Neurotrophic Action and Signalling of Epidermal Growth Factor in PC12h-R Cells and Cultured Cerebral Cortical Neurons

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上皮成長因子の神経栄養因子作用機序の解析

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Contents

Abbreviations

AChE	acetylcholinesterase
bFGF	basic fibroblast growth factor
CNS	central nervous system
CPTcAMP	8-(4-chlorophenylthio)-cyclic adenosine 3,5-monophosphate
BW248C51	1,5-bis(4-allyldimethyl-ammoniumphenyl)-pentan-3-one-dibromide
DMEM	Dulbecco's modified Eagle's medium
EGF	epidermal growth factor
EDTA	ethylenediamine tetraacetic acid
ERK	extracellular signal-regulated kinase
GFAP	glial fibrillary acidic protein
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
HS	horse serum
MAP	mitogen-activated protein
MAP2	microtubule-associated protein 2
MBP	myeline basic protein
MEM	minimum essential medium
MTT	3-(4,5--dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide
NGF	nerve growth factor
PBS	phosphate-buffered saline
PMSF	phenylmethanesulfonyl fluoride
PNCS	precolostrum newborn calf serum

SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
TGF α	transforming growth factor α
TH	tyrosine hydroxylase
WGA	wheat germ agglutinin

要　旨

上皮成長因子 (EGF) は、種々の細胞に対し細胞増殖促進の作用を示すことが知られている。一方、中枢神経系においては、大脳皮質ニューロン等に対し神経栄養因子として働き、生存維持や突起伸展の作用を持つことが知られている。本研究において、私は、ラット副腎髄質クロム親和細胞腫由来の PC12h-R 細胞が、EGF に応答して神経細胞様に分化すること、および培養大脳皮質ニューロンにおいて高酸素負荷にともなうアポトーシスを EGF が抑制することを見いだした。そして、これらの系を用い、EGF の神経栄養因子作用を伝達する細胞内シグナルの解析を行った。

PC12h-R 細胞を用いた解析

PC12 細胞は、EGF に対して細胞増殖促進の応答を示すことが知られている。一方、神経栄養因子の代表的存在である神経成長因子 (NGF) に対しては神経細胞様分化応答を示す。EGF と NGF の作用は、EGF と NGF がそれぞれのレセプターである EGF レセプターと $p140^{trkA}$ に結合することにより始まる。EGF レセプターと $p140^{trkA}$ はともにレセプター型チロシンキナーゼであり、リガンドの結合にともない活性化され、レセプター自身のチロシン残基のリン酸化、即ち自己リン酸化を引き起こす。EGF と NGF はともに、こうしたレセプター型チロシンキナーゼの活性化を介して、MAP (mitogen-activated protein) キナーゼやその他のシグナル蛋白質を活性化することが知られている。しかし、上述の様に PC12 細胞に対して、EGF は増殖促進、NGF は分化誘導という全く異なる作用をもたらすにもかかわらず、その両者のシグナル伝達機構は極めて似かよったものであることが判明している。

私は、PC12 細胞の亜株である PC12h-R 細胞が、EGF に応答して NGF の時と同様に神経突起の伸展、細胞増殖能の低下、チロシン水酸化酵素の発現上昇およびアセチルコリンエステラーゼの活性上昇を示し、神経細胞様に分化することを見いだした。続いて私は、PC12h-R 細胞における EGF 応答の原因を探る目的で、EGF に

よる細胞内シグナルの解析を、PC12h-R 細胞の親株で EGF に対して分化応答を示さないPC12h細胞と比較することにより行った。その結果、PC12h-R細胞では、EGFによる EGF レセプターのチロシンリン酸化および MAP キナーゼの活性化が PC12h細胞と比べ顕著に持続していることを見いだした。また、PC12h-R 細胞では、EGF添加にともなう EGF レセプター量の減少速度が低下していることを観察した。EGFレセプターは、EGF の結合にともない細胞内に取り込まれリソソームで分解を受けることにより down-regulation されることが知られており、PC12h-R 細胞では、そのEGF レセプターの down-regulation 能 が低下しているものと思われる。また、私は、PC12h 細胞において、NGF による $p140^{trkA}$ のチロシンリン酸化が、EGF による EGFレセプターのチロシンリン酸化と比べ顕著に持続していることを観察した。それに対し、PC12h-R 細胞における EGF による EGF レセプターのチロシンリン酸化は顕著に持続しており、PC12h 細胞での NGF による $p140^{trkA}$ のチロシンリン酸化と類似の経時変化を示した。MAP キナーゼの活性化の経時変化においても、レセプターのチロシンリン酸化の持続性に対応する結果が得られた。以上のことから、PC12 細胞の神経細胞様分化応答は、レセプター型チロシンキナーゼの活性化の持続、およびそれにともなう MAP キナーゼの持続的活性化により誘導されるものと考えられる。即ち、レセプター型チロシンキナーゼの活性化の持続時間が、PC12 細胞における分化と増殖を決定しているのかもしれない。また、PC12h-R 細胞では、EGF レセプターのdown-regulation 能が低下しているため、EGF レセプターの活性化が持続し、その結果、EGF に対し分化応答を示すのではないかと考えられる。

培養大脳皮質ニューロンを用いた解析

私は、胎生20日齢ラット大脳皮質ニューロンを 高酸素濃度 (50% O_2) 下で培養した際に起こる細胞死を、EGF が抑制することを見いだした。この高酸素負荷による神経細胞死は、RNA 合成阻害剤であるアクチノマイシン D および蛋白質合成阻害剤であるシクロヘキシミドにより抑えられることを観察した。さらに、高酸素濃度下

で培養した大脳皮質ニューロンにおいてクロマチンの凝集が観察されることから、この神経細胞死がアポトーシスの性質を示すことがわかった。EGF は、この神経細胞死を濃度依存的に抑制し、その EC_{50} 値は 0.3 ng/ml であり、EGF レセプターの Kd 値とほぼ同様の値を示した。また、培養大脳皮質ニューロン中に、抗 EGF レセプター抗体によって染色されるニューロンおよび EGF に応答して抗 c-Fos 抗体によって染色されるニューロンを観察した。培養は、細胞分裂阻害剤であるシトシンアラビノシド存在下で行っており、しかも抗グリア繊維性酸性蛋白質抗体で染色される細胞数の割合は、抗微小管関連蛋白質 2 抗体によって染色されるニューロン数の 0.5% 以下であった。以上のことから、培養大脳皮質ニューロンに対する EGF の効果は、グリア細胞を介した二次的なものではなく、ニューロンへの直接作用によって発揮されていると考えられる。次に私は、細胞内シグナルの持続性に注目し、培養大脳皮質ニューロンにおける、EGF による EGF レセプターのチロシンリン酸化および MAP キナーゼ活性化の経時変化を調べた。その結果、培養大脳皮質ニューロンでは、PC12h-R 細胞の場合と同様、EGF による EGF レセプターのチロシンリン酸化が顕著に持続していた。また、MAP キナーゼの活性化においても持続傾向があることを観察した。これらのことから、EGF は、大脳皮質ニューロンにおいて、その細胞内シグナルを持続させることによって、神経栄養因子作用を発揮していると考えられる。

以上のことから、EGF による細胞内シグナルの持続、特に EGF レセプターの持続的活性化が、EGF の神経栄養因子作用機序のひとつの大きな特徴であると考えられる。また、PC12h-R細胞は、大脳皮質ニューロンと非常によく似た EGF に対する細胞内シグナルの持続性を示すことから、EGF の神経栄養因子作用機序をより詳細に解明するためのよいモデル系となると考えられる。

Summary

Epidermal growth factor (EGF), a conventional mitogenic factor, stimulates the proliferation of various types of cells including epithelial and fibroblast cells. In a rat pheochromocytoma PC12 cell line, EGF also promotes cell proliferation. On the other hand, nerve growth factor (NGF), a well-known neurotrophic factor, induces neuronal differentiation. EGF and NGF bind to and activate receptor tyrosine kinases, the EGF receptor and $p140^{trkA}$, respectively. The both activated receptor tyrosine kinases are autophosphorylated on tyrosine residues and initiate intracellular signalling. EGF and NGF activate identical signalling elements including phospholipase C γ (PLC γ), phosphatidylinositol-3 (PI-3) kinase, $p21^{ras}$, and mitogen-activated protein (MAP) kinases.

PC12h-R is a variant isolated from long-term cultured PC12h cells, a subclone of PC12 cells. I found that PC12h-R cells exhibited a neuron-like phenotype, including neurite outgrowth, attenuation of cell proliferation, and increased level of tyrosine hydroxylase protein synthesis and of acetylcholinesterase activity, in response to EGF as well as to NGF. PC12h cells do not show EGF-induced neuronal differentiation. I found that EGF evoked prolonged tyrosine phosphorylation and activation of MAP kinases in PC12h-R cells, compared with those in PC12h cells. EGF induced sustained tyrosine phosphorylation and a decreased rate of down-regulation of the EGF receptor in PC12h-R, but not in PC12h cells. Therefore, decreased down-regulation of the EGF receptor might result in its prolonged activation, leading to the sustained activation

of MAP kinases and neuronal differentiation in PC12h-R cells. I suggest that the PC12h-R cell line is a useful model for elucidating the molecular mechanisms of the neurotrophic action of EGF.

In addition, the duration of NGF-induced tyrosine phosphorylation of $p140^{trkA}$ in PC12h cells was much longer than that of EGF-induced tyrosine phosphorylation of the EGF receptor in PC12h cells, although the duration of NGF-induced phosphorylation of $p140^{trkA}$ in PC12h cells was similar to that of EGF-induced tyrosine phosphorylation of the EGF receptor in PC12h-R cells. The duration of the activation of MAP kinases was basically dependent on that of the activation of the EGF receptor or $p140^{trkA}$. I suggest that the sustained activation of the receptor tyrosine kinase results in the sustained activation of MAP kinases and also the neuronal differentiation of PC12 cells. Namely, the duration of the receptor tyrosine kinase activity is thought to determine the cellular responses between the differentiation and the proliferation in the PC12 cell lineage.

