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# Expression of Neuregulins and Their Receptors During the Differentiation of Rat Hippocampal HiB5 Cells

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**Differentiating HiB5 cells, a rat hippocampal cell line, expressed neuregulins and showed constitutive activation of a neuregulin receptor, ErbB2, suggesting development of a neuregulin autocrine loop. RT-PCR analyses indicated that HiB5 cells produced SMDF and NDF, but not GGF, during the differentiation. None of neuregulin isoforms were detected in proliferating HiB5 cells. The neuregulins in HiB5 cells, at least in part, are the β-isoforms of which the most of neuronal neuregulin isoforms are. The expression of SMDF and NDF was enhanced by PDGF and bFGF that promote cell survival and differentiation, suggesting a close relationship between the synthesis of neuregulins and the differentiation process. HiB5 cells have ErbB2 and ErbB4, but not ErbB3 receptors. Constitutive tyrosine phosphorylation of ErbB2 was detected in HiB5 cells that had not been exposed to exogenous GGF.**

The proper function of nervous system depends on precise interconnections of millions of neurons originated from proliferating neural stem cells during the developmental process. Following the guides of external cues, neural stem cells leave the cell cycle, migrate into the proper places, and differentiate into neurons. The survival of differentiating and terminally differentiated neurons depends also on factors provided from surrounding cells. Therefore, many studies have been devoted to elucidate the identity and the function of stem cell-fate determining factors or target-derived neuronal survival factors. Several polypeptide growth factors such as neurotrophic factors and other growth factors have been identified as soluble factors influencing many aspects of the development of nervous system.

Neuregulins (NRG, also known as neu differentiation factor (NDF), acetylcholine receptor inducing activity (ARIA), glial growth factor (GGF), or heregulins) are a group of polypeptide growth factors that is widely expressed in the developing heart and nervous systems (Orr-Urtreger et al., 1993; Meyer & Birchmeier, 1994; Meyer et al., 1997). In the heart, neuregulins are expressed in mesenchymal cells, where they mediate mesenchyme-epithel interactions. The function of neuregulins is essential for the development of heart since NRG-/- embryos fail to form ventricular trabeculae and die in mid-gestation (Meyer & Birchmeier, 1995). In neurons, neuregulins are secreted from the presynaptic terminals in an activity-dependent manner and regulate the expression of several genes in the target cells. Neuregulins enhance the expression of nicotinic acetylcholine receptor (AchR) (Martinou et al., 1991; Duclert et al., 1996; Burden, 1998) and voltage-gated sodium channels (Corfas & Fischbach, 1993) of muscle cells. The promotion of the expression of the N-methyl-D-aspartate (NMDA) receptor NR2C subunits in cerebellar granule cells (Ozaki et al., 1997) and the neuronal AchR α7 subunit in the superior cervical ganglia (Yang et al., 1998) by neuregulins was also reported. In both cases, neuregulins augment nerve-muscle or nerve-nerve interactions. In addition, neuregulins play as a local signaling molecule to promote survival or differentiation of neighboring cells. For example, neuregulins promote maturation of astroglia and Schwann cells (Pinkas-Kramarski et al., 1994) as well as oligodendrocytes (Vartanian et al., 1994) in a paracrine manner. Neuregulins also facilitate myogenic differentiation or muscle fiber survival in autocrine manners (Moscoso et al., 1995; Trachtenberg, 1998; Kim et al., 1999).

Alternative splicing generates dozens of neuregulin isoforms from a single gene located on human chromosome 8. Three major classes of neuregulins have been characterized by distinct amino terminals. The neu differentiation factor (NDF; also called as heregulin or ARIA) belongs to the type 1, glial growth factor (GGF) to the type 2, and sensory and motor neuron-derived factor (SMDF; n-ARIA) to the type 3 (Marchionni et al., 1993; Carraway & Burden, 1995 for review). All the types have the EGF-like domain in common, but the types 1 and 2 have the immuno-

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globulin-like domain in addition. They can be also classified into two variants: α and β depending on the variable segments of their EGF-like domain. Neuregulin α-isoforms are found mostly in non-neuronal cell types and neuregulin β-isoforms in neuronal cell types.

