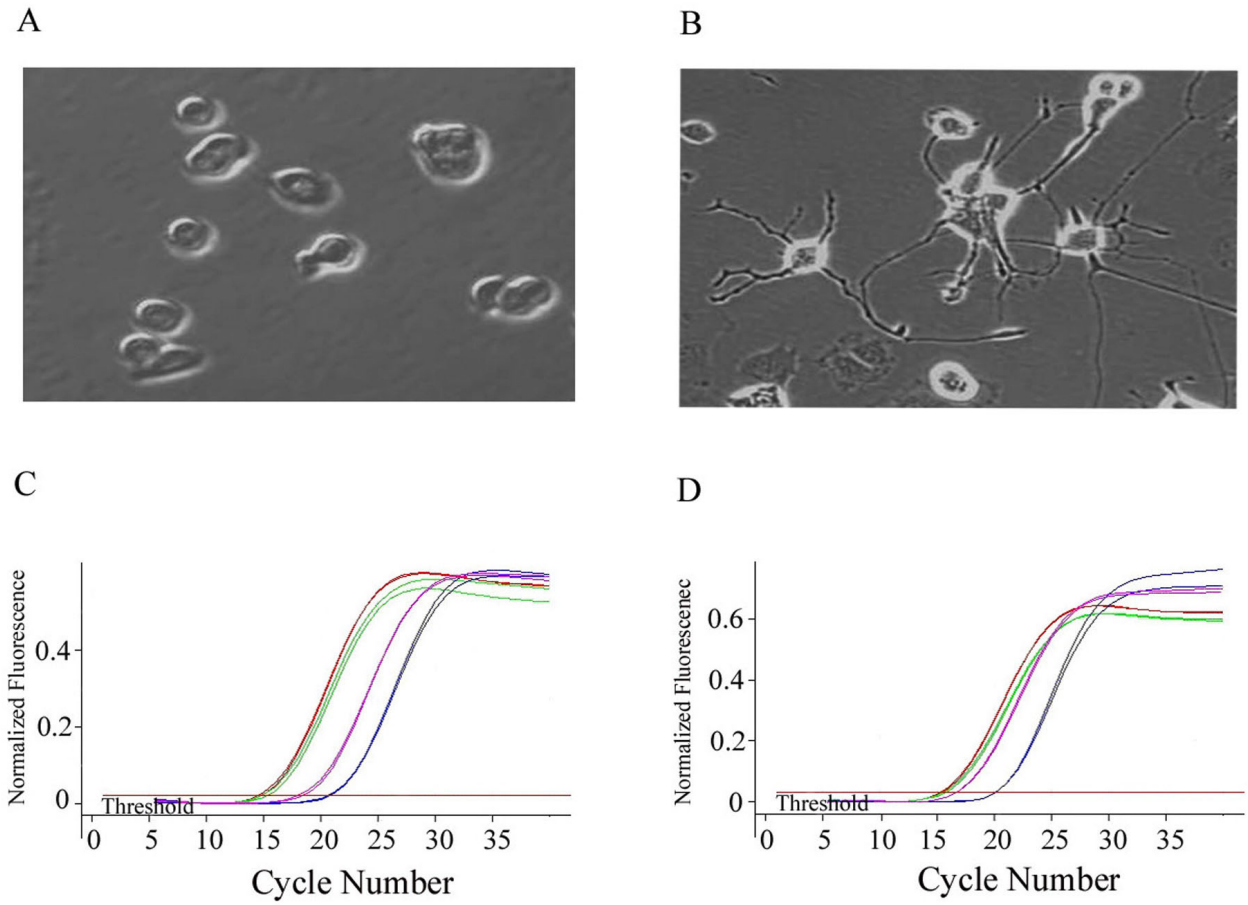

Fig. 1. Neuronal characteristics of PC12 cells incubated with NGF. Photomicrographs of PC12 cells cultured in the absence (A) or presence (B) of NGF (100 ng/ml) for 5 days. The real-time PCR analysis for thymosin β4 (C) and Na+/K+ ATPase β1 (D). The y-axis represents the fluorescence intensity and the x-axis the PCR cycle number. There was a 7.3-fold and 16.7-fold increase in thymosin β4 and Na+/K+ ATPase β1 expression, respectively, in NGF-treated cells as calculated from the average threshold cycle values. The target gene expression was normalized by a housekeeping gene expression (GAPDH). The samples were performed in duplicate. Red, control GAPDH; green, NGF-treated GAPDH; blue, control target; pink, NGF-treated target.