In the central nervous system, EGF acts as a neurotrophic factor on cultured cerebral cortical, subneocortical telencephalic and cerebellar neurons, enhancing neurite-outgrowth and cell survival. I found that EGF prevented the death of rat cerebral cortical neurons cultured in 50% oxygen atmosphere. The oxygen-induced neuronal death showed the features of apoptotic cell death; that is, chromatin condensation in nuclei and blockade by inhibitors of RNA or protein synthesis. As I found in PC12h-R cells, the cortical neurons showed the EGF-induced sustained tyrosine phosphorylation of the EGF receptor and the sustained activation of MAP kinase. EGF might exert the neurotrophic effect

through the sustained activation of the EGF receptor which leads to the prolonged activation of MAP kinase.

Duration of EGF- and NGF-induced signalling in PC12h and PC12h-R cells and cerebral cortical neurons

Response	PC12h		PC12h-R		Ctx[a]
	EGF	NGF	EGF	NGF	EGF
Tyrosine phosphorylation of EGF receptor or p140^{trkA} [b]	+	++	++	+++	++
Activation of MAP kinases [b]	+	++	+++	+++	++
Neuronal differentiation [c]	−	+	+	+	

[a] cultured cerebral cortical neurons
[b] +, transient; ++, sustained; +++, strongly sustained
[c] −, does not induce; +, induce

Part 1:

PC12h-R Cell, a Subclone of PC12 Cells, Shows EGF-induced Neuronal Differentiation and Sustained Signalling

Abstract

Epidermal growth factor (EGF) does not induce neuronal differentiation unlike nerve growth factor but promotes the growth of the rat pheochromocytoma PC12 cells. I found that PC12h-R, a subclone of PC12 cells, differentiated into neuron-like cells in response to EGF as well as to NGF. PC12h-R cells treated with EGF extended neurites, attenuated cell proliferation, and increased the levels of tyrosine hydroxylase protein synthesis and of acetylcholinesterase activity as those treated with NGF. The EGF-induced differentiation of PC12h-R cells was not mediated by the indirect activation of $p140^{trkA}$ by EGF. I found that EGF induced the sustained tyrosine phosphorylation of the EGF receptor, Shc proteins and mitogen-activated protein (MAP) kinases, and the prolonged activation of MAP kinases in PC12h-R cells compared with those in the parent PC12h, which does not show EGF-induced differentiation. In addition, the rate of EGF-induced down-regulation of the EGF receptor was decreased compared with that in PC12h cells. Furthermore, I found that the duration of EGF-induced tyrosine phosphorylation of the EGF receptor in PC12h-R cells was similar to that of NGF-induced $p140^{trkA}$ in PC12h cells. The EGF-induced phosphorylation of the EGF receptor in PC12h cells was less sustained than that of $p140^{trkA}$ by NGF. These findings suggest that the EGF-induced neuronal differentiation of PC12h-R cells is due to the sustained signalling, resulting from the decreased down-regulation of the EGF receptor, and that the duration of the receptor tyrosine kinase activity determines the cellular responses of the PC12 cell lineage. I conclude that the sustained

activation of the receptor tyrosine kinase induces neuronal differentiation in the PC12 cell lineage, whereas the transient activation promotes proliferation.

Introduction

Nerve growth factor (NGF) is known to be a neurotrophic factor for sympathetic, sensory, basal forebrain cholinergic, and striatal cholinergic neurons (Thoenen and Barde, 1980; Hartikka and Hefti, 1988; Hatanaka *et al.*, 1988; Abiru *et al.*, in press). Various peptide growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor and insulin-like growth factor, also act as neurotrophic factors, enhancing the survival and differentiation of post-mitotic neurons, as well as do as mitogens on mitotic cells (Hefti *et al.*, 1993). For example, EGF, a potent mitogen in a variety of cell types (Carpenter and Cohen, 1979), promotes the survival and process outgrowth of cultured subneocortical telencephalic, cerebral cortical and cerebellar neurons (Morrison *et al.*, 1987; Morrison *et al.*, 1988; Kornblum *et al.*, 1990). Studies of mechanisms by which these growth factors act on the neurons are proceeding, however the effects of neurotrophic factors are relatively well understood.

The rat pheochromocytoma PC12 cell line is a useful model system with which to elucidate the mechanisms of action of neurotrophic factors (Greene and Tischler, 1976). PC12 cells differentiate into sympathetic neuron-like cells in response to NGF. PC12 cells treated with NGF show dramatic cellular changes, including extension of neurites, cessation of mitosis, increased neurotransmitter biosynthesis, and development of Na^+ and Ca^{2+} channels (Halegoua *et al.*, 1991). On the other hand, EGF promotes cell growth but does not induce neuronal differentiation in PC12 cells (Huff *et al.*, 1981).

NGF and EGF bind to their receptors, p140^{trkA} and the EGF receptor, respectively, which possess the tyrosine kinase domains (Schlessinger and Ullrich, 1992). Their binding to these receptors is essential for their functions. These tyrosine kinase receptors activated by ligand binding initiate intracellular signalling, activating several enzymes and effectors, including phospholipase C γ (PLC γ) (Kim *et al.*, 1991; Vetter *et al.*, 1991), phosphatidylinositol-3 (PI-3) kinase (Ohmichi *et al.*, 1992; Soltoff *et al.*, 1992), Shc proteins, p21^{ras}, Raf protein, S6 kinase, and mitogen-activated protein (MAP) kinases or extracellular signal-regulated kinases (ERKs) (Blenis, 1993; Nishida and Gotoh, 1993; Schlessinger, 1993). Shc protein, Grb2 protein, Sos protein, p21^{ras}, Raf protein, MAP kinase kinase, and MAP kinase compose the Ras-MAP kinase cascade initiated by the tyrosine kinase receptors. Shc protein and MAP kinase are phosphorylated on tyrosine residues by tyrosine kinase receptors and on tyrosine/threonine residues by MAP kinase kinase, respectively. Although NGF and EGF activate almost the same signals including the Ras-MAP kinase cascade, they exert quite different effects on PC12 cells as described above (Chao, 1992; Schlessinger and Ullrich, 1992). Recently, it has been reported that the NGF-induced activation of p21^{ras} and MAP kinase is sustained as compared with that induced by EGF (Qiu and Green, 1992; Traverse *et al.*, 1992). In addition, PC12 cells exhibit neuronal differentiation when the Ras-MAP kinase cascade is constitutively activated (Guerrero *et al.*, 1988; Wood *et al.*, 1993; Cowley *et al.*, 1994). These results indicate that the duration of the factor-induced signalling determines the cellular response of PC12 cells between cell prolifera-

tion and neuronal differentiation.

PC12h is a subclone of PC12 cells, which differentiates into neuron-like cells in response to NGF but not to EGF, as PC12 cells do (Hatanaka, 1981). Here, I report that PC12h-R (Hatanaka and Tsukui-Tamagawa, 1984; Ikenaka *et al.*, 1990; Yamada *et al.*, 1994), a subclone isolated from long-term cultured PC12h cells, shows neuronal differentiation in response to EGF as well as to NGF. I also found that EGF induced the sustained tyrosine phosphorylation of the EGF receptor, Shc proteins and MAP kinases, and the sustained activation of MAP kinases in PC12h-R cells in comparison with those in PC12h cells. In this study, I examined relationship between the factor-induced neuronal differentiation and the duration of the induced signalling in the PC12 cell lineage.

Materials and Methods

Cell culture

PC12h and PC12h-R cells were maintained in 75 cm^2 flasks (Costar) using Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 5% (v/v) precolostrum newborn calf serum (PNCS, Mitsubishi Kagaku) and 5% (v/v) heat-inactivated (56℃, 30 min) horse serum (HS, Gibco). The cells were cultured in 100-mm collagen-coated Falcon dishes (surface area, 55 cm^2; to examine the tyrosine phosphorylation and the amount of $p140^{trkA}$), 60-mm collagen-coated Falcon dishes (surface area, 21 cm^2; to detect the tyrosine phosphorylation of total cellular proteins and MAP kinase, to assay MAP kinase activity, to determine the number of viable cells, and to examine the amount of TH protein), 35-mm collagen-coated Falcon dishes (surface area, 9 cm^2; to determine the number of neurite-bearing cells, to assay AChE activity, and to examine the amount of EGF receptor protein), or in collagen-coated 24-well Costar plates (surface area, 2 cm^2; to observe neurite outgrowth). Cells were cultured in DMEM supplemented with 5 μg/ml human transferrin, 5 μg/ml bovine insulin, and 20 nM progesterone (TIP/DMEM) (Hatanaka, 1983) (to observe neurite outgrowth, to determine the number of neurite-bearing cells, to examine the level of TH protein, and to assay AChE activity), or in the DMEM containing serum (to detect protein tyrosine phosphorylation and MAP kinase, to assay of MAP kinase activity, to determine the number of viable cells, and to examine the amounts of EGF receptor protein and $p140^{trkA}$). To detect protein tyrosine phosphorylation and MAP kinase, and to assay MAP kinase activity, the medium was changed to that without serum 2 hr before adding NGF. TH protein and $p140^{trkA}$ were metabolically labeled with [^{35}S]methionine by incubating PC12h and PC12h-R cells at 37℃ for 2.5 hr in methionine-free DMEM containing 100 μCi/ml [^{35}S]Translabel (ICN).