The effect of neuregulin is mediated by ErbB2, ErbB3, and ErbB4, the members of a subfamily of receptor tyrosine kinases (Carraway & Burden, 1995 for review). All of the neuregulin isoforms have a EGF-like domain that directly binds to ErbB3 and ErbB4 receptors, but not to ErbB2 receptor. Since ErbB3 receptor is devoid of catalytic function, upon ligand binding, heterodimerization of ErbB3 with ErbB2 that has a high kinase activity leads to the initiation of intracellular signaling events (Tzahar et al., 1996). ErbB4 with a tyrosine kinase activity forms homo- or heterodimer with ErbB2.

HiB5 cells, a neuronal precursor cell line of hippocampus, was established by immortalizing the primary cultured cells of rat embryonic hippocampus by a temperature-conditional SV40 large T antigen (Renfranz et al., 1991). They have a stem cell marker, nestin intermediate filament, and differentiate into neuronal and glial cells in vivo when implanted into rat brain. At a permissive temperature of 32℃, these cells proliferate. When the temperature is shifted to 39℃, they lose the precursor properties and acquire morphological and biochemical characteristics of differentiated cells. During this process, many cells do not survive but undergo programmed cell death. The survival and neurite outgrowth of these neuronal precursor cells are facilitated by locally supplied growth factors such as bFGF and PDGF BB, probably through the increased expression of BDNF of target cells (Renfranz et al., 1991; Kwon, 1997).

In order to investigate whether neuregulins participate as stem cell-fate determining factors or survival factors during differentiation of hippocampal stem cells, the expressions of neuregulin isoforms and their receptors were examined during differentiation of HiB5 cells. SMDF and NDF but not GGF became expressed as HiB5 cells preceded the differentiation process, and the expression of neuregulin was stimulated by bFGF and PDGF BB, the growth factors facilitating the survival and neurite outgrowth of HiB5 cells. HiB5 cells also expressed ErbB2 and ErbB4, but not ErbB3. Constitutive activation of ErbB2 was also detected. These results provide evidence for the existence of neuregulin autocrine loop during the differentiation of HiB5 cells.

## Materials and Methods

### *Cell culture*

Immortalized cell line HiB5 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.11 g/L sodium pyruvate, 3.7 g/L $NaHCO_3$, 0.39 g/L HEPES, penicillin, streptomycin, and 10% (v/v) fetal calf serum (Sigma). The cells were maintained at the permissive temperature, 32℃,in 5% $CO_2$. The cell differentiation was induced by switching the medium to the chemically defined media N2 (GibcoBRL) and incubation at the non-permissive temperature, 39℃. When required, PDGF or bFGF were treated at the concentrations of 30 ng/ml or 5 ng/ml, respectively.

### *Immunoprecipitation and Immunoblotting analysis*

Cells were washed twice with ice cold PBS, and then lysed with NP40-Tris buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, and 1% NP40) containing protease inhibitors (10 μg/ml aprotinin, 20 μM leupeptin, and 1 mM PMSF) and phosphatase inhibitors (1 mM sodium orthovanadate, 4 g/L sodium fluoride, and 8.8 g/L sodium pyrophosphate decahydrate). The lysate was incubated for 20 min on ice and precleared by centrifugation. Proteins in the lysate were resolved on SDS-PAGE and subjected to immunoblotting analysis using an enhanced chemiluminescence method. The anti-ErbB2 antibody was purchased from Zymed Lab., the anti-ErbB3 antibody (C-17) and the anti-ErbB4 antibody from Santa Cruz Biotechnology.

To measure the tyrosine-phosphorylation of ErbB2, the precleared lysates were incubated with the anti-ErbB2 antibody (Oncogene Sci.) for 2 h at 4℃, followed by addition of 50 μl of protein-A Sepharose beads (10% (v/v) suspension). After incubation for 1 h at 4℃, the beads were washed three times with NP40-Tris buffer. Bound proteins were subjected to immunoblotting analysis using an anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). To investigate the effect of GGFII on the tyrosine phosphorylation of ErbB2, GGFII (5 ng/ml) was treated to HiB5 cells for 5 or 10 min before lysis.

