Insulin-like Growth Factor-I-Dependent Signal Transduction Pathways Leading to the Induction of Cell Growth and Differentiation of Human Neuroblastoma Cell Line SH-SY5Y: The Roles of MAP Kinase Pathway and PI 3-Kinase Pathway

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Abstract. IGF-I regulates cell growth, differentiation, and survival in many cultured nerve cell lines. The present study was undertaken in the human neuroblastoma cell line, SH-SY5Y, to elucidate whether there are differences in the IGF-dependent signal transduction pathways that stimulate proliferation compared to those that induce differentiation. Quiescent SH-SY5Y cells were treated with IGF-I in the presence or absence of PD98059 (an inhibitor of MEK, a MAP kinase kinase) or LY294002 (an inhibitor of PI 3-kinase). Cell growth was assessed by measuring [3H]thymidine incorporation into DNA and cell number. Cell differentiation was assessed by measuring mRNA levels of NPY and neurite outgrowth. IGF-I both induced cell proliferation and differentiation. It stimulated tyrosine phosphorylation of the type I IGF receptor (IGF-IR), subunit, IRS-1, IRS-2, and Shc, and these changes were associated with activation of Erk and Akt. PD98059 inhibited activation of Erk and LY294002 repressed activation of Akt in response to IGF-I, but did not affect tyrosine phosphorylation of the IGF-IR, IRS-1, IRS-2, or Shc. Each PD98059 and LY294002 inhibited IGF-I-dependent cell proliferation in a concentration-dependent manner. In contrast, each of these inhibitors only partially depressed NPY gene expression induced by IGF-I and slightly inhibited IGF-I-mediated neurite outgrowth; however, when both PD98059 and LY294002 were present, IGF-I-dependent NPY gene expression and neurite outgrowth were abolished completely. These results suggest that in these nerve cells, 1) the IGF-I signals through the MAP kinase pathway and PI 3-kinase pathway are independently essential to induce IGF-I-dependent growth, and 2) alternate activation of the MAP kinase pathway and PI 3-kinase pathway is sufficient for the cells to undergo IGF-I-dependent differentiation.

Key words: IGF-I, Growth, Neuronal differentiation, MAP kinase, PI 3-kinase

(Endocrine Journal 47: 739–751, 2000)
IGF-I binding to its cognate receptor, the type I IGF receptor (IGF-I receptor), results in receptor tyrosine kinase activation followed by phosphorylating intracellular proteins, such as Shc, insulin receptor substrate (IRS)-1 and IRS-2 [4]. Phosphorylated Shc and IRS by receptor kinase bind to Grb2-SOS complex [5]. This complex stimulates the exchange GDP to GTP on p21ras. Activated p21ras then associates with Raf (MAP kinase kinase kinase) prior to its activation. Raf then activates MEK (MAP kinase kinase), which in turn activates extracellular signal-regulated protein (Erk; MAP kinase) [5, 6]. Activation of the MAP kinase pathway leads to the expression of the immediate early genes, such as c-fos, c-jun and c-myc and next they induce to express the late response genes [1]. As a result, various biological activities, including cell proliferation or cell differentiation, are induced. By contrast, phosphorylated IRS by receptor kinase also binds to the 85 kDa regulatory subunit of PI 3-kinase and activates the 110 kDa catalytic subunit of PI 3-kinase [7, 8]. Activated PI 3-kinase produces PI3P, which leads to activation of the downstream kinases, such as PKB/Akt and aPKC [9]. Activation of the PI 3-kinase pathway is also known to induce proliferation, differentiation, and survival [10, 11].

SH-SY5Y is one of the several established human neuroblastoma cell lines. This line can be induced to differentiate further along a sympathetic neuronal lineage, providing model systems for study of mechanisms regulating neuronal development [12-14]. IGF-I and basic fibroblast growth factor (bFGF) stimulate the proliferation of this cell line [15], and some growth factors, including IGF-I, can induce differentiation to a neuronal phenotype [16-18]. It has been shown that IGF-I acts through IGF-I receptor for its biological effects in SH-SY5Y cells, but it is unclear what signaling pathways stimulated by the type I IGF receptor lead to proliferation and/or differentiation. However, there are reports suggesting that the association of Shc with Grb2 is essential for IGF-I-mediated neurite outgrowth, and that an IRS-2-PI 3-kinase interaction may regulate growth cone extension and membrane ruffling [19].