Antibodies, factors, and reagents

Anti-p140^{trkA} antiserum was raised in rabbits against a synthetic peptide corresponding to the 14 carboxyl-terminal amino acids of human p140^{trkA}, conjugated to keyhole limpet hemocyanin (Calbiochem) using glutaraldehyde according to Harlow and Lane (1988). Affinity-purified anti-p140^{trkA} antibodies were obtained by applying the anti-p140^{trkA} antisera to Affi-Gel 10 (BioRad) coupled with the antigen peptide. The anti-TH monoclonal antibody, PCTH-7, was generated as described previously (Hatanaka and Arimatsu, 1984). Anti-phosphotyrosine monoclonal antibody (4G10) and anti-EGF receptor antibody were purchased from Upstate Biotechnology Inc. Anti-MAP kinase antibody was obtained from Santa Cruz Biotechnology Inc. NGF (2.5S-form) was prepared from male mouse submandibular glands according to the method of Bocchini and Angeletti (1969) with the modification of Suda et al (1978). EGF from mouse submaxillary grands and human recombinant transforming growth factor α (TGF α) were purchased from Toyobo Co., Ltd. and Otsuka pharmaceutical Co., Ltd., respectively. 8-(4-chlorophenylthio)-cAMP (CPTcAMP) and 1,5-bis(4-allyldimethyl-ammoniumphenyl)-pentan-3-one-dibromide (BW248C51) were purchased from Sigma.

Viable cell counting

The number of viable PC12h and PC12h-R cells cultured in the absence or presence of 50 ng/ml NGF or EGF was determined in a hemocytometer by means of nigrosin dye exclusion. The cultured cells were completely dissociated with 0.1% trypsin (Difco) for a few minutes .

Acetylcholinesterase assay.

The cells were washed once with phosphate-buffered saline and lysed in ice-

cold 20 mM Tris-HCl pH 7.5 containing 0.5% Triton X-100, 10 mM $MgCl_2$ and 150 mM NaCl. The lysates were assayed for acetylcholinesterase (AChE) activity, using 0.5 mM acetylthiocholine (Sigma) and 0.4 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (Ellman *et al.*, 1961). The enzyme reaction proceeded at 37℃ and was stopped with an equal volume of 5% SDS. The absorbance was measured photometrically at 412 nm. The specific activity was calculated on the basis of protein concentrations in the enzymatically assayed samples determined by the BCA protein assay (Pierce).

Immunoblotting

Cells were washed once with ice-cold Tris-buffered saline (TBS), lysed in a buffer containing 1% SDS, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM Tris-HCl pH 7.5, and 1 mM PMSF to detect EGF-induced tyrosine phosphorylation, or in that containing 1% SDS, 5 mM EDTA, 10 mM Tris-HCl pH 7.5 and 1mM PMSF to quantify the EGF receptor, and boiled for 5 min. The protein concentration was determined using the BCA protein assay, then 20 μg (for detection of the tyrosine phosphorylation and MAP kinase) or 40 μg (for detection of the EGF receptor) of protein were resolved by electrophoresis on 9.5% (for detection of the tyrosine phosphorylation), 10% (for detection of MAP kinase) and 7.5% (for detection of the EGF receptor) polyacrylamide gels according to Laemmli (1970). Proteins were transferred to a nitrocellulose membrane in 0.1 M Tris base, 0.192 M glycine, and 20% (v/v) methanol using a semi-dry electrophoretic transfer system. The membrane was blocked with 5% (w/v) nonfat dry milk and 0.1% Tween 20 in TBS at room temperature for at least 1hr, washed three times in 0.1% Tween 20/TBS (T-TBS), then incubated with 1 μg /ml anti-phosphotyrosine antibody 4G10, 25 ng/ml anti-MAP kinase antibody or 1 μg /ml anti-EGF receptor antibody in T-TBS at room temperature for 1-2 hr. After 3 washes with T-TBS, the

membrane was incubated with horseradish peroxidase-coupled sheep anti-mouse IgG secondary antibody (Amersham) (for detection of tyrosine phosphorylation) or horseradish peroxidase-coupled goat anti-rabbit IgG secondary antibody (Cappel) (for detection of MAP kinase) diluted to 1:1,000 with T-TBS at room temperature for 1 hr. The EGF receptor was detected by means of the ABC kit (Vector Labo.) after the incubation with the primary antibody. The membranes were then washed four times with T-TBS, and visualized using the ECL chemiluminescence system (Amersham).

Immunoprecipitation and WGA-agarose precipitation

PC12 cells were washed once with ice-cold TBS, and lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 20 mM Tris-HCl pH 7.5, 1 mM PMSF, and 5 μg/ml aprotinin to detect $p140^{trkA}$ tyrosine phosphorylation, or in a buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl pH 7.5, 1 mM PMSF, and 5 μg/ml aprotinin to quantify the EGF receptor, $p140^{trkA}$, and TH proteins. Lysates were centrifuged at 10,000 $\times$ g at 4℃ for 30 min, then the protein concentration of the clarified lysate was determined by the BCA protein assay. To quantify $p140^{trkA}$ and TH proteins, the clarified lysates were rotated with Protein G-Sepharose (Pharmacia) for 1 hr at 4℃, then separated by centrifugation at 10,000 $\times$ g at 4℃ for 1 min. Antibodies were added to the lysates at an excess amount; i.e., anti-EGF receptor antibody (5 μg) for detection of EGF receptor tyrosine phosphorylation, anti-$p140^{trkA}$ antiserum (10 μl) for detection of $p140^{trkA}$ tyrosine phosphorylation, affinity-purified anti-$p140^{trkA}$ antibody for quantification of $p140^{trkA}$ protein, or the anti-TH monoclonal antibody (20 μg) were added and incubated at 4℃ for 1-3 hr. Protein G-Sepharose (10-15 μl) was then added, and rotated at 4℃ for 30-60 min. To immunoprecipitate TH protein, rabbit anti-mouse IgG antibody (40

μg IgG: Cappel) was added prior to the Protein G-Sepharose. The immune complexes were pelleted by centrifugation at 10,000 × g at 4℃ for 1 min, then washed 3-5 times with the lysis buffer. The immune complexes were eluted with sample buffer (0.125 M Tris-HCl pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS, and 10% (v/v) β-mercaptoethanol), boiled for 3 min, then recovered by centrifugation for 5 min in a microfuge. To precipitate the EGF receptor, the clarified lysate was incubated with WGA-agarose (25 μl, Vector Labs.) for 2 hr at 4℃, then centrifuged at 10,000 × g at 4℃ for 1 min. The precipitated WGA-agarose was washed five times with the lysis buffer, heated to 56℃ for 30 min in the sample buffer, then the eluates were recovered after centrifugation for 5 min in a microfuge. The eluates were resolved by electrophoresis on 7.5, 10 and 7% SDS-polyacrylamide gels to analyze $p140^{trkA}$, TH and the EGF receptor, respectively. The gels were then immunoblotted with the anti-phosphotyrosine antibody to detect $p140^{trkA}$ tyrosine phosphorylation, or fluorographed with EN^3HANCE (NEN) to quantify $p140^{trkA}$ and TH proteins.

Assay of MAP kinase activity in renatured gels

Samples (20 μg of protein) were prepared as described above for the detection of tyrosine phosphorylation of total cellular proteins. MAP kinase activity was analyzed by means of the kinase renaturation gel assay according to Gotoh *et al.*(1990) with some modifications. Briefly, the samples were resolved by electrophoresis on a 12.5% SDS-polyacrylamide gel containing 0.5 mg/ml myelin basic protein (MBP). Thereafter SDS was removed by washing with 20% (v/v) 2-propanol and 50 mM Tris-HCl pH 8.0, then the gel was denaturated with 6 M guanidine-HCl in 50 mM Tris-HCl pH 8.0 and 5 mM β-mercaptoethanol. The gel was renatured at 4℃ overnight in a buffer containing 0.04% (w/v) Tween 40, 50 mM Tris-HCl pH 8.0, and 5 mM β-mercaptoethanol, then incubated at 22℃ for

1hr in 40 mM Hepes-NaOH pH 7.5 containing 0.1 mM EGTA, 20 mM $MgCl_2$, 2 mM dithiothreitol, and 25 μM [γ-^{32}P] ATP (25 μCi : Amersham). The gel was washed with 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate and dried. The MAP kinase activity was quantified using the FUJIX Bio-imaging Analyzer (Fuji Film Co. Ltd.), then the gel was exposed to X-ray film (Kodak).

Results

EGF-induced neurite outgrowth

As shown in Fig. 1, PC12h-R cell, a variant of PC12 cells, extended neurites in response to EGF as well as to NGF. EGF induced short processes within one day of exposure, and long, branched processes and neurite networks after five days. To examine the dose dependence of this effect of EGF, I measured the percentage of the cells bearing neurites that were longer than twice the cell body, following exposure to various concentrations of EGF for two days. As a result, EGF induced neurite outgrowth in a concentration-dependent manner (Fig. 2). The effect was maximal at an EGF concentration of 1 ng/ml, and half maximal at 0.03 ng/ml.

EGF-induced attenuation of cell proliferation

NGF attenuates the proliferation of PC12 cells when it induces their neuronal differentiation (Halegoua *et al.*, 1991). To investigate the effect of EGF on the growth of PC12h-R cells, I counted the number of PC12h-R cells exposed or not, to NGF or EGF for 6 days. PC12h-R cell proliferation decreased in response to EGF, as they do to NGF (Fig. 3A). The number of PC12h-R cells treated with EGF was only half that of the untreated cells.