### *RNA analysis by RT-PCR*

RNA was isolated from HiB5 cells using acid-phenol method at the proper time intervals. First-strand synthesis was performed on 1 μg of RNA in a final volume of 20 μl using random primers and reverse transcriptase. One microliter of the sample was subjected to PCR using the proper primer sets. The specific primer sets used are GGF pair (1187, 5'-AACCTCAAGAAGGAGGTCAG-3'; 1165, 5'-CCATTCACACAGAAAGTTTT-3'), SMDF pair (1445, 5'-GCATACACTTCACCTGTCTC-3'; 1165, 5'-CCATTCACACAGAAAGTTTT-3'), NDF pair (1440, 5'-TGTGCCGAGAAGGAGAAAACTTT-3'; 1165, 5'-CTCACTGAATGAGGTTCTCCTC-3'), NRG pair (663, 5'-TCTGGAGAGTATATGTGCAAAGTGATCAGC-3'; 1114, 5'-GCAGTAGGCCACCACACACATGATGCC-3'), and NRGβ pair (1442, 5'-TGTGCCGAGAAGGAGAAAACTTT-3'; 1447, 5'-CTGAGTTTTGGCAACGATC-3').

### *MTT assay*

Cells cultivated in a 35 mm culture dish were incubated

with 1.5 ml of MTT solution (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide, 0.5 mg/ml in the culture medium) at 37℃ for 4 h. After the MTT solution was removed, the cells were incubated with 1 ml of solubilization buffer (10% SDS and 50% dimethyformaldehyde, pH 4.7) at 37℃ for 24 h under dark condition. Cell lysates were transferred to a 96 well plate and $OD_{595}$ was read in ELISA reader.

## Results

The expression pattern of neuregulin isoforms was investigated during differentiation of HiB5 cells. The differentiation of HiB5 cells was induced by incubation at the nonpermissive temperature (39℃) in the serum free-N2 media for 2 d. The HiB5 cells in this condition left the cell cycle and underwent the differentiation process. During this process, however, majority of the cells died through a process known as apoptosis. The cells survived showed the extension of neurite and expressed the neuronal cell markers such as neurofilament, GAP43, and beta-tubulin type III (data not shown). Total RNAs from the proliferating HiB5 cells and the differentiated HiB5 cells were prepared, and examined the expression of GGF, SMDF, and NDF by

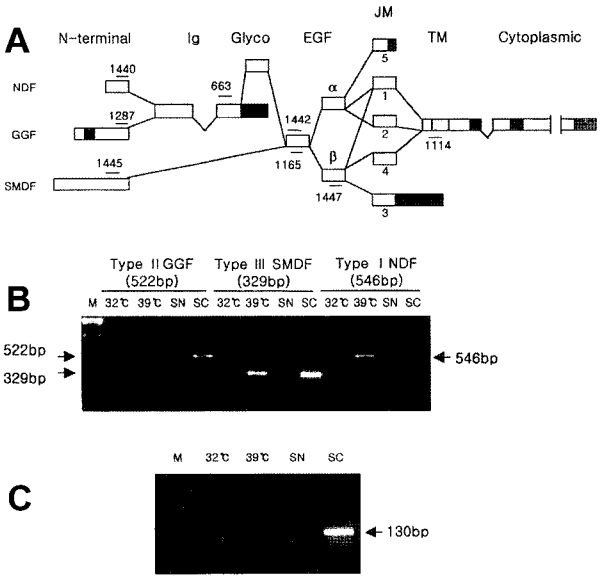


**Fig. 1.** RT-PCR analysis of neuregulin mRNA in HiB5 cells. A, A diagram showing neuregulin splicing variants as in Marchionni et al. (1993). The location of 5' (upstream) primers specific to NDF (1440), GGF (1287), SMDF (1445), NRGβ (1442), and NRG (663) are shown on top of the boxes denoting exons. The location of 3' (downstream) primers specific to NDF/GGF/SMDF (1165), NRGβ (1447), and NRG (1114) are shown on below of the boxes denoting exons. B, RT-PCR products of RNA extracted from the proliferating HiB5 cells (32℃), the differentiating HiB5 cells (39℃), sciatic nerves (SN), and spinal cord (SC). RT-PCR with the primers of GGF pair, SMDF pair, or NDF pair was expected to give a 522 bp, 329 bp, or 546 bp product, respectively. M, size markers. C, RT-PCR was performed with RNA extracted from the proliferating HiB5 cells (32℃), the differentiating HiB5 cells (39℃), sciatic nerves (SN), and spinal cord (SC). The 3' primer specific to the β-exon (1447) was employed in order to get RT-PCR product only from β-isoforms. RT-PCR with the primers of 1442 and 1447 was expected to give a 130 bp product. M, size markers.