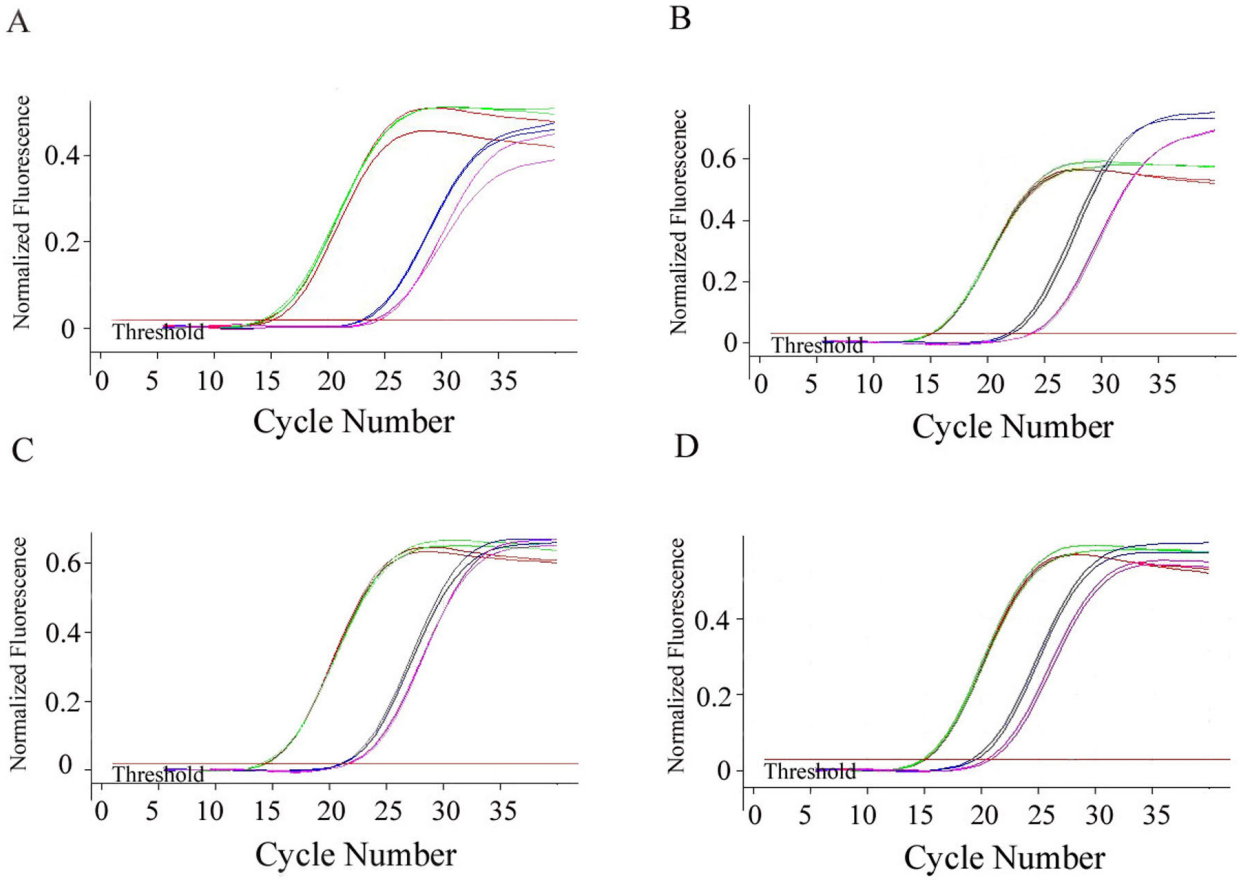

Fig. 2. NGF-mediated decrease in chemokine receptor mRNA levels in PC12 cells. RT/real-time PCR analyses for CCR3 (A), CXCR4 (B), CCR5 (C) and CCR7 (D). After NGF treatment, there were 3.5-, 4.2-, 1.8-, and 2.7-fold decreases in CCR3, CXCR4, CCR5, and CCR7 expression, respectively. Red, control GAPDH; green, NGF-treated GAPDH; blue, control target; pink, NGF- treated target.

these NGF-treated and untreated cells and analyzed by the real-time PCR analysis. To validate neuronal nature of cells we obtained from NGF treatment, we tested for the induction of thymosin β4 and Na+/K+ ATPase β1 peptide which have been known to be increased in NGF-treated PC12 cells (Leonard et al., 1987; Angelastro et al., 2000). Our real-time PCR results indicated that the level of thymosin β4 and Na+/K+ATPase β1 peptide increased significantly in NGF-treated cells (Fig. 1C, D). Chemokines and their receptors are classified into four groups, XC, CC, CXC, and CX3C, based on the configuration of their N-terminal cysteines (Rossi and Zlotnik, 2000). Our real-time PCR showed that the levels of CCR3, CCR5, CCR7, and CXCR4 mRNAs were decreased after culture with NGF for 5 d (Fig. 2). The expression of these mRNAs was similarly decreased in cells treated with NGF for 1 d (data not shown). The mRNAs for CCR1, CXCR1, CXCR2 and CXCR3 were barely detected in both untreated and NGF-treated PC12 cells (data not shown). Although the roles of altered chemokine receptors in neuronal differentiation are unidentified, the results overall extend the list of NGF-modulated genes in PC12 cells, and for the first time highlight possible implications of certain chemokine receptors in NGF-driven neuronal changes.