EGF-induced increase of TH protein synthesis

We previously reported that NGF induces the increase of protein synthesis

of TH in PC12h-R cells (Yamada *et al.*, 1994). I examined whether EGF also induces the increase of synthesis of TH protein. Using the anti-TH monoclonal antibody, TH protein was immunoprecipitated from lysates of biosynthetically [^{35}S] methionine-labeled PC12h-R cells cultured in the absence or presence of EGF or NGF for 5 days (Fig. 3B). There was one radio-labeled protein with an apparent molecular weight of 60 kDa, which agreed with that of TH protein. The level of TH protein increased in response to EGF as well as to NGF in PC12h-R cells. The amount of TH proteins in EGF- and NGF-treated PC12h-R cells was about 1.8 times more than that in the untreated cells.

cAMP-induced neurite outgrowth

In PC12 cells, analogs of cyclic AMP (cAMP) or cAMP-enhancing reagents induce only short processes which are different from those induced by NGF (Greene and Shooter, 1980). PC12h-R cells extended long neurites in response to 8-(4-chlorophenylthio)-cAMP (CPTcAMP), an analog of cAMP (Fig. 4). However, CPTcAMP-induced morphological change was distinct from that induced by NGF or EGF. Many of PC12h-R cells incubated with CPTcAMP became flat and the number of neurites per cell body increased.

EGF-induced increase of acetylcholinesterase activity.

In PC12 cells, acetylcholinesterase (AChE) activity increases in response to NGF (Greene and Rukenstein, 1981). AChE activities in PC12h-R cells incubated with NGF, EGF, or CPTcAMP also increased (Fig. 5A). However, the amount of

AChE activity was increased more by NGF or EGF than by CPTcAMP. The AChE activity measured in PC12h-R cells was almost entirely due to true AChE, since over 94% of the activities disappeared in the presence of a specific inhibitor of AChE, BW248C51.

EGF induced the increase of AChE activity in a dose-dependent manner (Fig. 5B). The effect was half maximal at an EGF concentration of 0.03 ng/ml, and the concentration-dependence curve was similar to that of neurite outgrowth induced by EGF.

These results indicate that PC12h-R cells incubated with EGF extende neurites and increase AChE activities in a similar manner to those exposed to NGF, but differently from those incubated with CPTcAMP. In summary, EGF induced extension of neurites, attenuation of cell growth, and increase of TH protein synthesis and AChE activity in PC12h-R cells in a similar manner to that of NGF, and the PC12h-R cells treated with EGF differentiated into neuron-like cells.

Participation of $p140^{trkA}$ in the response of PC12h-R cells to EGF

The activation of $p140^{trkA}$, the NGF receptor possessing a tyrosine kinase domain, is essential to the NGF response in PC12 cells (Barbacid, 1993). $p140^{trkA}$ is autophosphorylated on tyrosine residues following its activation by NGF-binding. To examine whether or not the response to EGF in PC12h-R cells is mediated indirectly by $p140^{trkA}$, it was immunoprecipitated with the anti-$p140^{trkA}$ antiserum from lysates of PC12h-R cells treated or not with EGF or NGF for 5 min

and 4 hr, and probed using the anti-phosphotyrosine monoclonal antibody in Western blot analysis (Fig. 6). I observed that $p140^{trkA}$ was tyrosine phosphorylated in PC12h-R cells treated with NGF, but not in those exposed to EGF for 5 min and 4 hr. Therefore, EGF did not induce the tyrosine phosphorylation of $p140^{trkA}$ in PC12h-R cells.

K252a is a kinase inhibitor of $p140^{trkA}$ and it blocks NGF-induced neuronal differentiation of PC12 cells (Koizumi *et al.*, 1988; Knüsel and Hefti, 1992). PC12h-R cells were cultured with or without 50 ng/ml NGF or EGF in the presence or absence of 200 nM K252a (Fig. 7). In PC12h-R cells, K252a inhibited NGF-induced neurite outgrowth but not that induced by EGF. Inversely, K252a enhanced EGF-induced neurite extension. PC12h-R cells incubated with both EGF and K252a showed long and branched processes and a dense network of neurites compared with those exposed to EGF alone. K252b, an analog of K252a (Knüsel and Hefti, 1992), also failed to block the EGF-induced neurite outgrowth like K252a. K252b-induced enhancement of neurite extension in PC12h-R cells exposed to EGF was much weaker than that induced by K252a (data not shown). That is, EGF-induced neurite outgrowth of PC12h-R cells was not due to the activation of $p140^{trkA}$.

In addition, the EGF-induced neurite extension in PC12h-R cells was not blocked by anti-NGF serum (data not shown). These results indicated that the response of PC12h-R cells to EGF is not mediated by either NGF or $p140^{trkA}$.

TGF α -induced neurite outgrowth

Transforming growth factor α (TGFα) binds to and activates the EGF receptor (Derynck, 1988). To examine whethere or not TGFα induces neurite outgrowth of PC12h-R cells as well as EGF does, PC12h-R cells were cultured with or without TGFα for 5 days (Fig. 8). In PC12h-R cells, TGFα induced neurite outgrowth, as EGF or NGF did. This result indicated that the EGF-induced differentiation of PC12h-R cells is elicited by the activation of the EGF receptor.

EGF-induced tyrosine phosphorylation of cellular proteins

The neuronal differentiation of the PC12 cell lineage might require the sustained activation of the Ras-MAP kinase cascade (Qiu and Green, 1992; Traverse *et al.*, 1992). In the Ras-MAP kinase cascade initiated by tyrosine kinase receptor, several proteins including tyrosine kinase receptor, Shc protein and MAP kinase are phosphorylated on tyrosine residues when they are activated (Nishida and Gotoh, 1993; Schlessinger, 1993). I investigated whether the EGF-induced tyrosine phosphorylation signal was sustained in PC12h-R cells. For comparison, I used PC12h cells (the parent of PC12h-R cells) which are not induced to differentiate by EGF, as controls. Lysates prepared from PC12h and PC12h-R cells after exposure to EGF for various periods, were subjected to Western blot analysis using the anti-phosphotyrosine monoclonal antibody (Fig. 9). EGF induced the tyrosine phosphorylation of at least five cellular proteins (180, 52, 46, 44, and 42 kDa) in both cell lines and it reached the maximum within at least 5 min after exposure to EGF. In PC12h cells, the level of tyrosine

phosphorylation of the 180 kDa protein and that of the 52, 46, 44, and 42 kDa proteins declined nearly to the basal level within 1 hr and 30 min, respectively. However, in PC12h-R cells, the tyrosine phosphorylation of these five proteins was detected even 3 hr after exposure to EGF. From their molecular weights, it was thought that the 180 kDa protein was the EGF receptor, the 52 and 46 kDa proteins were Shc proteins, and the 44 and 42 kDa proteins were MAP kinases (ERK 1 and 2, respectively). These results show that the tyrosine phosphorylation of the cellular proteins induced by EGF, is sustained in PC12h-R cells, but not in PC12h cells.

EGF-induced mobility sift of MAP kinases

When MAP kinases are phosphorylated and activated, they show the shifts in electrophoretic mobility (Wood *et al.*, 1992; Okumura *et al.*, 1994). I also investigated time course of the EGF-induced phosphorylation of MAP kinases by examining their EGF-induced mobility sifts (Fig. 10). In both PC12h and PC12h-R cells, the mobility sifts of 44 and 42 kDa MAP kinases, ERK 1 and 2, respectively, were observed by the EGF treatment. The sifts in PC12h-R cells were remarkably sustained in comparison with those in PC12h cells. These results indicate that the EGF-induced phosphorylation of MAP kinases are prolonged in PC12h-R cells more than in PC12h cells.

EGF-induced activation of MAP kinases

To examine the duration of EGF-induced activation of MAP kinases, lysates

of PC12h and PC12h-R cells treated with EGF for various periods were analyzed by means of the kinase renaturation gel assay using myelin basic protein (MBP) as the substrate (Fig. 11A). I observed three kinases with apparent molecular weights of 85, 44 and 42 kDa which were activated by EGF in both cell lines. The activities of the 44 and 42 kDa kinases were not detected in the control kinase assay in polyacrylamide gel without MBP, but that of the 85 kDa kinase was detected (data not shown). These results indicate that the 44 and 42 kDa kinases phosphorylate MBP and that the activity of the 85 kDa kinase reflects its autophosphorylation activity. The 44 and 42 kDa kinases are 44 and 42 kDa MAP kinases (ERK 1 and 2), respectively, but the 85 kDa kinase remains unknown.

I quantified the activities of the 85 kinase and 44 and 42 kDa MAP kinases using the Fuji Bio-imaging analyzer (Fig. 11B,C,D). In both PC12h and PC12h-R cells, the activities of these kinases reached the maximum within at least 5 min of exposure to EGF. In PC12h cells, the activities of them rapidly declined nearly to the basal level at 30 min after EGF exposure. However, in PC12h-R cells, the 42 and 44 kDa MAP kinase activities were sustained at relatively high level, and slowly declined (Fig. 11B, C). The activity of the 85 kDa kinase in PC12h-R cells was remarkably sustained at a high level even 3 hr after exposure to EGF (Fig. 11D). Therefore, EGF induced the prolonged activation of 42 and 44 kDa MAP kinases and 85 kDa kinase in PC12h-R cells, but not in PC12h cells.

Amount of EGF receptor protein

PC12 cells overexpressing the EGF receptor show EGF-induced neuronal

differentiation and sustained activation of MAP kinases (Traverse *et al.*, 1994). To examine whether or not the responses to EGF in PC12h-R cells are due to increases in the amount of EGF receptor protein, lysates and wheat germ agglutinin (WGA) precipitates prepared from PC12h and PC12h-R cells were analyzed by Western blot analysis using the anti-EGF receptor antibody (Fig. 12). WGA binds to oligosaccharides on the EGF receptor. There were two protein bands with molecular weights of 180 and 130 kDa in the lysates and the WGA precipitates of both cell lines. The band of 130 kDa protein in the WGA precipitates was much weaker than that in the lysates. The 180 kDa protein was considered to be the fully glycosylated mature EGF receptor. The 130 kDa protein might be a non-glycosylation form or a degradation product of the EGF receptor. Both proteins were present at similar levels in the lysates (and the WGA precipitates) from PC12h-R cells and PC12h cells. Therefore, I conclude that the amounts of EGF receptor protein in PC12h and PC12h-R cells are similar.