IGF-I is quite unique among the many growth factors, in that it is well known to induce both cell growth and differentiation of nerve cells. As we introduced here, it remains to be elucidated what signaling pathways among the classically described IGF-I signaling pathways, such as the MAP kinase and PI 3-kinase pathways, are required for each process. The present study was undertaken to elucidate whether there are differences in the IGF-dependent signal transduction pathways that induce cell growth and differentiation. To this end we utilized the MEK inhibitor, PD98059, and the PI 3-kinase inhibitor, LY294002, in the human neuroblastoma cell line, SH-SY5Y. We assessed cell growth by measuring [3H]thymidine incorporation into DNA and cell number, while differentiation was assessed by measuring neurite outgrowth and mRNA levels of neuropeptide Y (NPY).

Materials and Methods

Materials

Recombinant human IGF-I (hIGF-I) was kindly donated by Dr. Toshiaki Okuma (Fujisawa Pharmaceutical Co., Osaka, Japan). Leupeptin and pepstatin were generously gifted from Dr. Takaaki Aoyagi (Institute of Microbial Chemistry, Tokyo, Japan). Anti-phosphotyrosine monoclonal antibody was kindly provided by Dr. Takao Yamori (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan). Anti-IGF-I receptor β-subunit and Shc antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) and Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.), respectively. Polyclonal anti-IRS-1 antibody and anti-IRS-2 antibody were prepared by immunizing rabbits with synthetic peptides according to Ogihara et al. [20]. Anti-phospho-Erk polyclonal antibody (#9101S) and anti-phospho-Akt polyclonal antibody (#9271S) were obtained from New England Biolabs (Beverly, MA, U.S.A.). [Methyl-3H] thymidine (6.5 mCi/mmol), and [α-32P] dCTP (3,000 Ci/mmol) were purchased from Amersharm (Arlington Heights, IL, U.S.A.). Other chemicals were of the reagent grade available commercially.

Cell Cultures

The SH-SY5Y cells, a subclone of the human neuroblastoma cell line SK-N-SH, were kindly provided
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by Dr. Kimihiko Sano (School of Medicine, University of Kobe, Hyogo, Japan). Cells were cultured in RPMI1640 medium with 10% fetal calf serum (FCS, Nichirei Co., Tokyo), penicillin (100 U/ml), and streptomycin (50 µg/ml) in 150 cm² flasks with 5% CO₂ at 37°C in humidified incubator according to Pahlman et al. [14]. The medium was replaced every 3 days and the cells were passed every 7 days before they reached confluence. Prior to each experiment, cells were basically passed at 1 × 10⁴ cells /cm² and cultured in the RPMI1640 medium with 10% FCS for 48 h. They were washed with Hanks’ balanced salt solution (HBSS) and starved for 24 h to achieve quiescence under serum-free conditions, SHTE-medium (RPMI1640 medium containing 30 nM selenium, 10 nM hydrocortisone, 30 µg/ml transferrin and 10 nM β-estradiol, and the same antibiotics as described above). The cells were further incubated in the SHTE-medium containing various agents. In the case of inhibitor studies, cells were pretreated with various inhibitors 30 min before the addition of IGF-I.

Measurement of Thymidine Incorporation into DNA

Cells (2.0 × 10⁴) were plated in 24 well plates and cultured as described above to achieve quiescence. They then were incubated with or without IGF-I for additional 24 h and labeled with [methyl-³H]thymidine for the last 4 h, and [³H]thymidine incorporation into DNA was measured as described previously [21]. In all experiments, each experimental point represents the mean of three replicate wells.

Quantification of Cell Numbers

Cells were sparsely plated in 35 mm dishes and cultured as described above to achieve quiescence. Cultures were treated with or without IGF-I from 1 to 4 days, and harvested by trypsinization, and cells excluding trypan blue were counted. In all experiments, each experimental point represents the mean of three replicate dishes.

