

Platelet-derived growth factor가
dopaminergic 신경세포 분화에
미치는 영향

연세대학교 대학원

의과학사업단

손 인 숙

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지도 연 동 수 부교수

이 논문을 박사 학위논문으로 제출함

2000년 12월 일

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손인숙의 박사 학위논문을 인준함

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2000년 12월 일

감사의 글

본 논문이 나오기까지 모든 지도와 수고를 아끼지 않고 도움을 주신 많은 분들께 감사를 드립니다. 특히 처음부터 끝까지 가르쳐 주시고 이끌어 주신 연동수 교수님께 충심으로 감사드리며 동시에 본 연구와 논문을 위하여 조언과 격려를 아끼지 않으신 백광세 교수님, 남택상 교수님, 임중우 교수님, 최재원 교수님께 깊은 감사를 드립니다. 아울러 본 실험의 전 과정에 걸쳐 수고와 가르침을 준 박정선 선생님, 이진주 선생님과 생리학교실 여러 선생님께도 큰 고마움을 전하는 바입니다. 끝으로 오늘의 제가 있도록 키워 주신 부모님과 따뜻하게 격려해 주신 시부모님, 항상 곁에서 용기를 주고 조언을 해 준 남편과 사랑하는 아이들에게 이 논문을 드립니다.

저 자 씬

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Platelet-derived growth factor가 dopaminergic

가 , peptide growth factor
 cytokine
 platelet-derived growth factor (PDGF)
 가 , ciliary neurotrophic factor (CNTF) triiodothyronine (T₃) 가
 cytokine dopamine

1. Epidermal growth factor (EGF)
 가

2. GABA substance P
 tyrosine hydroxylase (tyrosine hydroxylase immuno-reactive, TH-ir)
 serotonin

3. PDGF TH-ir EGF,
 basic fibroblast growth factor, nerve growth factor, brain-derived neurotrophic factor, CNTF,
 glial cell-derived neurotrophic factor TH-ir

4. PDGF가 TH-ir tyrosine hydroxylase
 western blot
 PDGF TH-ir
 TH-ir TH-ir 가

: , dopamine , platelet-derived growth factor, epi-
 dermal growth factor,

Platelet-derived growth factor가 dopaminergic

< >

I.

(ventricular zone) ^{1,2}
(neuronal stem cell)가
, ³(extracellular matrix) ⁴
epidermal growth factor (EGF),^{5,6} transforming growth factor- (TGF),⁷ basic fibroblast growth
factor (bFGF)⁸ peptide growth factor ⁹
radial glial cell ¹⁰
acetylcholine
가
(neurotrophic factor)가 ¹¹
가 , ,
cytokine ¹²
protein (GFAP) ^{13,14} glial fibrillary acidic

14 laminin (optic stalk)

15 laminin 10 ,

12 laminin

laminin , laminin 16 가

laminin , , , 가 laminin

가 17 laminin GABA

18 subtype

cytokine 19,20 EGF

brain-derived neurotrophic factor (BDNF), platelet-derived growth factor (PDGF) 가 ,

ciliary neurotrophic factor (CNTF) triiodothyronine (T₃) 가 20 가

dopamine

II.

1.

Reynolds Weiss 21 , 1 2

Ca⁺⁺/Mg⁺⁺-free Hanks' balanced salts solution (HBSS, Gibco, Grand Island, NY, USA)

10 ml

trypsin 0.13% 가 37°C 15 incubation . Incubation

Nitex filter (Tetko, Elmsford, NY, USA) #210 #130 ,

100×g 5 . Trypsin HBSS

, Nitex filter #130

EGF가 (serum-free medium, SFM)

Nitex filter #40

hemocytometer 6-well 2.5×10⁵ cells/ml 1 ml

plate . 5 7
 (cell clusters) . SFM 50 nM hydrocortisone (Sigma, St
 Louis, MO, USA), 100 µM putrescine (Sigma), 30 nM selenium (Sigma), 20 µg/ml transferrin
 (Sigma), 10 µg/ml insulin (Sigma), 20 ng/ml EGF (Sigma) 5 mM HEPES (Sigma)
 Dulbecco's modified Eagle's medium (Gibco) .

2.

poly-L-lysine (PLL) chambered
 1 chambered ,
 nestin 가 (micro-
 tubule) tau GFAP
 galactocerebroside (GC)가
 가
 PLL chambered 7 EGF가
 SFM tau, GFAP GC .
 7 dopamine tyrosine hydroxylase neurotransmitter
 -aminobutyric acid (GABA), substance P, serotonin
 가 .