EGF-induced tyrosine phosphorylation of the EGF receptor

As described above, EGF induced the sustained tyrosine phosphorylation and activation of MAP kinases in PC12h-R cells. I considered that the prolonged activation of MAP kinases might be caused by the sustained activation of the EGF receptor. I examined the time course of EGF-induced tyrosine phosphorylation of the EGF receptor in PC12h-R and PC12h cells. Lysates prepared from the cells after exposure to EGF for various periods and immunoprecipitates from the lysates with the anti-EGF receptor antibody were Western blotted using the anti-

phosphotyrosine monoclonal antibody (Fig. 13). In both cell lines, tyrosine phosphorylation of the EGF receptor (a 180 kDa protein) was induced and reached the maximum within at least 5 min after exposure to EGF. The level of tyrosine phosphorylation of the EGF receptor was sustained in PC12h-R cells, whereas it rapidly declined in PC12h cells. The phosphorylation of the EGF receptor in PC12h-R cells was detected even 1-2.5 hr after EGF addition, however it declined to the basal level within 1 hr in PC12h cells.

EGF-induced down-regulation of the EGF receptor

The EGF receptor is endocytosed and degraded in lysosomes, following its activation (Soderquist and Carpenter, 1986). I considered that the prolonged activation of the EGF receptor in PC12h-R cells might result from the decreased rate of the down-regulation of the EGF receptor by internalization. I investigated the levels of the EGF receptor after adding EGF. The lysates and the immunoprecipitates with the anti-EGF receptor from PC12h-R and PC12h cells incubated with EGF for various periods were immunoblotted using the anti-EGF receptor antibody (Fig. 13). In both cell lines, the amount of the 180 kDa EGF receptor decreased in response to EGF. The EGF receptor in PC12h cells was scarcely detected 1 hr after EGF exposure. In contrast, that of PC12h-R cells remained to be detected even 2.5 hr after exposure to EGF. These results indicated that the down-regulation of the EGF receptor in PC12h-R cells was reduced in comparison with that in PC12h cells. These results suggest that the EGF-induced sustained tyrosine phosphorylation of the EGF receptor is due to the

decreased rate of down-regulation of the EGF receptor in PC12h-R cells.

NGF-induced tyrosine phosphorylation of p140^{trkA}

In PC12h-R cells, the EGF-induced tyrosine phosphorylation of the EGF receptor was sustained. To examine whether or not the prolonged duration of the signalling in PC12h-R cells is observed only with EGF, I compared the duration of NGF-induced tyrosine phosphorylation of p140^{trkA} in PC12h-R and PC12h cells. I produced an anti-p140^{trkA} antibody using the C-terminal 14 amino acid synthetic peptide of human p140^{trkA} as an antigen. The p140^{trkA} protein immunoprecipitated with the anti-p140^{trkA} antiserum from lysates of both cells exposed to NGF for various periods was Western blotted with the anti-phosphotyrosine antibody (Fig. 14). NGF-induced tyrosine phosphorylation of p140^{trkA} was clearly observed even 2.5 hr after NGF exposure in PC12h-R cells, although it declined nearly to the basal level within 2.5 hr in PC12h cells. That is to say, NGF induced the prolonged tyrosine phosphorylation of p140^{trkA} in PC12h-R cells in compared with that in PC12h cells. In addition, the duration of NGF-induced tyrosine phosphorylation of p140^{trkA} was longer than that of EGF-induced tyrosine phosphorylation of the EGF receptor in both cell lines. However, the duration of the EGF-induced phosphorylation of the EGF receptor in PC12h-R cells was similar to that of the NGF-induced p140^{trkA} phosphorylation in PC12h cells.

NGF-induced tyrosine phosphorylation of cellular proteins

It is known that tyrosine phosphorylation of several cellular proteins, including

PLC γ-1 (Kim *et al.*, 1991; Vetter *et al.*, 1991), PI-3 kinase (Ohmichi *et al.*, 1992; Soltoff *et al.*, 1992) and MAP kinases (Boulton *et al.*, 1991), is induced following the activation of $p140^{trkA}$ by NGF as well as following that of the EGF receptor by EGF. To investigate whether or not the tyrosine phosphorylation of cellular proteins lasts as $p140^{trkA}$ in PC12h-R cells, I examined the duration of NGF-induced tyrosine phosphorylation of total cellular proteins in both PC12h and PC12h-R cells (Fig. 15). The lysates prepared from both cells after exposure to NGF were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted using the anti-phosphotyrosine antibody 4G10. In these cells, tyrosine phosphorylation of at least three cellular proteins (54 kDa, 44 kDa and 42 kDa proteins) was induced upon treatment with NGF. The tyrosine phosphorylation level of these proteins reached the maximum within 5 min after NGF treatment. In PC12h cells, the tyrosine phosphorylation level of the 54 kDa, 44 kDa and 42 kDa proteins declined nearly to the basal level at 1 hr after exposure to NGF. In PC12h-R cells, however, the tyrosine phosphorylation of the 54 kDa, 44 kDa, and 42 kDa proteins was evident even 2 hr after NGF treatment. From their molecular weights, it was thought that the 52 kDa protein was Shc protein, and the 44 and 42 kDa proteins were MAP kinases (ERK 1 and 2, respectively). In addition, sustained tyrosine phosphorylation of 75 kDa and 90 kDa proteins was also observed following NGF treatment in PC12h-R cells, but not in PC12h cells. Thus, the NGF-induced tyrosine phosphorylation of the cellular proteins in PC12h-R cells was sustained as that of $p140^{trkA}$.

NGF-induced activation of MAP kinases

I examined the duration of NGF-induced activation of MAP kinases in PC12h and PC12h-R cells by means of the kinase renaturation gel assay using MBP as a substrate (Fig. 16). NGF induced the activation of 44 and 42 kDa MAP kinases (ERK1 and 2, respectively) in both cells. The activities of these kinases reached the maximum within at least 5 min after NGF exposure, then declined gradually. The activities of MAP kinases at 1 hr after exposure to NGF in PC12h-R cells were higher than those in PC12h cells. Thus, PC12h-R cells showed the NGF-induced sustained activation of MAP kinases in comparison with PC12h cells. In addition, the NGF-induced activation of MAP kinases was prolonged compared with that induced by EGF in PC12h cells. In PC12h-R cells, the duration of the MAP kinase activation induced by NGF was similar to that induced by EGF.

Amount of $p140^{trkA}$ protein

It has been reported that PC12 cells overexpressing $p140^{trkA}$ showed prolonged tyrosine phosphorylation of cellular proteins, including $p140^{trkA}$ and MAP kinase, in response to NGF (Hempstead *et al.*, 1992). To investigate whether or not the NGF-induced sustained signalling observed in PC12h-R cells is due to an increased amount of $p140^{trkA}$ protein, I examined the quantity of $p140^{trkA}$ protein in PC12h and PC12h-R cells (Fig. 17). $p140^{trkA}$ protein was immunoprecipitated in crude lysates of biosynthetically [^{35}S]methionine-labeled cells. Radiolabeled proteins with apparent molecular weights of 110 kDa and 130 kDa were observed in both PC12h and PC12h-R cells. Because neither of these

proteins was detected in immunoprecipitations in the presence of the immunizing peptides, they were thought to be p140^{trkA} or its derivatives. The 130 kDa protein was considered to be mature p140^{trkA} protein that was fully glycosylated, and the 110 kDa protein was thought to be a precursor of the p140^{trkA} protein with lesser glycosylation (Martin-Zanca *et al.*, 1989). Both proteins were present in nearly the same amounts in PC12h-R cells as in PC12h cells. In order to immunoprecipitate p140^{trkA} completely and compare the amount of p140^{trkA} exactly, immunoprecipitation was carried out further from the supernatant of the first immunoprecipitation. In the second immunoprecipitation, equal amounts of p140^{trkA} protein were also detected. It was thought that p140^{trkA} was almost completely immunoprecipitated in the first immunoprecipitation, because the amount of p140^{trkA} protein detected in the second immunoprecipitation was much less than that in the first. These results indicate that the NGF-induced sustained tyrosine phosphorylation of p140^{trkA} and several cellular proteins, and the prolonged activation of MAP kinases observed in PC12h-R cells are not simply due to an increased amount of p140^{trkA} protein.

Discussion

I showed that PC12h-R, a subclone of PC12 cells, differentiates into neuron-like cells in response to EGF as well as to NGF. It has been reported that PC12D, a subline of PC12 cells, shows weak neurite outgrowth in response to EGF (Sano and Kitajima, 1992). In contrast to PC12D cells, PC12h-R cells started to extend the neurites early, and elaborated a neurite network consisting of long and branched processes in response to EGF as well as to NGF. This neurite network was stable in the presence of EGF. In addition, EGF attenuated the growth of PC12h-R cells. To my knowledge, PC12h-R is the first of the PC12 cell lineage in which EGF induces the attenuation of cell proliferation. EGF increased the synthesis of TH protein and AChE activity in PC12h-R cells in the same manner as NGF did. I consider that EGF induces similar neuronal differentiation as NGF.

CPTcAMP, an analog of cAMP, evoked neurite outgrowth of PC12h-R cells, although the cells incubated with CPTcAMP displayed a different morphology from the cells exposed to EGF or NGF. In addition, CPTcAMP induced little increase of the AChE activity in comparison with EGF or NGF. These results suggest that PC12h-R cells possess several independent pathways for the induction of neurite outgrowth. EGF and NGF are thought to activate a similar pathway, inducing similar neuronal differentiation in PC12h-R cells. EGF and NGF activate the receptor tyrosine kinases, but CPTcAMP is thought to activate protein kinase A. Distinct pathways, including the receptor tyrosine kinase and

protein kinase A, may induce different phenotypic changes, although they evoke similar changes in terms of neuronal differentiation. Namely, differences in neuronal phenotypes induced by neurotrophic agents might be due to the activated pathways.

