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The Biologic Role of Ganglioside in Neuronal Differentiation

– Effects of GM_1 Ganglioside on Human Neuroblastoma SH-SY5Y cells –

Min-Cheol Lee, M.D., Woo-Seok Lee, M.D., Chang-Soo Park, M.D.,
and Sang-Woo Juhng, M.D.

*Department of Pathology, Chonnam National University Medical School
Kwangju, Korea 501-190*

Human neuroblastoma SH-SY5Y cell is a cloned cell line which has many attractive features for the study of neuronal proliferation and neurite outgrowth, because it has receptors for insulin, IGF-I and PDGF. Gangliosides are sialic acid containing glycosphingolipids which form an integral part of the plasma membrane of many mammalian cells. They inhibit cell growth mediated by tyrosine kinase receptors and ligand-stimulated tyrosine kinase activity, and autophosphorylation of EGF(epidermal growth factor) and PDGF receptors. The experiment was designed to study the effects of GM_1 ganglioside on growth of human neuroblastoma SH-SY5Y cells stimulated with trophic factor in vitro. The cells were plated in Eagle's minimum essential medium without serum. The number and morphologic change of SH-SY5Y cells were evaluated in the serum free medium added GM_1 ganglioside with insulin or PDGF. SH-SY5Y cells were maintained for six days in serum-free medium, and then cultured for over two weeks in serum-free medium containing either insulin or PDGF. The effect of insulin on cell proliferation developed earlier and was more potent than that of PDGF. These proliferative effects were inhibited by GM_1 ganglioside, and the cells showed prominent neurites outgrowth. These findings suggest that GM_1 ganglioside inhibits the cell proliferation mediated by tyrosine kinase receptors and directly induces neuritogenesis as one of the neurotrophic factors.

Key Words : *SH-SY5Y cell, Insulin, PDGF, GM_1 ganglioside, Neuritogenesis.*

INTRODUCTION

Gangliosides are sialic acid containing glycosphingolipid and are presented at an outer leaflet of the plasma membrane of many mammalian cells. They are amphophilic in nature because they have both lipids and carbohydrates. The lipid portion of the molecule is embedded in the hydrophobic part of the membrane and its carbohydrate portion extends into the extracellular environment. Topologically, gangliosides are ideally situated to modulate biologic effects on cells through their interactions with various ligands(Yavin and Habig, 1986 ; Mugnai et al., 1988). When administered exogenously, either in vivo or in vitro, they affect several different cellular events(Yamakawa and Nagai, 1978 ; Yates, 1986) including cell proliferation(Yates et al., 1985 ; Spiegel et al., 1985 ; Icard-Liepkalns et al., 1982). Exogenously added gangliosides inhibit proliferation of several cell types, but this inhibitory effect varies among different types of gangliosides and cell-

Address for correspondence : *Min-Cheol Lee, M.D., Department of Pathology, Chonnam National University Medical School, 5 Hakdong, Dongku, Kwangju, Seoul 501-190, Korea. Tel : (062) 232-1246, Fax : (062) 225-0480*

s(Keenan et al., 1975 ; Bremer and Hakomori, 1982).

The SH-SY5Y cell line, originally derived from a human neuroblastoma SK-N-SH(Biedler et al., 1978), is an excellent in vitro model to study neuronal proliferation, differentiation and neurite regeneration(Ishii and Mill, 1987 ; Ishii et al., 1987). SH-SY5Y cells are of human origin from a neuronal rather than chromaffin cell lineage. It is easy to maintain them in a serum-free medium(SFM). In addition, these cells have receptors for insulin, insulin-like growth factor-I (IGF-I)(Wang et al., 1992) and platelet-derived growth factor (PDGF ; unpublished data of Yates et al.).

A ganglioside, GD_{1a}, inhibits autophosphorylation of PDGF receptor tyrosine kinase of cell proliferation(Beckmann et al., 1988). From this, it is beneficial to study the biological mechanism of the action of gangliosides using SH-SY5Y neuroblastoma cells. This experiment was designed to determine the effects of GM_1 ganglioside on proliferation and morphologic changes of SH-SY5Y cells stimulated by insulin or PDGF.