3. Tyrosine hydroxylase

cytokine tyrosine hydroxylase (TH immuno-
 reactive. TH-ir) . ,
 PLL chambered
 20 ng/ml EGF, 20 ng/ml bFGF (Sigma), 100 ng/ml nerve growth factor (NGF, Sigma), 10
 ng/ml BDNF (Sigma) 10 ng/ml CNTF (Sigma), 1 ng/ml glial cell-derived neurotrophic factor
 (GDNF, Sigma), 10 ng/ml PDGF (Sigma) 7 .
 cytokine 가 .
 7 TH-ir 가 tyrosine hydroxylase
 western blot .

4.

4% parafor-
maldehyde 20 mM phosphate buffered saline (PBS, pH 7.4) 4°C 20
. PBS , 95% ethanol 4°C 10
. PBS 37°C 30 10% 20
mM PBS (blocking) incubation . Blocking
1 37°C 60 incubation . 1
PBS 2 37°C 30 incubation . 2
PBS , 50% glycerol 0.05 mg/
ml propyl gallate 7† PBS mount . Kodak
Ektachrome 400 .

Rabbit anti tau IgG (Sigma); 1 : 100, rabbit anti GFAP IgG (Sigma); 1 : 100, rabbit anti GC IgG (Sigma); 1 : 100, rabbit anti substance P IgG (Boehringer Mannheim, Indianapolis, IN, USA); 1 : 200, mouse anti tyrosine hydroxylase IgG (Boehringer Mannheim); 1 : 500, mouse anti GABA IgG (Boehringer Mannheim); 1 : 500, mouse anti serotonin IgG (Boehringer Mannheim); 1 : 10, mouse anti neurofilament IgG (SMI-31, Boehringer Mannheim); 1 : 1,000.

2 . FITC conjugated sheep anti mouse IgG (Boehringer Mannheim); 1 : 400, rhodamine conjugated goat anti rabbit IgG (Boehringer Mannheim); 1 : 400.

5. Western blot

Tyrosine hydroxylase western blot .
tyrosine hydroxylase Zhou²² 10% SDS-PAGE gel
, gel Towin nitrocellulose (NC)
²³ NC 3% BSA/TBS 1 30
. NC TBS 3 0.5% BSA/TBS mouse anti-
TH IgG 1 : 500 2 . TBS 3
horseradish-peroxidase conjugated goat anti-mouse IgG (Jackson, West Grove, Pennsylvania, USA) 0.5% BSA/TBS 1 : 5000 1 30 . TBS
3 enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA)

III.

1.