The EGF-induced neurite outgrowth of PC12h-R cells was enhanced by K252a, although that induced by NGF was inhibited. K252a enhances the EGF-induced increase of ornithine decarboxylase activity and neurite outgrowth in PC12 cells (Koizumi *et al.*, 1988; Isono *et al.*, 1994). Our results agree with these reports. Nevertheless, it remains unclear how K252a potentiates the EGF responses. EGF-induced tyrosine phosphorylation of MAP kinases is prolonged in the presence of K252a, which may be involved in regulation of MAP kinases (Isono *et al.*, 1994). K252b, a close analog of K252a (Knüsel and Hefti, 1992), did not enhance the EGF-induced neurite outgrowth of PC12h-R cells so much as K252a did, although it blocked that induced by NGF in the same way as K252a. This may be due to differences in the targets of these compounds. Alternatively, the weak enhancement of the EGF-induced neurite outgrowth by K252b may result from its hydrophilicity. Being more hydrophilic than K252a, K252b does not freely pass the cell membrane. Therefore, the target of K252a that enhances the EGF-induced neurite extension may be found in the cytosol.

The Ras-MAP kinase cascade, consisting of Shc protein, $p21^{ras}$, Raf protein, MAP kinase kinase, MAP kinase, and so on, is an important signalling pathway induced by many factors (Nishida and Gotoh, 1993; Schlessinger, 1993). This cascade participates in the regulation of cell proliferation and differentiation of a

variety of cells. EGF promotes the proliferation but NGF induces the neuronal differentiation in PC12 cells, although both factors activate the Ras-MAP kinase cascade in a similar manner (Chao, 1992; Schlessinger and Ullrich, 1992). The mechanism of determination of the cellular response between proliferation and differentiation in PC12 cells has remained unknown. Recently, it has been reported that NGF-induced activation of $p21^{ras}$, MAP kinase kinase, and MAP kinase is sustained in comparison with that induced by EGF in PC12 cells (Qiu and Green, 1992; Traverse *et al.*, 1992). In addition, the constitutively activated forms of $p21^{ras}$, Raf protein, or MAP kinase kinase induce neuronal differentiation in PC12 cells (Guerrero *et al.*, 1988; Wood *et al.*, 1993; Cowley *et al.*, 1994).

Here, I found that PC12h-R cell, a subclone of PC12 cells, showed the neuronal differentiation in response to EGF, as well as the sustained tyrosine phosphorylation of the EGF receptor, Shc proteins and MAP kinases, and the sustained activation of MAP kinases. In PC12h-R cells, the duration of tyrosine phosphorylation induced by EGF was similar to that induced by NGF. On the other hand, the NGF-induced tyrosine phosphorylation was sustained more than that induced by EGF in PC12h cells which did not display EGF-induced neuronal differentiation. These results indicated that the duration of the factor-induced activation of Ras-MAP kinase cascade is important for specifying the cellular response in the PC12 cell lineage. Recently, Traverse *et al.* (1994) have reported that in PC12 cells overexpressing the EGF receptor, EGF induces the sustained activation of MAP kinase kinase and MAP kinase, as well as neuronal differentiation. Their report may support our conclusion.

The EGF-induced tyrosine phosphorylation of the EGF receptor was remarkably sustained in PC12h-R cells. As the level of tyrosine phosphorylation of the EGF receptor is correlated with the tyrosine kinase activity of the EGF receptor (Ullrich and Schlessinger, 1991), the activity of the EGF receptor is thought to be sustained in PC12h-R cells. Some tyrosine residues on the EGF receptor, the phosphorylation of which is induced by the EGF binding, are essential to signal transduction from the EGF receptor to SH2-containing proteins including Shc protein, PLC γ , PI-3 kinase, and so on (Pawson and Schlessinger, 1993). In PC12h-R cells, the sustained tyrosine phosphorylation of the EGF receptor probably results in the sustained tyrosine phosphorylation of Shc proteins and MAP kinases, and therefore in the sustained activation of MAP kinases downstream of the EGF receptor in the EGF-signalling pathway. In this context, the regulation of the duration of signalling on the EGF receptor is thought to be important to the duration of the EGF-induced signalling in PC12h-R cells. As mentioned above, PC12 cells that overexpress the EGF receptor show the sustained activation of MAP kinase (Traverse *et al.*, 1994). However, the amount of the EGF receptor protein in PC12h-R was the same as that in PC12h cells. The sustained signalling in PC12h-R cells was not simply due to an increased level of the EGF receptor protein. I found that the rate at which the amount of the EGF receptor decreased after EGF exposure was slower in PC12h-R than in PC12h cells. This finding suggests that the decreased down-regulation of the EGF receptor prolongs the activation of the EGF receptor, leading to the sustained activation of MAP kinases and the neuronal differentiation in PC12h-R

cells. The EGF receptor to which the ligand binds is internalized in endocytic vesicles and degraded in lysosomes (Soderquist and Carpenter, 1986). In PC12h-R cells, a deficiency in the degradation of the EGF receptor through endocytosis may change the response to EGF. In addition, the NGF-induced tyrosine phosphorylation of $p140^{trkA}$ in PC12h-R cells was also sustained compared with that in PC12h cells. It remains to be examined whether or not the sustained phosphorylation of $p140^{trkA}$ is also due to a decreased rate of down-regulation in PC12h-R cells. It appears that the defect in PC12h-R cells is at a molecule involved in the regulation of both the EGF receptor and $p140^{trkA}$ activities.

In PC12 cells, the NGF-induced activation of the Ras-MAP kinase cascade is sustained in comparison with that induced by EGF (Marshall, 1995). Here, I found that the NGF-induced tyrosine phosphorylation of $p140^{trkA}$ was sustained compared with that of the EGF receptor induced by EGF. Therefore, the prolonged activation of $p140^{trkA}$ might lead to the sustained activation of the Ras-MAP kinase cascade and neuronal differentiation in PC12 cells. In addition, the EGF-induced tyrosine phosphorylation of the EGF receptor in PC12h-R cells was prolonged in a similar manner to the NGF-induced phosphorylation of $p140^{trkA}$ in PC12h cells. These findings indicate that the duration of the receptor tyrosine kinase activity may determine the cellular response of the PC12 cell lineage. Namely, the sustained activation of the receptor tyrosine kinase may induce neuronal differentiation through the sustained activation of the Ras-MAP kinase cascade in PC12 cells. I do not know and intend to examine how the NGF-

induced tyrosine phosphorylation of p140^{trkA} is sustained. The difference in the duration of the tyrosine phosphorylation between p140^{trkA} and the EGF receptor may be due to the presence of tyrosine phosphatase which dephosphorylates the activated EGF receptor, but not p140^{trkA}. Alternatively, it is possible that rate of down-regulation of p140^{trkA} mediated by endocytosis and degradation in lysosomes, is slower than that of the EGF receptor. I believe that these studies on the regulation of the receptor tyrosine kinase activity are important for elucidating the mechanisms by which the cellular responses of PC12 cells are determined.

Previously, I reported that PC12h-R cells rapidly differentiate into neuron-like cells in response to NGF in comparison with PC12h cells (Yamada *et al.*, 1994). The NGF-induced tyrosine-phosphorylation of the cellular proteins, including p140^{trkA}, Shc protein and MAP kinase, was sustained for longer in PC12h-R, than in PC12h cells. In addition, it has been reported that PC12 cells that overexpress p140^{trkA}, display a rapid response to NGF and the sustained NGF-induced tyrosine phosphorylation of p140^{trkA}, PLC γ, and MAP kinase (Hempstead *et al.*, 1992). Therefore, the speed of the differentiation may depend upon the duration of the signalling. Recently, it has been reported that PC12 cells possess the potential to differentiate into neuron-like cells in response to EGF, but this process is very slow in comparison with that induced by NGF (Nakafuku and Kajiro, 1993). I also observed that PC12h cells and other subclone of PC12 cells extended short neurites in long-term cultures in the presence of EGF (data not shown). In PC12 cells, the transient signalling induced by EGF may cause the slow response which

leads to the observation that EGF does not induce neuronal differentiation in the short-term culture.

In the central nervous system, EGF acts as a neurotrophic factor (Hefti *et al.*, 1993). For example, EGF promotes the neurite outgrowth and cell survival of cultured cerebral, subneocortical telencephalic and cerebellar neurons (Morrison *et al.*, 1987; Morrison *et al.*, 1988; Kornblum *et al.*, 1990). In part 2, I report that EGF prevents high oxygen-induced apoptosis and induces prolonged tyrosine phosphorylation of the EGF receptor in primary cultured rat cerebral cortical neurons. It remains unclear whether or not the phosphorylation of the EGF receptor in the cortical neurons is sustained by the same mechanism as that in PC12h-R cells. I suggest that EGF exerts neurotrophic effects through the sustained activation of the EGF receptor. PC12h-R cells may be a useful model to elucidate the molecular mechanisms of the neurotrophic action of EGF.

References

Abiru, Y., Nishio, C. and Hatanaka, H. The survival of striatal cholinergic neurons cultured from postnatal 2-week-old rats is promoted by neurotrophins. *Dev. Brain Res.*, in press.

Barbacid, M. (1993) Nerve growth factor: a tale of two receptors. *Oncogene*, **8**, 2033-2042.

Barde, Y. -A. (1989) Trophic factors and neuronal survival. *Neuron*, **2**, 1525-1534.