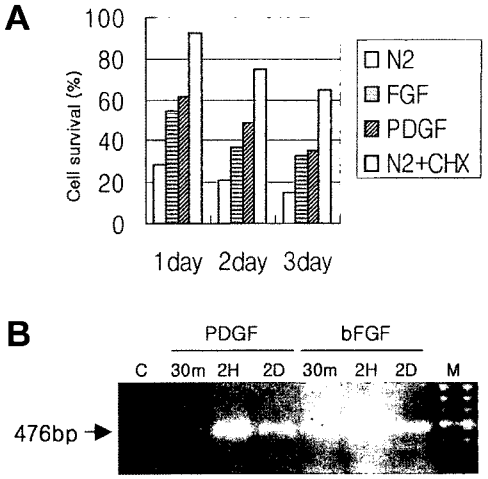


**Fig. 2.** Expression of neuregulins in HiB5 cells was promoted by PDGF or bFGF. A, Survival values of HiB5 cells at the differentiation condition, the incubation at 39℃ in the serum free-N2 media, relative to the proliferation condition. Cell survival was measured by MTT assay. N2, N2 media only; FGF, N2 media plus 5 ng/ml bFGF; PDGF, N2 media plus 30 ng/ml PDGF; N2+CHX, N2 media plus 100 nM cycloheximide. B, Promotion of the neuregulin mRNAs by PDGF or bFGF measured by RT-PCR. At the differentiation condition, PDGF (30 ng/ml) or bFGF (5 ng/ml) was added to N2 media. At the indicated time points, RNA was extracted and RT-PCR was performed with the primers of NRG pair that amplify NDF and GGF isoforms. RT-PCR product was expected to be 476 bp. C, before the addition of PDGF or bFGF; 30 m, 30 min; 2H, 2 h; 2D, 2 d; M, size markers.

RT-PCR (Fig. 1B). The expressions of neuregulin isoforms in sciatic nerve and spinal cord were also compared. All of the neuregulin isoforms examined were not expressed in the proliferating HiB5 cells. On the other hand, the expressions of SMDF and NDF were observed in the differentiated HiB5 cells. GGF was not expressed in the differentiated HiB5 cells. All three isoforms were expressed in spinal cord but not in sciatic nerve. When RT-PCR was performed with the primer pair specific to the β-exon of neuregulins, the 130 bp amplified product was observed in the differentiated HiB5 cells, but not in the proliferating HiB5 cells (Fig. 1C). As expected, spinal cord, but not sciatic nerve, expressed neuregulin β-isoforms.

As mentioned before, majority of HiB5 cells died in the differentiation condition through apoptosis (more than 80% after 2 d at 39℃ in the serum free-N2 media), which could be suppressed by cycloheximide (Fig. 2A). In this condition, the presence of growth factors such as bFGF and PDGF increased cell survival (Fig. 2A) and also promoted the differentiation (Kwon, 1997). In order to examine whether the expressions of neuregulin isoforms respond to bFGF and PDGF in the differentiation condition, the levels of neuregulin mRNA at 30 min, 2 h, and 2 d after the addition of bFGF or PDGF were compared by RT-PCR (Fig. 2B). In this experiment, the NRG primer pair recognizing both NDF and GGF was employed. Neuregulin mRNA significantly increased at 2 h after the addition of bFGF or PDGF and returned to the control level after 2 d. Taken together, these results indicate

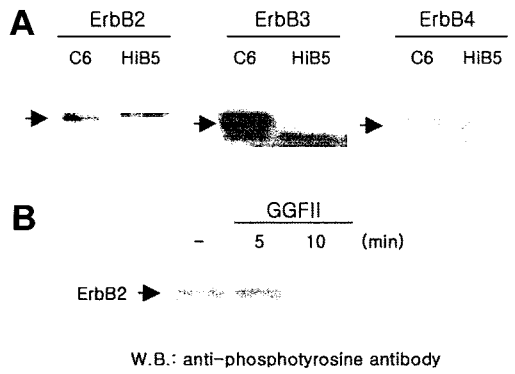


**Fig. 3.** Constitutive activation of ErbB2 in HiB5 cells. A, Expression of ErbB receptors in HiB5 cells. Total cell extracts of C6 glioma cells (C6) or HiB5 cells (HiB5) was analyzed by immunoblot with an anti-ErbB2 (ErbB2), an anti-ErbB3 (ErbB3), or an anti-ErbB4 (ErbB4) antibody. Differentiation of HiB5 cells was induced for 2 d before the analysis. B, Activation of ErbB2 receptors expressed in HiB5 cells. ErbB2 in the extracts of HiB5 cells was immunoprecipitated with an anti-ErbB2 antibody. Phosphorylation levels of ErbB2 were then determined by immunoblot with an anti-phosphotyrosine antibody 4G10. GGFII (5 ng/ml) was treated for 5 or 10 min before lysis. −, before the addition of GGFII.

that the differentiated HiB5 cells express SMDF and NDF, and their expressions can be enhanced by bFGF or PDGF that promote cell survival and differentiation.