Total RNA Preparation

Cells (7.85 × 10⁵) were plated in 100 mm dishes, and after culture as described above, they were treated with or without IGF-I for 4 days. Total RNA was prepared as described previously by Puisant and Houdebine [22] with a slight modification. The cells were harvested in 6 ml of a solution (solution D, containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol). To extract total RNA, 0.6 ml of 2 M sodium acetate, pH 4.0, 6 ml phenol (which had been saturated with water), 1.2 ml of chloroform-isooamylalcohol (49 : 1) were added and the mixture was shaken vigorously at each addition. The mixture was centrifuged at 10,000 × g for 10 min at 4°C. The water phase was saved and mixed with an equal volume of isopropanol. The mixture was kept at 4°C for more than 2 h, and the precipitated RNA was centrifuged at 3,000 × g for 10 min at −20°C. The precipitate was redissolved in 0.4 ml of a solution containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% SDS. This solution was mixed with 0.4 ml of chloroform-isooamylalcohol (49 : 1) again and centrifuged at 3,000 × g for 10 min at 4°C. The water phase was mixed with 0.4 ml of isopropanol and 0.04 ml of 2 M sodium acetate, pH 5.0. The precipitated RNA in the mixture was recovered by centrifugation at 15,000 × g for 10 min at 4°C, washed with 0.3 ml of 70% ethanol, dried in vacuo, and dissolved in a solution containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. By determining the absorbance at 260 and 280 nm, the purity of RNA was confirmed and the concentration of RNA in the solution was calculated.

Northern Blot Analysis

Northern blot analysis was performed as described previously [23] with a slight modification. For each sample 15 µg of total RNA was dissolved in 20 µl of a solution containing 50% deionized formamide, 3% formaldehyde, 40 µg/ml of ethidium bromide and 1/10 volume of 10 × loading buffer containing 50% glycerol, 1 mM EDTA, pH 7.5, 0.4% bromophenol blue and 0.4% xylene cyanol in 3-(N-morpholino) propanesulphonic acid (MOPS) buffer solution and heated at 65°C for 15 min. The denatured RNA was electrophoresed on a 1.5% agarose and 3% formaldehyde gel. After electrophoresis, the
amount of RNA in the gel was assessed with a transilluminator. The RNA in the gel was transferred to a nylon membrane (GeneScreen Plus, NEN Life Science Products, Boston, MA, U.S.A.), and hybridized with cDNA probes for neuropeptide tyrosine (NPY) (Health Science Research Resources Bank, Osaka, Japan) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Clontech, Palo Alto, CA, U.S.A.). The probes were labeled with [α-32P]dCTP (3,000 Ci/mmol; Amersham, Bucks, UK) using Amersham’s multiprime DNA labeling systems according to the manufacturer’s protocol. Each band was quantified by Bio-Imaging Analyzer System, Fujix Bas 2000 (Fuji Film Co., Tokyo, Japan).

**Assessment of Neurite Outgrowth**

For analysis of neurite outgrowth, the cells (0.9 x 10⁵) were plated on 35 mm dishes and cultured as described above. Twenty-four hours after the addition of IGF-I, the number of neurite bearing cells among a total of three hundred cells was counted. Processes longer than the cell body were considered neurites. In all experiments, each experimental point represents the mean of three replicate dishes.

**5-Bromo-2-deoxyuridine (BrdU) Assay**

Cells (2.0 x 10⁴) were plated in 24 well plates, cultured as described above, and treated with or without IGF-I for 4 days. BrdU was added 1 h before the termination of each experiment and BrdU assay was performed using In Situ Cell Proliferation Kit, AP (Boehringer Mannheim, Germany) according to the manufacturer’s protocol. Cultures were photographed in a phase-contrast microscope (Olympus, Tokyo, Japan).