MATERIALS AND METHODS

Cell culture and treatment with insulin, PDGF and GM_1 ganglioside

SH-SY5Y cells (obtained from Dr. Allan J. Yates ; Ohio State University, Columbus, OH, U.S.A.) were maintained in Eagle's minimum essential medium-(MEM, Gibco Laboratories Inc., Grand Island, NY, U.S.A.) supplemented with 15% fetal bovine serum, fungizone (0.22ug/ml) and penicillin-streptomycin (80 units/ml-80 ug/ml) in plastic tissue culture flasks. Cells were incubated in a humidified atmosphere of 95% air and 5% carbon dioxide at 37℃. Every second day two-thirds of the medium was replaced.

To determine cell growth, SH-SY5Y cells were harvested with 0.2ml trypsin solution (trypsin, 0.01% and EDTA, 0.02%) and mechanically dissociated into a single cell suspension for determination of cell numbers using a Coulter counter. Cells were plated in 1 ml serum-free media with a density of 10,000 cells/well into 24 well plates (Corning, NY, U.S.A., 1.88cm^2) on day-4. The media was changed every other day.

Insulin(Sigma Chemical Co., St. Louis, MO, U.S.A.) with the concentration of 1, 5, 10, 25, 50ug/ml and PDGF (Amgen Biologicals, Thousand Oaks, CA, U.S.A.) with the concentration of 10, 20, 30ng/ml were initially added to fresh culture media on day-6. To study the effect of GM_1 ganglioside on growth of insulin or PDGF-stimulated SH-SY5Y cells, GM_1 ganglioside (Sigma Chemical Co. St. Louis, MO, U.S.A.) was added at the following concentrations : 0.01, 0.1, 1.0, 10 and 100 uM within an hour on the SH-SY5Y cells cultured in SFM with insulin(50ug/ml) or PDGF(20ng/ml).

Determination of cell proliferation and morphologic changes

SH-SY5Y cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and treated with 0.2ml trypsin solution for two minutes at room temperature. Trypsin was inactivated by the addition of serum-containing medium to a final volume of 1ml. Cells were counted using a Coulter counter every other day in the insulin treated-group and every fourth day in the PDGF-treated group.

Values presented as means(mean±SD) are the averages of three readings from each well at three times of the experiments simultaneously. The morphologic changes are evaluated under phase-contrast microscopy at the 10th day in the insulin treated-group and the 20th day in PDGF-stimulated group. The experimental schedule is summarized in table 1.

Table 1. Experimental schedule

Day	Procedure
0	Culture SH-SY5Y cells in MEM with 15% FBS
2	Change medium
4	Plate cells in SFM
6	Add insulin or PDGF two days after change to SFM ; Add GM_1 ganglioside within an hour after insulin and PDGF treatment
8-28*	Count cells and evaluate morphologic changes

* Day-2 to 20 after insulin, PDGF and/or GM_1 ganglioside treatment

RESULTS

Effect of GM_1 ganglioside on growth of insulin-stimulated SH-SY5Y cells

Cells were maintained relatively well in serum free medium (SFM) for six days, however, the numbers of cells were significantly decreased after eight days indicating degeneration. When insulin added in SFM, insulin stimulated the growth of SH-SY5Y cells dose-dependently(Fig. 1).

The effect of GM_1 ganglioside on this insulin-stimulated growth was studied on the 50 ug/ml insulin-treated group. Inhibitory effect of GM_1 ganglioside on cell growth stimulated by insulin became prominent, 40 to 50% of control group, over an 8 day period in 0.01 M, and moreso, over 50% of control group, in 1μM or 10μM concentration(Fig. 2).

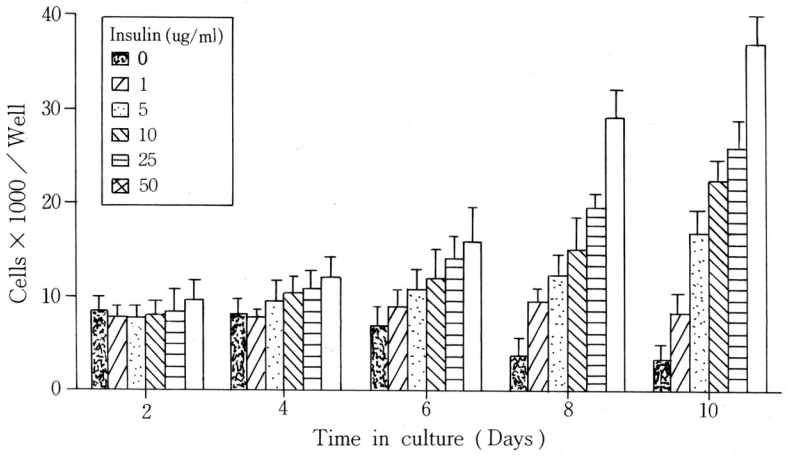

Fig. 1. Effect of insulin(1 to 50ug/ml) on growth of SH-SY5Y cells(10×1000/well) in serum free medium. Cells were seeded on day-0. Different doses of insulin were added daily, and media were changed on every other day.