In several instances, neuregulins act in an autocrine manner. In order to examine this possibility, the expressions of ErbB2, ErbB3, and ErbB4 were examined by immuno-blotting analysis. ErbB2 and ErbB4, but not ErbB3, were expressed in HiB5 cells (Fig. 3A). In order to test whether neuregulin receptors in HiB5 cells are functionally active, ErbB2 was immunoprecipitated with an anti-ErbB2 antibody from the total cell lysate of HiB5 cells, and the phosphorylation of tyrosine residues of ErbB2 was examined by immuno-blotting analysis with an anti-phosphotyrosine antibody (Fig. 3B). The tyrosine- phosphorylation of ErbB2 was observed even before the addition of GGFII. The addition of GGFII from the external source did not considerably increase the phosphorylation of ErbB2. The neuregulin isoforms synthesized from HiB5 cells are likely to be responsible for the constitutive phosphorylation of ErbB2, and it suggests that some autocrine loop of neuregulin may exist in the differentiated HiB5 cells.

## Discussion

In this study, an autocrine loop of neuregulin was proposed to develop during differentiation of hippocampal embryonic stem cell HiB5. The HiB5 cells synthesized SMDF and NDF when they differentiated. Before the differentiation, none of neuregulin isoforms was expressed. The increase of neuregulin mRNA in response to PDGF or bFGF that promote the survival and differentiation of HiB5 cells further supports the close relationship between the differentiation process and the expression of neuregulins.

The HiB5 cells have ErbB2 and ErbB4, but not ErbB3. Therefore, homodimerization of ErbB4 or heterodimerization of ErbB2/ErbB4 upon binding of neuregulin with ErbB4 may lead to the initiation of intracellular signaling. The phosphorylation of tyrosine residues of ErbB2 of HiB5 cells proposes that ErbB2 is functionally active. Interestingly, the phosphorylation of ErbB2 was detected even before the addition of GGFII and was not largely increased by GGFII either. The constitutive activation of ErbB2 is consistent with the finding that HiB5 cells synthesize neuregulins by themselves. Taken together, these results suggest that an autocrine loop of neuregulin develops during the differentiation of HiB5 cells. The function of this autocrine loop remains to be determined. In the preliminary experiment, the addition of GGFII did not increase the survival of HiB5 cells during differentiation. It will be interesting to see whether blocking of ErbB receptors lead to impairment of the survival or the differentiation of HiB5 cells.

There are several instances where neuregulins act in an autocrine manner. Schwann cells secrete SMDF/n-ARIA and NDF (Rosenbaum et al., 1997). ErbB2 and ErbB3 are expressed in Schwann cells and the proliferation and the survival of Schwann cells are enhanced by GGF and NDF. Furthermore, mitogenic responses of Schwann cells to bFGF, HGF, and TGF-1β were suppressed by neuregulin-neutralizing antibodies, supporting the role of neuregulin autocrine loop in Schwann cell responses to other growth factors. During myogenesis of rat L6 cells, L6 cells produce both neuregulin and its receptors, ErbB2 and ErbB3. Analogous to Schwann cells, neuregulin-neutralizing antibodies halted the progress of myogenesis at the point somewhere after the expression of myogenin and cell cycle arrest (Kim et al., 1999).

Although HiB5 cells express their own neuregulin receptors, ErbB2 and ErbB4, the action of neuregulin expressed in HiB5 cells may not be limited to an autocrine loop. It has been well established that neuregulins mediate neuron-glia interactions in the peripheral nervous system and the central nervous system, where neuronally produced neuregulins induce the proliferation or survival of Schwann cells and astrocytes (Pinkas-Kramarski et al., 1994; Morrissey et al., 1995). Furthermore, neuronally produced neuregulins are effective in the induction of radial glia formation that is critical for the migration of granule cells along glial fibers in the developing cerebellum (Rio et al., 1997). Therefore, neuron-glia interactions mediated by neuregulins may work for the formation of glial cells around the developing neural cells or the migration of neuronal cells along glial cells in hippocampus.

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