		EGF가	SFM	1
(Fig. 1A).		2	2	(Fig. 1B),
7	50		(Fig. 1C).	
PLL	chambered			

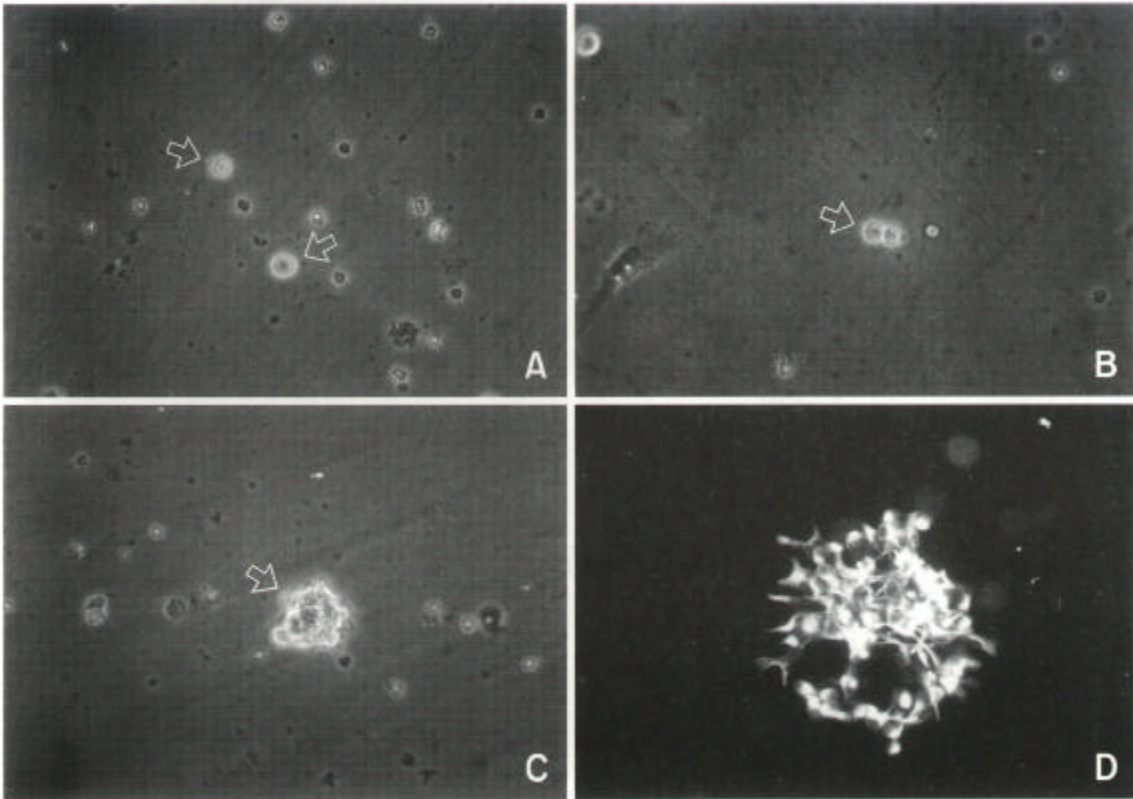


Fig. 1. Light microscopic appearance of EGF-responsive neuronal stem cells from newborn rat forebrains. (A) single neuronal stem cell (arrows) after 1 day in primary culture ($\times 200$). (B) Dividing neuronal stem cells (arrow) after 2 days in primary culture ($\times 200$). (C) A cluster of cells (arrow) after 7 days in primary culture ($\times 200$). (D) Floating cell clusters were replated on poly-L-lysine coated slides and allowed to attach for 1 hr: indirect immunocytochemistry was then performed using anti-nestin antibody. Virtually all cells expressed nestin ($\times 400$).

nestin, GFAP, tau, GC . 가 nestin
 (Fig. 1D), GFAP, tau, GC .
 , , ,
 , fibronectin OX-42 . Chambered
 가 가 가

2.