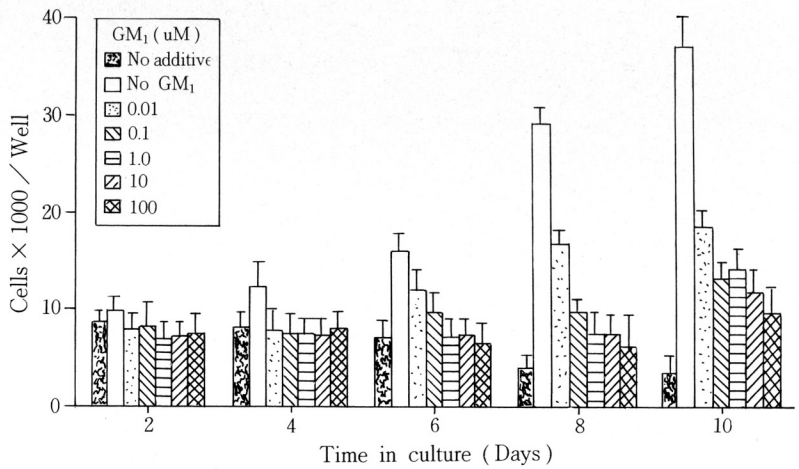

Fig. 2. Effect of GM_1 ganglioside(0.01-100uM) on growth of SH-SY5Y cells(10×1000/well) stimulated with insulin(50ug/ml).

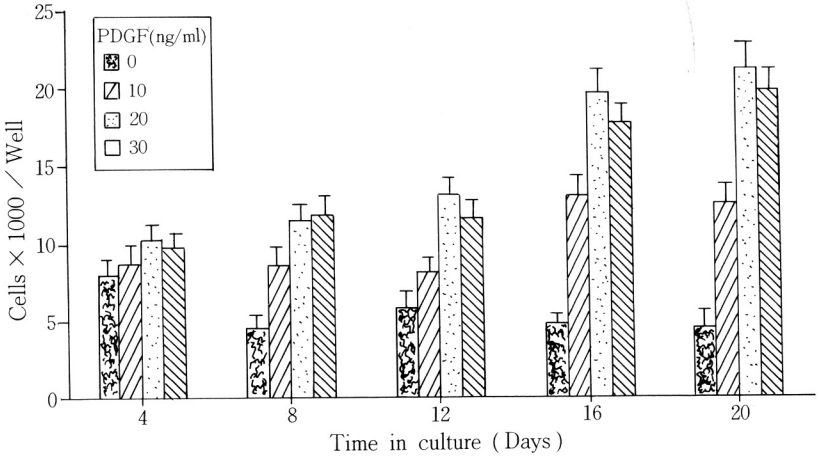

Fig. 3. Effect of PDGF(10-30ng/ml) on growth of SH-SY5Y cells(10X1000/well) in serum free medium.

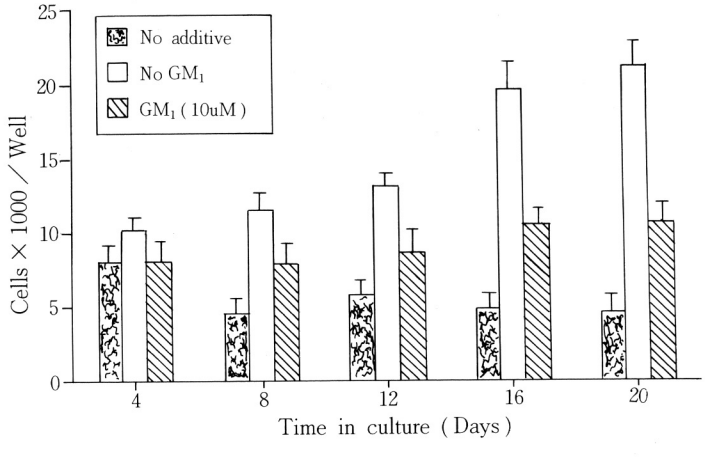

Fig. 4. Effect of GM_1 ganglioside(10uM) on PDGF-stimulated(20ng/ml) growth of SH-SY5Y cells.