**Immunoblot Analysis using Anti-phosphotyrosine Antibody, Anti-phospho-Erk Antibody or Anti-phospho-Akt Antibody**

Cells (7.85 x 10⁵) were plated on 100 mm dishes and cultured as described above until they became quiescent. The cells were treated with or without IGF-I for 1 min or several time points. The cells were then harvested at 0°C in 400 µl of immunoprecipitation buffer containing 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM NaF, 10% glycerol, 1% NP-40, 1 mM Na3VO4, 100 KIU/ml aprotinin, 20 µg/ml PMSF, 10 µg/ml leupeptin, 5 µg/ml pepstatin, 10 mg/ml PNPP. The lysates were centrifuged at 14,000 x g for 20 min at 4°C. The supernatant was incubated with anti-IRS-1 antibody (10 µl), anti-IRS-2 antibody (10 µl), anti-IGF-I receptor β-subunit antibody (1 µg) or anti-Shc antibody (10 µg) for 12 h at 4°C; 40 µl of protein A-Sepharose [50% (v/v); Pharmacia Biotech, Sweden] was then added and incubation was continued for 2 h. Under 4°C, the immunoprecipitates were collected by centrifugation, washed three times with washing buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM NaF, 10% glycerol, 0.1% NP-40 and boiled for 5 min in a mixture of 60 µl of immunoprecipitation buffer and 30 µl of 3 x Laemmli’s sample buffer (9% SDS,
15% glycerol, 30 mM Tris-HCl, pH 7.8, 0.05% bromophenol blue, 6% 2-mercaptoethanol). These samples were then stored at -80°C until electrophoresis. A fraction of each sample that contained the same amount of proteins was subjected to immunoblotting using anti-phosphotyrosine antibody as described above.

Results

IGF-I Promotes Both Proliferation and Differentiation of SH-SY5Y Cells

To investigate the effects of IGF-I on SH-SY5Y cell proliferation, we measured [3H]thymidine incorporation into DNA after IGF-I treatment for 24 h and cell number after 1 to 4 days of IGF-I exposure. As

![Graphs](image-url)
shown in Figs. 1a and 1b, IGF-I promoted DNA synthesis as well as cell proliferation. In addition, to assess differentiation, we determined mRNA levels of NPY after 4 days incubation of IGF-I and neurite outgrowth after 24 h of IGF-I treatment. IGF-I induced the expression of NPY gene and neurite outgrowth in a concentration-dependent manner (Figs. 1c, 1d). To examine whether IGF-I induced both cell growth and differentiation in the same cells, cells were incubated with or without IGF-I (100 ng/ml) for 4 days and then both BrdU incorporation and neurite outgrowth were assessed. Compared with control cells, IGF-I stimulated BrdU incorporation in cells that exhibited neurites (Figs. 2a, 2b). These results clearly showed that a part of SH-SY5Y cells were induced to proliferate and differentiate simultaneously by IGF-I.

**IGF-I Induces Tyrosine Phosphorylation of IGF-I Receptor, IRSs and Shc, Resulting in Activation of Erk and Akt Phosphorylation in SH-SY5Y Cells**

We first assessed tyrosine phosphorylation of intracellular proteins in response to IGF-I. IGF-I increased tyrosine phosphorylation of intracellular proteins that migrated with apparent molecular masses of 175–185 kDa, 120–125 kDa and 90–100 kDa (Fig. 3a). Using immunoprecipitation with specific antibodies followed by immunoblotting with an anti-phosphotyrosine antibody, we found time-dependent and IGF-I-dependent increases in tyrosine phosphorylation of the IGF-I receptor β-subunit, IRS-1, IRS-2, and Shc (Fig. 3b). Although phosphorylated IRS-2 was dephosphorylated rapidly, the changes in IGF-I receptor autophosphorylation correlated well with the changes in tyrosine phosphorylation of 175–185 kDa proteins (Fig. 3a), which likely include IRS-1, as well as 66 kDa and 52 kDa Shc isoforms (Fig. 3b). As reported by others [18, 19, 26], IGF-I-induced activation of Erks was detected after 2 min by immunoblotting using an antibody against phosphorylated Erk (Fig. 3c). Activation of Akt, a downstream component of PI 3-kinase, in response to IGF-I also was observed after 2 min treatment, using an anti-phospho-Akt antibody (Fig. 3c). These results suggest that IGF-I activates sig-