Blenis, J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci. USA*, **90**: 5889-5892.

Bocchini, V. and Angeletti, P. U. (1969) The nerve growth factor: Purification as a 30,000-molecular weight protein. *Proc. Natl. Acad. Sci. USA*, **64**, 787-794.

Boulton, T. G., Ney, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., Depinho, R. A., Panayootatos, N., Cobb, M. H. and Yancopoulos, G. D. (1991) ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, **65**, 663-675.

Carpenter, G. J. and Cohen, S. (1979) Epidermal growth factor. *Annu. Rev. Biochem.*, **48**, 193-216.

Carpenter, G. (1987) Receptor for epidermal growth factor and other polypeptide mitogens. *Ann. Rev. Biochem.*, **56**, 881-914.

Chao, M. V. (1992) Growth factor signalling: where is the specificity? *Cell*, **68**, 995-997.

Cowley, S., Paterson, H., Kemp, P. and Marshall, C. J. (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell*, **77**, 841-852.

Derynck, R. (1988) Transforming growth factor α. *Cell*, **54**, 593-595.

Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M. (1961) A new

and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, 7, 88-95.

Enokido, Y. and Hatanaka, H. (1993) Apoptotic cell death occurs in hippocampal neurons cultured in a high oxygen atmosphere. *Neurosci.*, **57**, 965-972.

Gotoh, Y., Nishida, E., Yamashita, T., Kawakami, M. and Sakai, H. (1990) Microtubule-associated protein (MAP) kinase activated by nerve growth factor and epidermal growth factor in PC12 cells. *Eur. J. Biochem.*, **193**, 661-669.

Greene, L. A. and Tischler, A. S. (1976) Establishment of noradrenergic clonal cell line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA*, **72**, 2424-2428.

Greene, L. A. and Shooter, E. M. (1980) The nerve growth factor: biochemistry, synthesis and mechanism of action. *Ann. Rev. Neurosci.*, **3**, 353-402.

Greene, L. A. and Rukenstein, A. (1981) Regulation of acetylcholinesterase activity by nerve growth factor. *J. Biol. Chem.*, **256**, 6363-6367.

Guerrero, I., Pellicer, A. and Burstein, D. E. (1986) Dissociation of c-fos from ODC expression and neuronal differentiation in a PC12 subline stably transfected with an inducible N-ras oncogene. *Biochem. Biophys. Res. Commun.*, **150**, 1185-1192.

Halegoua, S., Armstrong, R. C. and Kremer, N. E. (1991) Dissecting the mode of action of neuronal growth factor. *Curr. Top. Microbiol. Immunol.*, **165**, 119-170.

Harlow, E. and Lane, D. (1988) *Antibodies*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 78-81.

Hartikka, J. and Hefti, F. (1988) Development of septal cholinergic neurons in culture: plating density and glial cells modulate effects of NGF on survival, fiber growth and expression of transmitter-specific enzymes. *J. Neurosci.* **8**, 2967-2985.

Hatanaka, H. (1981) Nerve growth factor-mediated stimulation of tyrosine hydroxylase activity in a clonal rat pheochromocytoma cell line. *Brain Res.*, **222**, 225-233.

Hatanaka, H. (1983) Nerve growth factor-mediated differentiation of a nerve cell line cultured in a hormone-supplemented serum-free medium. *Dev. Brain Res.*, **6**, 243-250.

Hatanaka, H. and Tsukui-Tamagawa, H. (1984) Rapid responsive induction of tyrosine hydroxylase in rat pheochromocytoma cells by nerve growth factor. (1984) *3rd Int. Congr. Biol. Tokyo, Japan.*

Hatanaka, H. and Arimatsu, Y. (1984) Monoclonal antibodies to tyrosine hydroxylase from rat pheochromocytoma PCl2h cells with the special reference to nerve growth factor-mediated increase of the immunoprecipitatable enzymes. *Neurosci. Res.*, **1**, 253-263.

Hatanaka, H., Tsukui, H. and Nihonmatsu, I. (1988) Developmental change in the nerve growth factor action from induction of choline acetyltransferase to promotion of cell survival in cultured basal forebrain cholinergic neurons from postnatal rats. *Dev. Brain Res.*, **39**, 85-95.

Hefti, F., Denton, T. L., Knüsel, B. and Lapchak, P. A. (1993) Neurotrophic factors:what are they and what are they doing? Pages 25-49, in Loughlin, S. E., and Fallon, J. H. (eds.), *Neurotrophic factors*, Academic Press, Inc., San Diego, California.

Hempstead, B. L., Rabin, S. J., Kaplan, L., Reid, S., Parada, L. F. and Kaplan, D. R. (1992) Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. *Neuron*, **9**, 883-896.

Huff, K., End, D. and Guroff, G. (1981) Nerve growth factor-induced alteration in the response of PC12 pheochromocytoma cells to epidermal growth factor. *J. Cell Biol.*, **88**, 189-198.

Ikenaka, K., Nakahira, K., Takayama, C., Wada, K., Hatanaka, H. and Mikoshiba,

K. (1990) Nerve growth factor rapidly induced expression of the 68 KDa neurofilament gene by posttranscriptional modulation in PC12h-R cells. *J. Biol. Chem.*, **265**, 19782-19785.

Isono, F., Widmer, H. R., Hefti, F. and Knüsel, B. (1994) Epidermal growth factor induces PC12 cell differentiation in the presence of the protein kinase inhibitor K-252a. *J. Neurochem.*, **63**, 1235-1245.

Kim, U. -H., Fink, D., Kim, H. S., Park, D. J., Contreras, M. L., Guroff, G. and Rhee, S. G. (1991) Nerve growth factor stimulates phosphorylation of phospholipase C-γ in PC12 cells. *J. Biol. Chem.*, **266**, 1359-1362.

Knüsel, B. and Hefti, F. (1992) K-252 compounds: modulators of neurotrophin signal transduction. *J. Neurochem.*, **59**, 1987-1996.

Koizumi, S., Contreras, M. L., Matsuda, Y., Hama, T., Lazarovici, P. and Guroff, G. (1988) K-252a: a specific inhibitor of the action of nerve growth factor on PC12 cells. *J. Neurosci.*, **8**, 715-721.

Kornblum, H. I., Raymon, H. K., Morrison, R. S., Cavanaugh, K. P., Bradshaw, R. A. and Leslie, F. M. (1990) Epidermal growth factor and basic fibroblast growth factor: effects on an overlapping population of neocortical neurons in vitro. *Brain Res.*, **535**, 255-263.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, **80**, 179-185.

Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T. and Barbacid, M. (1989) Molecular and biochemical characterization of the human *trk* proto-oncogene. *Mol. Cell. Biol.*, **9**, 24-33.

Morrison, R. S., Kornblum, H. I., Leslie, F. M. and Bradshaw, R. A. (1987) Trophic stimulation of cultured neurons from neonatal rat brain by EGF. *Science*,

238, 72-75.

Morrison, R. S., Keating, R. F. and Moskal, J. R. (1988) Basic fibroblast growth factor and epidermal growth factor exert differential trophic effects on CNS neurons. *J. Neurosci. Res.*, **21**, 71-79.

Nakafuku, M. and Kajiro, Y. (1993): Epidermal growth factor and transforming growth factor-α can induce neuronal differentiation of rat pheochromocytoma PC12 cells under particular culture conditions. *FEBS lett.*, **315**, 227-232.

Nishida, E. and Gotoh, Y. (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.*, **18**, 128-131.

Ohmichi, M., Decker, S. J. and Saltiel, A. R. (1992) Activation of phosphatidyl-inositol-3 kinase by nerve growth factor involves indirect couplings of the *trk* proto-oncogene with src homology 2 domains. *Neuron*, **9**, 769-777.

Okumura, N., Miyatake, Y., Takao, T., Tamaru, T., Nagai, K., Okada, M. and Nakagawa, H. (1994) Vasoactive intestinal peptide induces differentiation and MAP kinase activation in PC12h cells. *J. Biochem.* **115**, 304-308.

Pawson, T. and Schlessinger, J. (1993) SH2 and SH3 domains. *Curr. Biol.*, **3**, 434-442.

Qiu, M. -S. and Green, S. H. (1992) PC12 cell neuronal differentiation is associated with prolonged $p21^{ras}$ activity and consequent prolonged ERK activity. *Neuron*, **9**, 705-717.

Sano, M. and Kitajima, S. (1992) Activation of microtubule-associated protein kinase in PC12D cells in response to both fibroblast growth factor and epidermal growth factor and concomitant stimulation of the outgrowth of neurites. *J. Neurochem.*, **58**, 837-844.

Schlessinger, J. and Ullrich, A. (1992) Growth factor signalling by receptor tyrosine kinases. *Neuron*, **9**, 383-391.

Schlessinger, J. (1993) How receptor tyrosine kinases activate Ras. *Trends Biochem. Sci.*, **18**, 273-275.

Soderquist, A. M. and Carpenter, G. (1986) Biosynthesis and metabolic degradation of receptors for epidermal growth factor. *J. Memb. Biol.* **90**, 97-105.

Soltoff, S., Rabin, S. J., Cantley, L. and Kaplan, D. R. (1992) Nerve growth factor promotes the activation of phosphatidylinositol 3-kinase and its association with the *trk* tyrosine kinase. *J. Biol. Chem.* **267**, 17472-17477.

Suda, K., Barde, Y. -A. and Thoenen, H. (1978) Nerve growth factor in mouse and rat serum: Correlation between bioassay and radioimmunoassay determinations. *Proc. Natl. Acad. Sci. USA*, **75**, 4042-4046.

Thoenen, H. and Barde, Y. -A. (1980) Physiology of nerve growth factor. *Physiol. Rev.*, **60**, 1284-1335.

Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. *Biochem. J.*, **288**, 351-355.