Effect of GM_1 ganglioside on growth of PDGF-stimulated SH-SY5Y cells

A significant growth promoting effect was noted after 16 days by the addition of PDGF in SFM(Fig. 3). Cells cultured in SFM with PDGF showed maximal proliferation in the 20ng/ml treated group.

GM_1 gangliosides also inhibited the proliferation of PDGF-stimulated (20ng/ml) SH-SY5Y cells after 16 days, but there was no significant difference between the concentration of GM_1 ganglioside (Fig. 4).

Cytologic findings

Various neurites outgrowth, neuritogenesis, was noted. The degree of fiber outgrowth was semi-quantitatively assayed using the following criteria (Fig. 5) : grade 0 (no fiber outgrowth), grade 1 (induction of a few fibers no longer than the diameter

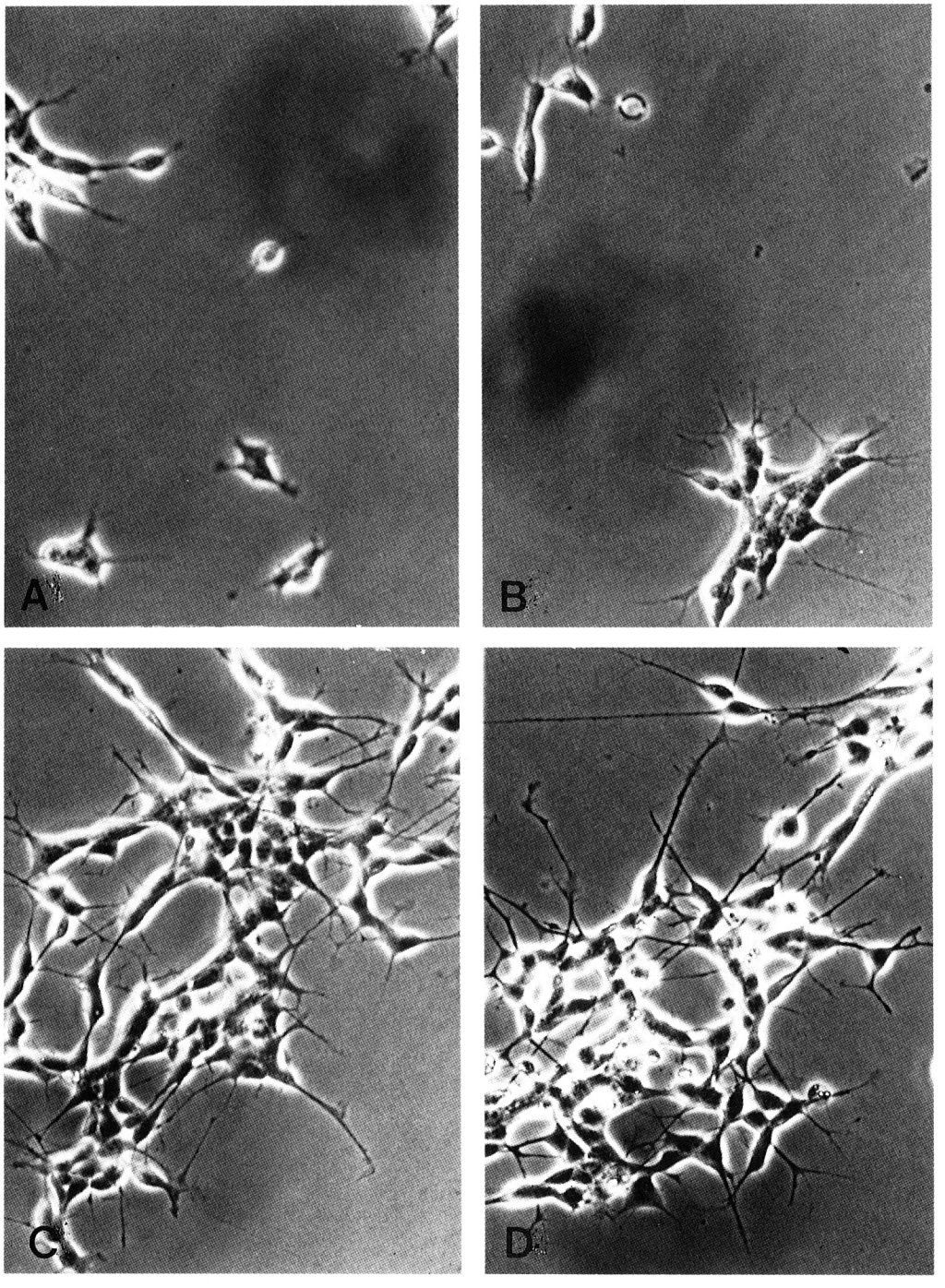

Fig. 5. Neuritogenic effects of GM_1 ganglioside on SH-SY5Y cells stimulated with insulin and PDGF ; induction of a few fibers no longer than the diameter of the cell body(A, grade 1), fibers the diameter of the cell body(B, grade 2), fibers twice the diameter of the cell body(C, grade 3), and three times the diameter of the cell body(D, grade 4).

Table 2. Neuritogenic effects of GM_1 ganglioside on SH-SY5Y cells stimulated with insulin and PDGF

	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Insulin	———	———			
PDGF	———	———			
Insulin+GM_1			———	———	———
PDGF+GM_1			———	———	———

Grade 0 : No fiber outgrowth
1 : Induction of a few fibers no longer than the diameter of the cell body
2 : Induction of fibers as long as the diameter of the cell body (20 - 40 μm)
3 : Induction of fibers twice the length of cell diameter
4 : Induction of fibers three times the length of the cell diameter

of the cell body), grade 2 (induction of fibers approximately as long as the cell body), grade 3 (induction of fibers twice the length of cell diameter), and grade 4 (induction of fibers three times longer than the cell diameter).

There were only a few, short fiber outgrowths in the insulin and PDGF stimulated groups. They were limited within grade 1. However, GM_1 ganglioside markedly promoted the fiber outgrowth of insulin or PDGF stimulated SH-SY5Y cells. No significant difference was noted between the two groups.

The cytologic findings were summarized in table 2.

DISCUSSION