![Fig. 2. Effects of IGF-I on DNA synthesis in the differentiated SH-SY5Y cells. (a and b) The cells were treated without (a) or with 100 ng/ml IGF-I (b) for 4 days and BrdU was added at 1 h before the termination of 4 days incubation without or with IGF-I. BrdU assay was performed using In Situ Cell Proliferation Kit, AP according to manufacturer's protocol. The cultures were then photographed under a phase-contrast microscope. These experiments were performed three times independently and a representative photograph is presented. Lower panels show the higher power view (×2) of the sites of dotted square in the upper panels. The arrows indicate the cells exhibiting neurite outgrowth, were stained by BrdU.](image-url)
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Because we utilized PD98059 (an MEK inhibitor) and LY294002 (a PI 3-kinase inhibitor) to elucidate the importance of each signaling pathway for induction of cell growth and differentiation, we firstly examined the effects of the inhibitors on activation of Erk or Akt in response to IGF-I. Addition of 100 \( \mu \)M PD98059 at the stimulation of IGF-I inhibited activation of Erk, but 5 \( \mu \)M LY294002 did not affect IGF-I-dependent activation of Erk (data not shown). On the other hand, addition of 100 \( \mu \)M PD98059 at the stimulation of IGF-I had no effects on IGF-I dependent Akt activation, whereas 5 \( \mu \)M LY294002 inhibited Akt activation (data not shown). These results indicated that PD98059 or LY294002 specifically inhibited the MAP kinase pathway or PI 3-kinase pathway activated by IGF-I, respectively.

**Blocking Either the MAP Kinase or PI 3-Kinase Pathway Completely Inhibits IGF-I induced Proliferation in SH-SY5Y Cells**

To investigate whether the activation of the MAP kinase or PI 3-kinase pathway is required for IGF-I-induced proliferation, cells were incubated with IGF-I in the presence of PD98059 or LY294002. Each of these agents inhibited both basal and IGF-I stimulated [3H]thymidine incorporation into DNA (Figs. 4a, 4b). After IGF-I treatment for 3 days in the absence or presence of PD98059 or LY294002, each agent also inhibited IGF-I-stimulated cell proliferation (Fig. 4c). These results suggest that activation of both MAP kinase and PI 3-kinase pathways is essential for IGF-I-induced SH-SY5Y cell growth.

**Blocking the MAP Kinase Pathway or PI 3-Kinase Pathway Only Partially Inhibits IGF-I-induced Differentiation in SH-SY5Y Cells**

We then analyzed the effects of each inhibitor on IGF-I-induced cell differentiation, as judged by NPY mRNA expression and neurite outgrowth. PD98059 only inhibited NPY expression minimally even at high concentrations (Fig. 5a). LY294002 partially inhibited NPY expression, but concentrations higher than those required to inhibit DNA synthesis, were required (Fig. 6a). PD98059 also had only a minimal
Fig. 4. Effects of PD98059 or LY294002 on DNA synthesis induced by IGF-I in SH-SY5Y cells. (a and b) Quiescent SH-SY5Y cells were pretreated without or with PD98059 (a) or LY294002 (b) at the indicated concentrations for 30 min and incubated with or without 100 ng/ml IGF-I. Measurement of [\( ^{3}H \)]thymidine incorporation into DNA after 24 h of incubation with 100 ng/ml IGF-I, as described in Materials and Methods. These experiments were performed three times independently, and a representative result is presented. (c) The cells were pretreated without or with PD98059 (100 \( \mu \)M) or LY294002 (5 \( \mu \)M) for 30 min and incubated with IGF-I (100 ng/ml) for 3 days. Cell number was then evaluated as described in Materials and Methods. Values are expressed as a relative increase compared to the cell number of day 0. These experiments were performed at least three times independently, and a representative result is presented.

Fig. 5. Effects of PD98059 on the expression of NPY and neurite outgrowth induced by IGF-I in SH-SY5Y cells. (a) Quiescent SH-SY5Y cells were pretreated without or with PD98059 at the indicated concentrations for 30 min and incubated with or without 100 ng/ml IGF-I. Northern blotting analysis after 4 days of incubation with 100 ng/ml IGF-I using \( ^{32}P \)-labeled NPY and G3PDH cDNAs, was performed as described in Materials and Methods. The amount of NPY mRNA is expressed as described in Fig. 1c. These experiments were performed three times independently and a representative blot is presented. (b) Quiescent SH-SY5Y cells were pretreated without or with PD98059 at the indicated concentrations for 30 min and incubated with or without 100 ng/ml IGF-I. Analysis of neurite outgrowth after 24 h of incubation with 100 ng/ml IGF-I was performed as described in Materials and Methods. These experiments were performed at least three times independently, and a representative result is presented.
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**Discussion**

The data presented here suggest that different components of classically described IGF-I signaling pathways have a role in determining cell growth or differentiation. Furthermore, we demonstrated that IGF-I promotes cell growth and differentiation simultaneously in SH-SY5Y cells.