7 EGF가 PLL chambered
 SFM .

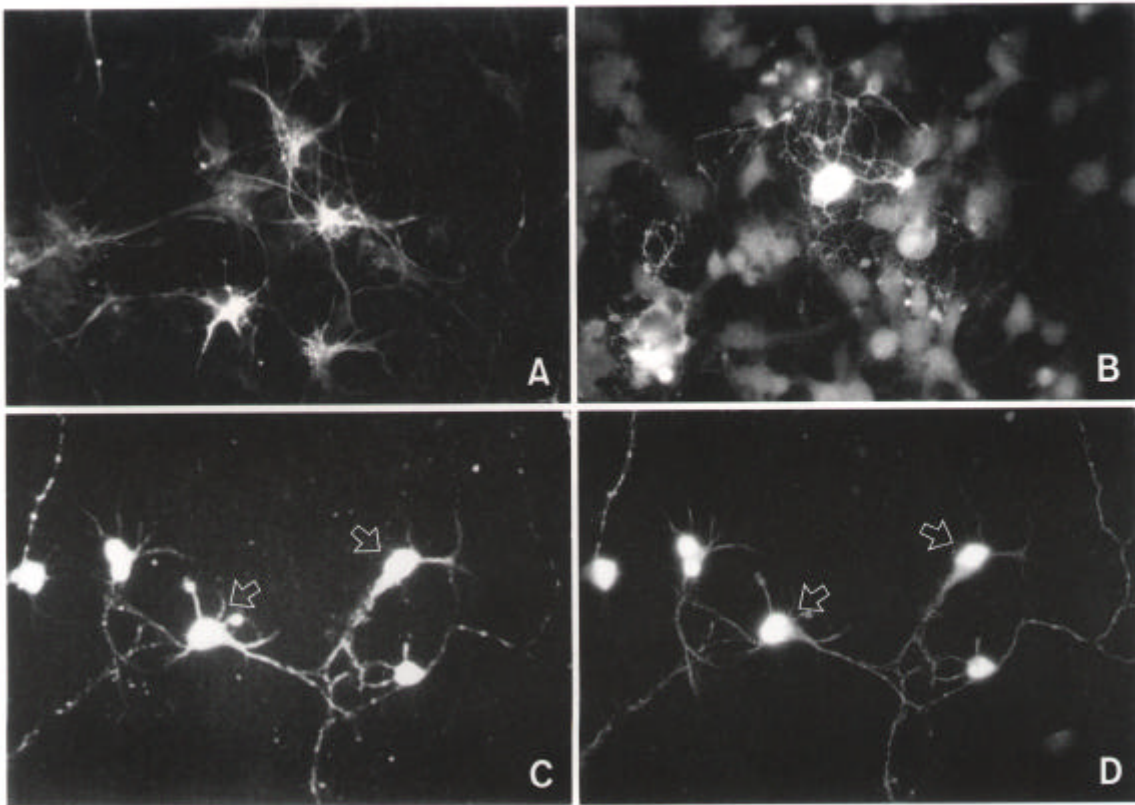


Fig. 2. Immunocytochemical characterization of different cellular types derived from EGF-responsive neuronal stem cells. After 1 week in secondary culture, cells were processed for indirect immunocytochemistry using anti GFAP and anti GC antibodies and for double immunocytochemistry using anti tau and anti neurofilament antibodies. (A) GFAP-positive cells with flat, polygonal morphology ($\times 400$). (B) GC-positive cells ($\times 400$). (C) Tau-positive cells ($\times 400$). (D) Neurofilament-positive cells ($\times 400$). Each arrow in C and D indicates an identical cell.

2, 7
 80% (Fig. 2A).
 (polygonal shape astrocyte)가 , 가
 가 (process bearing astrocyte)가 . 7 GC ,
 (Fig. 2B).
 . Tau (Fig. 2C) neurofilament (Fig. 2D) .
 가 가 . Fig.
 2C Fig. 2D tau neurofilament . 7
 EGF가 7
 가 , 가
 가
 tau dopamine tyrosine hydroxylase 가
 . GABA substance P (Fig. 3)
 neurofilament . TH-ir , serotonin

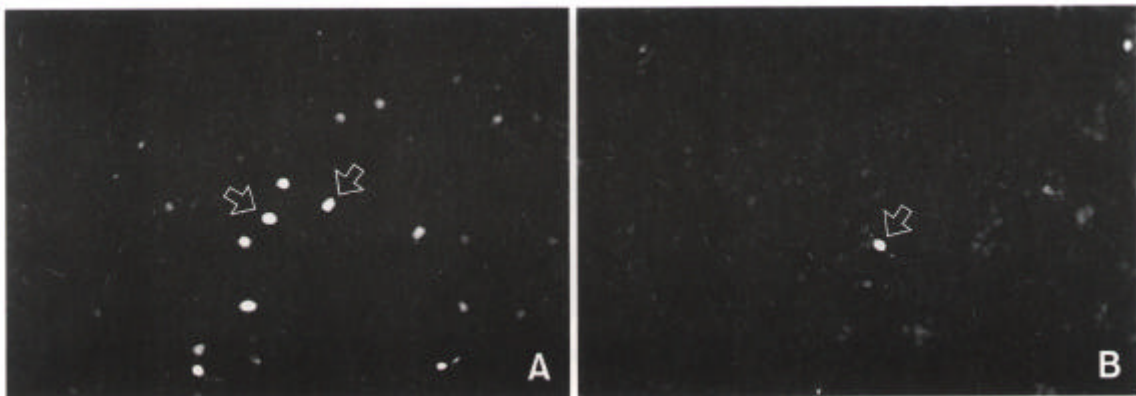


Fig. 3. Characterization of neuronal cells differentiated from EGF-responsive neuronal stem cells. After 1 week in secondary culture, cells were processed for indirect immunocytochemistry using several antibodies. Arrows indicate GABA-positive cells (A, $\times 200$) or substance P-positive cell (B, $\times 200$).

3. PDGF가 TH-ir

chambered PDGF가 SFM (Fig. 4C) cytokine SFM (Fig. 4A)