The elucidation of the molecular mechanisms with morphologic studies regulating proliferation and differentiation of neurons is a problem of fundamental importance to spinal cord regeneration. Growth factors play an important roles in proliferation and differentiation of neuronal cells(Sprugel et al., 1987 ; Holly, 1975), and gangliosides interact with and modify these processes(Beckmann et al.,1988 ; Dreyfus et al., 1980 ; Emerich and Walsh, 1989). However, it is not clear that the specific effects on molecular mechanisms through which these two different classes of biochemicals exert their effects and interact with each other. Identification of specific trophic factors and determinations of their effects on neurons are still in early stages of study. This experiment was designed to study the effect of GM_1 ganglioside on SH-SY5Y cells stimulated by insulin and PDGF.

The results from the present study showed three major points of interest. Firstly, SH-SY5Y cells can survive for six days in a serum-free medium, moreover, they can survive and proliferate for over

Table 3. Growth factors affecting neural development

Growth factor	Molecular mass(kDa)	Target cells[a]	Tissue source
Brain growth factor	12	N	Brain
Cholinergic neuronotrophic factor	20	N	Eye, sciatic nerve
Epidermal growth factor	6	A,N	Submaxillary gland
Fibroblast growth factor(acidic)	16	A,N	Brain, retina, hypothalamus
Fibroblast growth factor(basic)	16	A,O,N	Brain, retina, pituitary, hypothalamus
Glial growth factor	31	A,S	Pituitary, caudate nucleus,neuroma
Insulin-like growth factor I	7	O,N	Liver, other cells
Insulin-like growth factor II	7		Fetal liver, fetal brain
Interleukin I (α, β)	16	A,O	Macrophages, astrocytes, microglia
Interleukin II	15	O	T cells
Nerve growth factor(β NGF)	26	N	Submaxillary gland, placenta
Neuroleukin	56	N	Submaxillary gland
Platelet-derived growth factor	32	A,O	Platelets, placenta, astrocytes

[a](A) astrocytes ; (N), neurons ; (O), oligodendrocytes ; (S), schwann cells.
(Cited from [b]Siegel et al., 1989)

two weeks in a serum-free medium containing either insulin or PDGF. Secondly, the effect of insulin on cell proliferation appeared earlier and were more potent than that of PDGF. Thirdly, these proliferative effects are reversed by low concentrations of GM_1 ganglioside, and cells show prominent neurites outgrowth in the GM_1 ganglioside treatment group.