We have shown that known IGF-I signaling pathways are intact in these cells, that is IGF-I stimulates type I IGF receptor tyrosine kinase, followed by phosphorylation of receptor kinase substrates, such as IRS-1, IRS-2, and Shc. In addition, we observed rapid IRS-2 dephosphorylation within 10 min after IGF-I treatment. In contrast, tyrosine phosphorylation of IRS-1 and Shc remained for at least 60 min after IGF-I stimulation. These results suggest that tyrosine phosphorylation of these substrates are regulated in different manners. In L6 myotubes, IRS-2 dephosphorylation was observed to be more rapid than that of IRS-1 in response to insulin [20]. Our results are discrepant with those of Kim et al. who could not demonstrate IRS-1 expression in SH-SY5Y cells [26]. The reasons for this difference are
unclear. It is possible that the pattern of gene expression in these cells was changed during passage. Also culture conditions and experimental media utilized by Kim et al. differed from original conditions and ours [18].

As reported by others [18, 19, 26], IGF-I stimulates Erk and Akt, and these changes in activities correlate temporally with changes of tyrosine phosphorylation of IRS-1 and Shc, and imply that IRS-1 and Shc may play more important roles in IGF-I signal transduction than IRS-2. Another difference between our study and that of Kim et al. is their observation that treatment of SH-SY5Y cells with PI 3-kinase inhibitors, wortmannin and LY294002, increased both basal and IGF-I-induced tyrosine phosphorylation of IRS-2 [26]. In our study, PD98059 or LY294002 inhibited IGF-I-dependent Erk or Akt activation, respectively, without affecting IGF-I-dependent tyrosine phosphorylation (data not shown). These findings further suggest that the characteristics of their SH-SY5Y cells and ours are different. Because IRS-1 is shown to be important during nerve development [1], SH-SY5Y expressing IRS-1 could become the more suitable model to elucidate IGF-I action.

Our finding that activation of both the MAP kinase and PI 3-kinase pathway is necessary for IGF-I-induced cell proliferation is unique. In all reported studies of growth factor-induced cell proliferation, only one of these pathways has been found to be essential. For example, in PC12 neural cell line, Traverse et al. demonstrated that EGF induced cell proliferation through transient activation of MAP kinase [27], but not of PI 3-kinase. In oligodendrocytes, activation of MAP kinase is required for their proliferation induced by PDGF and bFGF [28]. Our results show that IGF-I-induced growth of SH-SY5Y cells can be regulated through several signaling pathways, and suggest that signaling cross-talk among signaling pathways is essential.

Our data also indicated that IGF-I can induce differentiation by alternative pathways. Again our findings differed somewhat from those of Kim et al. who reported that only activation of Erk was essential for IGF-I-stimulated neuronal differentiation in SH-SY5Y cells [18]. They found that the MEK inhibitor PD98059 inhibited IGF-I-induced neurite outgrowth and the expression of GAP-43 neuronal marker gene. They also found that LY294002 inhibited neuronal differentiation in response to IGF-I, and that this was associated with a decrease in IRS-2 tyrosine phosphorylation [26]. We have no explanation for these differences. In the case of PC12 cells, Traverse et al. reported that sustained activation of MAP kinase by NGF is necessary to induce differentiation [27]. In contrast, wortmannin inhibited NGF-induced neurite outgrowth in PC12 cells [29, 30]. While the MAP kinase and PI 3-kinase pathways may act independently in PC12 cells to induce neuronal differentiation, our results suggest that activation of either MAP kinase or PI 3-kinase is necessary in SH-SY5Y cells. In other words, these results suggest that in the case of IGF-I-induced neuronal differentiation, activation of some components of one pathway along the MAP kinase and PI 3-kinase pathways could compensate for those of another pathway. Taken together, these findings would indicate cross-talk among signaling pathways.