NGF, BDNF CNTF, GDNF SFM

cytokine SFM

, EGF bFGF SFM

TH-ir cytokine (Fig. 4B), EGF, bFGF, NGF, BDNF CNTF, GDNF가

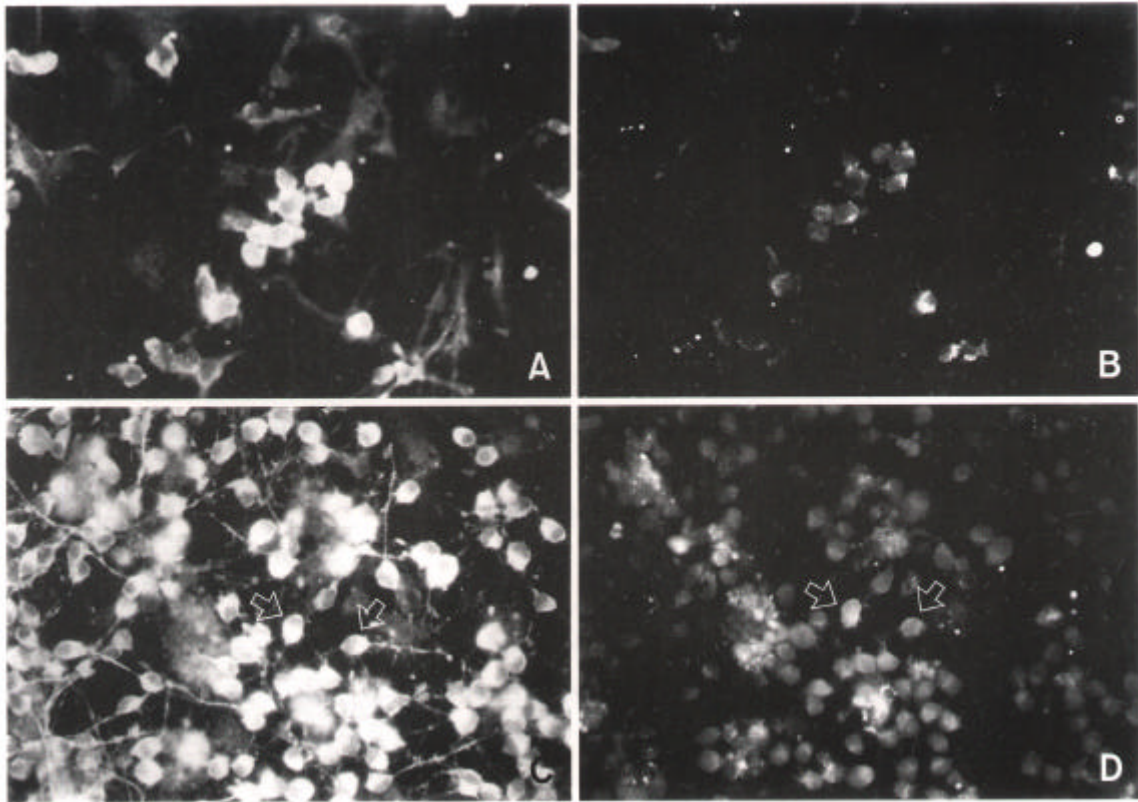


Fig. 4. Effects PDGF on neuronal cell differentiation from EGF-responsive neuronal stem cells. Cell clusters were replated and cultured in serum-free medium (SFM, B) and PDGF-containing SFM (C, D). After 1 week in secondary culture, cells were processed for double immunocytochemistry using anti tau (A, C, $\times 400$) and anti tyrosine hydroxylase (B, D, $\times 400$). There are more tau expressed neuronal cells in culture of PDGF-containing SFM (C) than in culture of SFM (A). Tyrosine hydroxylase immuno-reactive cells are seen in culture of PDGF-containing SFM (D, arrows). Each arrow in C and D indicates an identical cell.

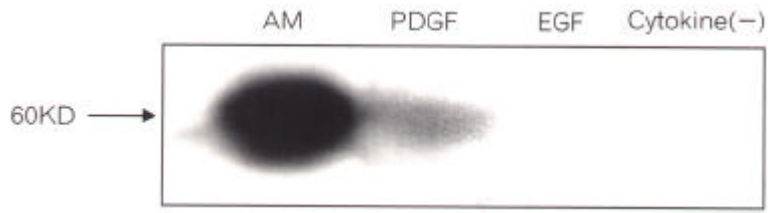


Fig. 5. Western blot analysis of tyrosine hydroxylase in neuronal cells differentiated from neuronal stem cells. AM: adrenal medulla for control. PDGF: cultivated cell in PDGF-containing SFM. EGF: cultivated cells in EGF-containing SFM. cytokine (-): cultivated cell in SFM without cytokine.

, PDGF가 (Fig. 4D).
 PDGF TH-ir
 TH-ir 가 tau 가
 (Fig. 4C, D).