Over the last ten years, there has been a tremendous increase in the study of the biologic factors which stimulate the growth of different cell types in the central nervous system(Table 3)([b]Siegel et al., 1989).

Some of these factors are components of sera and are essential for the survival and growth of cells in vitro(Furcht, 1986 ; Buck and Horwitz, 1987). Insulin, IGF-I(insulin-like growth factor I) and PDGF act on cells by binding to specific receptors on the extracellular surface of the plasmalemma(Deuel, 1987 ; Sporn and Roberts, 1988). Although the molecular mechanisms involved in signal transduction of these growth factors are not completely known, it is certain that the receptors for insulin, IGF-I and PDGF possess tyrosine kinase activities which are quickly activated by receptor-ligand interactions(Ross et al., 1986 ; Pierce et al., 1988). The NGF(nerve growth factor) receptor does not seem to have intrinsic tyrosine kinase activity(Yarden et al., 1986 ; Yan and Johnson, 1988), although NGF can produce protein-tyrosine phosphorylation(Kaplan et al., 1991) which involves activation of *trk* proto-oncogene product(Kaplan et al., 1991). Phosphorylation of specific substrates, including receptor autophosphorylation, is a critical component of the signal transduction process for the tyrosine kinase class of receptors(Chapron et al., 1989).

The SH-SY5Y cell is a clone of the human neuroblastoma cell line SK-N-SH and retains many neuronal charcteristics(Biedler et al., 1978). The cells have IGF-I receptors, and proliferate in a serum-free medium in response to IGF-I, and respond mitogenically to insulin, IGF-I and IGF-II(Wang et al., 1992). These growth factors also stimulate mitogenesis of normal sympathetic neuroblasts in the rat(Froesch et al., 1985). This experiment proves that PDGF as well as insulin stimulate SH-SY5Y cells to divide. However, the proliferative effect of PDGF on SH-SY5Y cells presents later and is less potent than that of insulin. It has been known for sometime that SH-SY5Y cells form neurites in response to insulin, IGF-II and NGF(Baskin et al., 1988 ; El-badry et al., 1989 ; Assouline et al., 1987). In this experiment, most of the cells stimulated with insulin or PDGF show no neurite outgrowth, but induction of a few fibers no longer than the diameter of the cell body is identifiable in a small portion of the cells.

Gangliosides are a sialic acid containing complex glycosphingolipids found in most of the vertebrate cells, and are present in high concentrations in the neuron-rich regions of the nervous systems([a]Siegel et al., 1989). They inhibit cell growth mediated by tyrosine kinase receptors, and also inhibit ligand-stimulated tyrosine kinase activity including autophsphorylation of EGF(epidermal growth factor) and PDGF receptors(Bremer and Hakomori, 1982 ; Bremer et al., 1984 ; Bremer et al., 1986). Whether gangliosides affect the tyrosine kinase activity of *trk* is not known. The growth of SH-SY5Y cells stimulated with insulin and PDGF is significantly decreased by administration of GM_1 ganglioside in this experiment. This result suggests that the mechanism of the growth inhibitory effect of gangliosides is possibly caused by insulin and PDGF receptor tyrosine kinase activities.

Considerable experimental evidence shows that exogenously administered gangliosides facilitate the recovery of damaged nerve tissue in vivo(Sparrow and Grafstein, 1982 ; Toffano et al., 1983) and stimulate neuritogenesis of many types of neurons in vitro(Ledeen, 1984 ; Spoerri and Roisen, 1988). Recent evidence indicates that the early administration of GM_1 ganglioside enhances recovery of neurological function(Mahadik and Karpiak, 1985) in the patients with spinal cord injury. Gangliosides added to culture media in the absence of NGF induce neuritogenesis in several neuroblastoma cell lines including SH-SY5Y cells, but not in PC 12 cells(Ferrari et al., 1983 ; Katoh-Semba et al., 1984). However, gangliosides enhance the degree of neurite formation induced by NGF in PC 12 cells and cultured dorsal root ganglion neurons without affecting the binding of NGF(Ferrari et al., 1983 ; Hashimoto, 1988). These findings can be interpreted that gangliosides are trophic factors which either directly induce neuritogenesis or facilitate NGF signal transduction mechanisms. The cytologic changes in this experiment suggest that the GM_1 ganglioside directly induces neuritogenesis as a neurotrophic factor.

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