In a number of cell types, IGF-I can induce both cell proliferation and differentiation, but apparently
by distinct pathways. For example, IGF-I promotes differentiation and mitosis in fetal rat brown adipocytes and L6A1 myoblasts [31, 32]. In these cells, IGF-I-dependent MAP kinase activation plays a primary role in proliferation, while an inhibition of IGF-I-dependent MAP kinase activation by PD98059 leads to an enhancement of differentiation. At the same time, however, IGF-I stimulation of PI 3-kinase activation results in differentiation, indicating that IGF-I activates different signaling pathways to induce proliferation or differentiation. SH-SY5Y cells, therefore, seem unique in that IGF-I-dependent activation of both the MAP kinase and PI 3-kinase pathways is important for both cell proliferation and neuronal differentiation. We need to further elucidate at which stages of induction of differentiation activation of the MAP kinase or PI 3-kinase pathway is required, and into which target molecules these two signaling pathways converge in the process of inducing cell growth.

Furthermore, our results demonstrate that IGF-I promotes proliferation and differentiation simultaneously in SH-SY5Y cells (Fig. 2). In general, nerve cells do not proliferate after neuronal differentiation. However, there appears to be exceptions. For example, in immature neurons from chick lumbosacral sympathetic ganglia, IGF-I induces DNA synthesis and neurite outgrowth at the same time [33]. It is possible that some nerve cells, which possess the capacity to proliferate in response to growth factors, such as neuroblastoma cells, can differentiate at least to some degree at the same time.

How do the nerve cells determine their fate in response to IGF-I, that is, whether to proceed to proliferation or differentiation? In SH-SY5Y cells, both TPA and bFGF are reported to potentiating cell differentiation induced by IGF-I [15]. In fact, we found that treatment with TPA or bFGF potentiated

Fig. 8. Effects of PD98059 and LY294002 on neurite outgrowth induced by IGF-I in SH-SY5Y cells. Quiescent SH-SY5Y cells were pretreated with or without 100 μM PD98059 and/or 5 μM LY294002 for 30 min and incubated without or with 100 ng/ml IGF-I. A representative phase contrast image of cells from the experiments above is shown. The insert graph shows the results of analyses of neurite outgrowth (Cont; Control, No; No inhibitors, PD; PD98059, LY; LY294002, and PD+LY; PD98059 and LY294002). These experiments were performed at least three times independently and a representative result is presented.
IGF-I-induced expression of NPY and neurite outgrowth in this cell (data not shown). These results indicate that signals in the PKC or FGF pathway converge with IGF-I signaling pathways. During *in vivo* neuronal development, it appears that a variety of extracellular signaling molecules, including IGF, interact at specific stages [1], and these events are likely essential for normal growth and development of the nerve system. Our findings that IGF-I can promote cell proliferation and differentiation using two distinct signaling pathways suggest that cell fate (proliferation or differentiation) and the magnitude of IGF-I activity is determined by how signaling of other factors modulates IGF-I signals of the MAP kinase and/or PI 3-kinase pathways. Clearly it is important to learn more about the mechanisms by which growth factor signals converge.

In summary, these data suggest that in SH-SY5Y cells: 1) both the MAP kinase and PI 3-kinase pathways appear essential for IGF-I-induced cell proliferation, and 2) activation of either the MAP kinase pathway and PI-3 kinase pathway is sufficient for IGF-I-dependent differentiation. Interaction between these classically described IGF-I signaling pathways and other growth factors’ signaling pathways possibly play important roles in determining cell growth or neuronal differentiation.

**Acknowledgments**

We wish to thank Dr. Kimihiko Sano (School of Medicine, University of Kobe, Japan) for the kind gift of SH-SY5Y cells. Recombinant human IGF-I was kindly provided by Dr. Toshiaki Okuma, Fujisawa Pharmaceutical Co. We also express our appreciation to Dr. Takaaki Aoyagi (Institute of Microbial Chemistry, Tokyo, Japan) for leupeptin and pepstatin and to Dr. Takao Yamori (Cancer Chemotherapy Center, Japan Foundation for Cancer Research, Tokyo, Japan) for anti-phosphotyrosine antibody. Finally, we wish to thank Dr. A. Joseph D’Ercole and Dr. Judson J. Van Wyk (University of North Carolina at Chapel Hill, NC, U.S.A.), Dr. Asako Takenaka (Yamagata University, Yamagata, Japan) and Dr. Masayuki Ito (National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan) for their helpful discussions during the performing of these experiments and the writing of this paper.

**References**