Neuronal expression of several chemokine receptors, including CCR3, CCR5, and CXCR4, has been reported in the human brain (Lavi et al., 1998). These receptors are involved in pathogenesis of HIV-associated neurological disease as co-receptors of CD4 for HIV infection (Klein et al., 1999). Further, up-regulation of these receptors has been found associated with AD pathological changes (Xia and Hyman, 1999). The role of CCR7 in the CNS has not been well characterized. Among chemokine receptors expressed in the brain, CXCR4 attracted

much attention. From studies with knockout mice, CXCR4 deficiency causes abnormally developed cerebellum and hippocampal dentate gyrus characterized by an altered migration of progenitor cells (Zou et al., 1998; Ma et al., 1998; Lu et al., 2002). In the absence of CXCR4, the number of dividing cells in the migratory stream and in the dentate gyrus itself is reduced, and cells differentiate prematurely before reaching their final position (Lu et al., 2002). CXCR4 mRNA expression in mouse brain is prominent in sites of neuronal and progenitor cell proliferation, and is progressively down-regulated postnatally (Tissir et al., 2004). Microarray analysis revealed that CXCR4 is expressed in neural stem cells but not detected in neuronal restricted progenitor cells in rat embryos (Luo et al., 2002). Based on these and other reported effects of chemokines in the developing nervous system, signaling through chemokine receptors is considered to ensure the continued proliferation of progenitor cell populations (Tran and Miller, 2003). Taken in the context of these reports, our findings support that general down-regulation of the chemokine receptors may play an important role in dropping out of proliferation and entering differentiation phase.

Other than chemoattractic roles in tissue positioning, scarce evidence has been given of the physiological roles of chemokine receptors in the CNS. In this regards, our results may give an insight into a previously uncharacterized mechanism of chemokine receptor action in acquiring neuronal phenotypes. Demonstration of their functional roles in PC12 cell differentiation will provide a more comprehensive view of the mechanisms involved in differentiation into neuronal cells.

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REFERENCES

Angelastro JM, Klimaschewski L, Tang S, Vitolo OV, Weissman TA, Donlin LT, Shelanski ML and Greene LA (2000) Identification of diverse nerve growth factor-regulated genes by serial analysis of gene expression (SAGE) profiling. *Proc Natl Acad Sci USA* 97:10424-10429.

Bajetto A, Bonavia R, Barbero S and Schettini G (2002) Characterization of chemokines and their receptors in the central nervous system: physiopathological implications. *J Neurochem* 82:1311-1329.

Brown AJH, Hutchings C, Burke JF and Mayne LV (1999) Application of a rapid method (targeted display) for the identification of differentially expressed mRNAs following NGF-induced neuronal differentiation in PC12 cells. *Mol Cell Neurosci* 13:119-130.

Gerard C and Rollins BJ (2001) Chemokines and disease. *Nat Immunol* 2:108-115.

Greene LA and Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA* 73:2424-2428.

Klein RS, Williams KC, Alvarez-Hernandez X, Westmoreland S, Force T, Lackner AA and Luster AD (1999) Chemokine receptor expression and signaling in macaque and human fetal neurons and astrocytes: implications for the neuropathogenesis of AIDS. *J Immunol* 163:1636-1646.

Lavi E, Kolson DL, Ulrich AM, Fu L and Gonzalez-Scarano, F (1998) Chemokine receptors in the human brain and their relationship to HIV infection. *J Neurovirol* 4:301-311.

Lee NH, Weinstock KG, Kirkness EF, Earle-Hughes JA, Fuldner RA, Marmaros S, Glodek A, Gocayne JD, Adams MD, Kerlavage AR, Fraser CM and Venter JC (1995) Comparative expressed-sequence-tag analysis of differential gene expression profiles in PC-12 cells before and after nerve growth factor treatment. *Proc Natl Acad Sci USA* 92:8303-8307.

Leonard DGB, Ziff EB and Greene LA (1987) Identification and characterization of mRNAs regulated by nerve growth factor in PC12 cells. *Mol Cell Biol* 7:3156-3167.

Lu M, Grove EA and Miller RJ (2002) Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. *Proc Natl Acad Sci USA* 99:7090-7095.

Luo Y, Cai J, Liu Y, Xue H, Chrest FJ, Wersto RP and Rao M (2002) Microarray analysis of selected genes in neural stem and progenitor cells. *J Neurochem* 83:1481-1497.

Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT and Springer TA (1998) Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci USA* 95:9448-9453.

Pfaffl MW, Horgan GW and Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30:e36.

Rossi D and Zlotnik A (2000) The biology of chemokines and their receptors. *Annu Rev Immunol* 18:217-242.

Tissir F, Wang CE and Goffinet AM (2004) Expression of the chemokine receptor Cxcr4 mRNA during mouse brain development. *Dev Brain Res* 149:63-71.

Tran PB and Miller RJ (2003) Chemokine receptors: signposts to brain development and disease. *Nat Rev Neurosci* 4: 444-455.

Tsai HH, Frost E, To V, Robinson S, Ffrench-Constant C, Geertman R, Ransohoff RM and Miller RH (2002) The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting

their migration. *Cell* 110:373-383.

Xia MQ and Hyman BT (1999) Chemokines/chemokine receptors in the central nervous system and Alzheimer's disease. *J Neurovirol* 5:32-41.

Zou YR, Kottmann AH, Kuroda M, Taniuchi I and Littman DR (1998) Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 393: 595-599.