4. Western blot

TH-ir 가 PDGF western blot (Fig.
 5). tyrosine hydroxylase
 PDGF가 western blot
 tyrosine hydroxylase band band가
 EGF cytokine band가
 bFGF, NGF, BDNF CNTF, GDNF가
 tyrosine hydroxylase band

IV.

subpendy-
 mal layer, ,²⁴
 EGF, bFGF, TGF α ,^{2,7,21,25}

subependymal layer

가²⁶ ,

EGF

PLL

chambered

cytokine PDGF 가 .
가 PDGF 가
, 가
. bFGF PDGF
가 ²⁰ PDGF 가
. insulin-like growth factor (IGF)-1
가 . , Brooker IGF-1
가 , IGF-1 가 가
IGF-1 가 ²⁷ .
GABA substance P
TH-ir serotonin . TH-ir serotonin
가 TH-ir
serotonin 가 .
, 가 ^{28,29} .
EGF
^{30,31,32} ,
TH-ir
(committed stem cell, progenitor cell)
^{33,34} . retrovirus-mediated
recombinant gene transfection , , optic tectum
, 가 ,
^{35,36,37} .
PDGF TH-ir , EGF, bFGF, NGF, BDNF,
CNTF, GDNF TH-ir . PDGF
가 가 , TH-ir
. Cytokine
가
가 . GDNF BDNF
TH-ir 가 ^{38,39} PDGF
GDNF BDNF TH-ir .

TH-ir leukin (IL)-1^{40,41} Ling cytokine PDGF inter-IL-1 TH-ir
 , IL-11 leukemia inhibitory factor 가 , dopamine
 , TH-ir 가⁴¹
 TH-ir cytokine ,
 (floor plate neural tube)
 choline
 TH-ir⁴² Cytokine
 TH-ir 가 , dopamine
 가
 TH-ir sonic hedgehog
 (SHH) 가 가⁴³ SHH 45kDa
 가 20 kDa SHH-N (amino-terminal cleavage product)
 SHH-C (carboxy-terminal cleavage product) , SHH-N
^{44,45} (9) SHH-N
 가 TH-ir 가 . SHH
 (notochord) , (floor plate cell)
 (neural tube) ,^{46,47} cyclic AMP-dependent protein kinase A (PKA)
 PKA 가 가 SHH^{48,49}
 Dopamine choline, GABA
 . choline -
 , NGF choline 가
 가 choline acetyltransferase 15 가⁵⁰
 choline .
 laminin GABA
¹⁷ 가¹⁸
 가
 PDGF TH-ir
 . TH-ir TH-ir 가

V.

가

cytokine

dopamine

1. EGF

가

2.

GABA

substance P

, tyro-

sine hydroxylase

(tyrosine hydroxylase immuno-reactive, TH-ir)

serotonin

3. PDGF

TH-ir

EGF, bFGF,

NGF, BDNF CNTF, GDNF

TH-ir

4. PDGF가

TH-ir

tyrosine hydroxylase

western blot

PDGF

TH-ir

. TH-ir

TH-ir

가

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Abstract

Effects of platelet-derived growth factor on dopaminergic neuron differentiation from neuronal stem cells *in vitro*

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*Department of Medicine
Brain Korea 21 Project for Medical Sciences*

(Directed by Associate Professor Dong-Soo Yeon)

It is generally acknowledged that extrinsic factors such as cytokines, cell-to-cell contact and extracellular matrix components are important in generating cellular diversities observed in the central nervous system. Johe *et al.* reported that platelet-derived growth factor (PDGF) enhanced neuronal differentiation and that ciliary neurotrophic factor acted on stem cells to generate astrocytes. This study was undertaken to investigate which cytokine can differentiate dopaminergic neurons from neuronal stem cells obtained from fetal rat fore-brains.

Results are summarized as follows.

1. Neuronal stem cells cultured in EGF-containing serum-free medium subsequently differentiate into neurons, astrocytes, and oligodendrocytes.
2. Differentiated neuronal cells were immuno-reactive for γ -aminobutyric acid or substance P, but not for serotonin and tyrosine hydroxylase.
3. PDGF converted neuronal stem cells into tyrosine hydroxylase immuno-reactive (TH-ir) cells, but epidermal growth factor, basic fibroblast growth factor, nerve growth factor, brain-derived neurotrophic factor, ciliary neurotrophic factor, or glial cell-derived neurotrophic factor did not.
4. PDGF-induced TH-ir cells from neuronal stem cells were confirmed using western blot.

These results suggest that PDGF may be an important regulator in differentiation of TH-ir cells from neuronal stem cells, and cytokines can affect neurotransmitter phenotype of the neurons generated from neuronal stem cells.

Key Words: differentiation, neuronal stem cell, dopaminergic neuron, platelet-derived growth factor, epidermal growth factor, neuron