Traverse, S., Seedorf, K., Paterson, H., Marshall, C. J., Cohen, P. and Ullrich, A. (1994) EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. *Curr. Biol.*, **4**, 694-701.

Ullrich, A. and Schlessinger, J. (1991) Signal transduction by receptors with tyrosine kinase activity. *Cell*, **61**, 203-212.

Vetter, M. L., Martin-Zanca, D., Parada, L. F., Bishop, J. M. and Kaplan, D. R. (1991) NGF rapidly stimulates tyrosine phosphorylation of phospholipase C-γ1 by a kinase activity associated with the product of the *trk* proto-oncogene. *Proc. Natl. Acad. Sci. USA*, **88**, 5650-5654.

Wood, K. W., Sarnecki, C., Roberts, T. M. and Blenis, J. (1992) *ras* mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. *Cell*, **68**, 1041-1050.

Wood, K. W., Qi, H., D'Arcangelo, G., Armstrong, R. C., Roberts, T. M. and Halegoua, S. (1993) The cytoplasmic *raf* oncogene induces a neuronal

phenotype in PC12 cells: a potential role for cellular raf kinases in neuronal growth factor signal transduction. *Proc. Natl. Acad. Sci. USA*, **90**, 5016-5020.

Yamada, M., Ikeuchi, T., Tsukui, H., Aimoto, S. and Hatanaka, H. (1994) Sustained tyrosine phosphorylation of $p140^{trkA}$ in PC12h-R cells responding rapidly to NGF. *Brain Res.* **661**, 137-146.

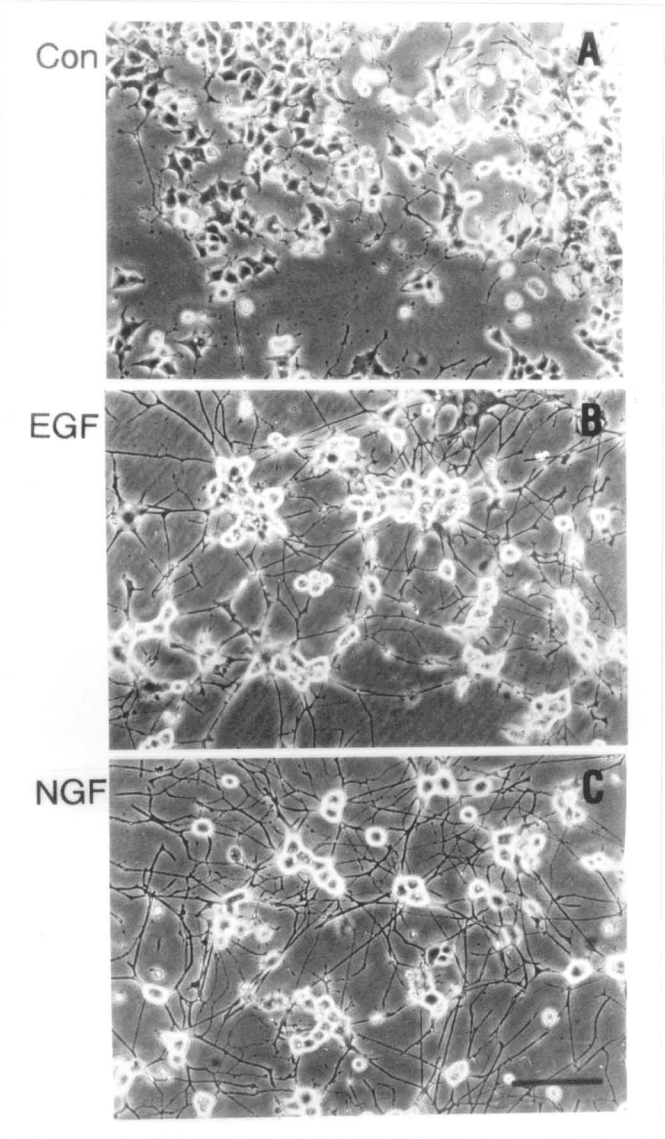

Fig. 1. EGF- and NGF-induced neurite extension in PC12h-R cells. PC12h-R cells were plated on the collagen-coated dishes and cultured in DMEM supplemented with 5 μg/ml human transferrin, 5 μg/ml bovine insulin and 20 nM progesterone (TIP/DMEM). PC12h-R cells were cultured for 9 days in the absence (A) or presence of 50 ng/ml EGF (B) and 50 ng/ml NGF (C). Bar represents 100 μm.

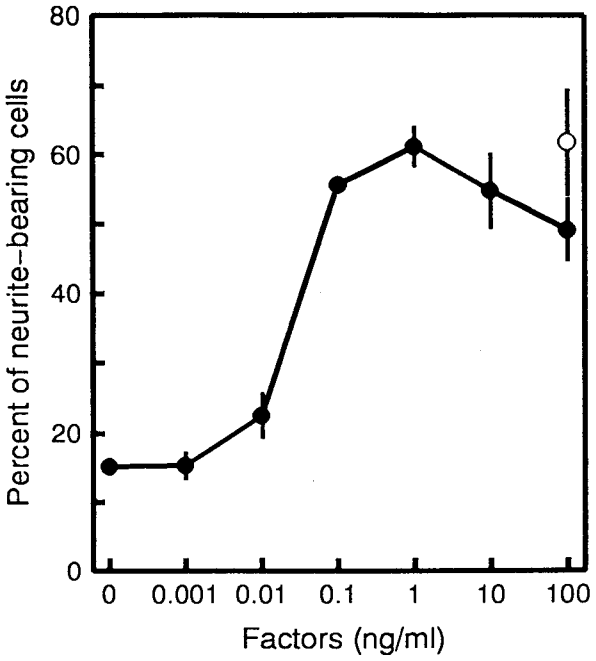

Fig. 2. Dependence of neurite extension on EGF dose in PC12h-R cells. PC12h-R cells were cultured for 2 days with 0, 0.001, 0.01, 0.1, 1, 10 and 100 ng/ml EGF (●) and 100 ng/ml NGF (○) in TIP/DMEM. The percentage of cells bearing neurites longer than twice the diameter of the cell body is shown. The values represent the means ± S.D. of four individual cultures.

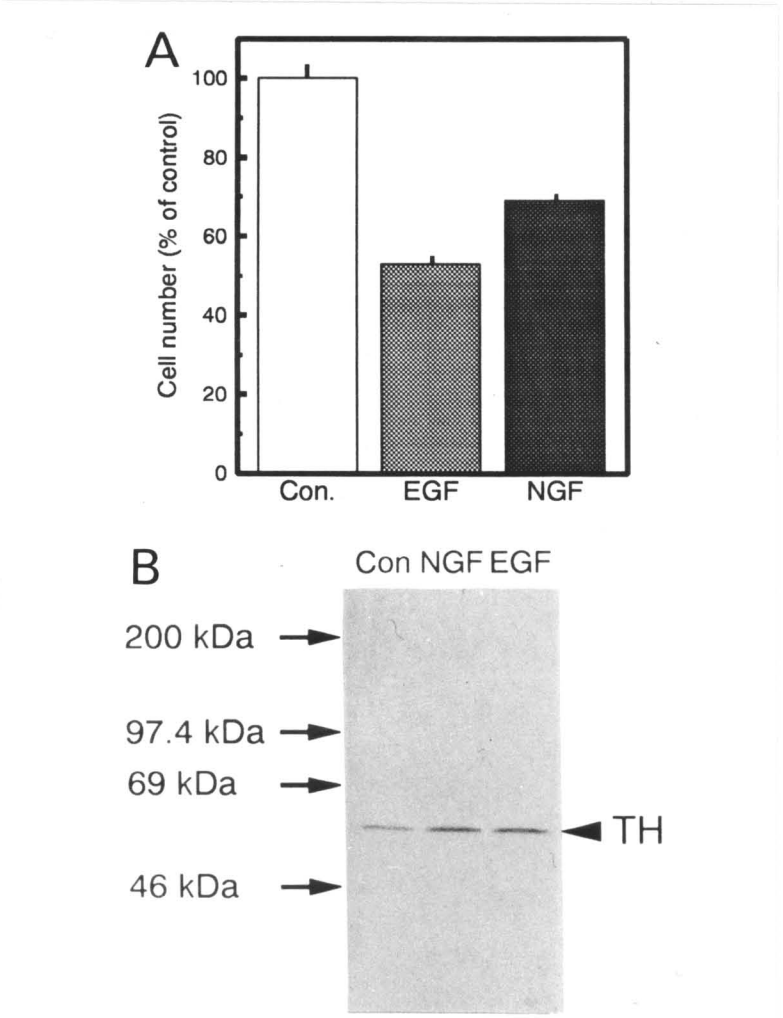

Fig. 3. EGF-induced attenuation of cell growth and increase of TH protein synthesis in PC12h-R cells. (A) Attenuation of cell proliferation in PC12h-R cells treated with EGF. PC12h-R cells were cultured in DMEM supplemented with 5% (v/v) precolostrum newborn calf serum and 5% (v/v) heat-inactivated horse serum. The PC12h-R cells cultured without (Con.) or with 50 ng/ml EGF (EGF) and 50 ng/ml NGF (NGF) for 6 days, were counted as described under "Materials and Methods". The values represent the means ± S.D. of four individual cultures. (B) EGF-mediated increase of TH protein synthesis in PC12h-R cells. PC12h-R cells were cultured without (Con) or with 100 ng/ml NGF (NGF) and 100 ng/ml EGF (EGF) for 5 days, and labeled with [^{35}S] methionine. The cells were lysed and immunoprecipitated with the anti-TH monoclonal antibody (PCTH-7). The immunoprecipitates were analyzed on 10% SDS-polyacrylamide gel. The positions of molecular weight markers are indicated on the left. The arrowhead indicates TH protein.

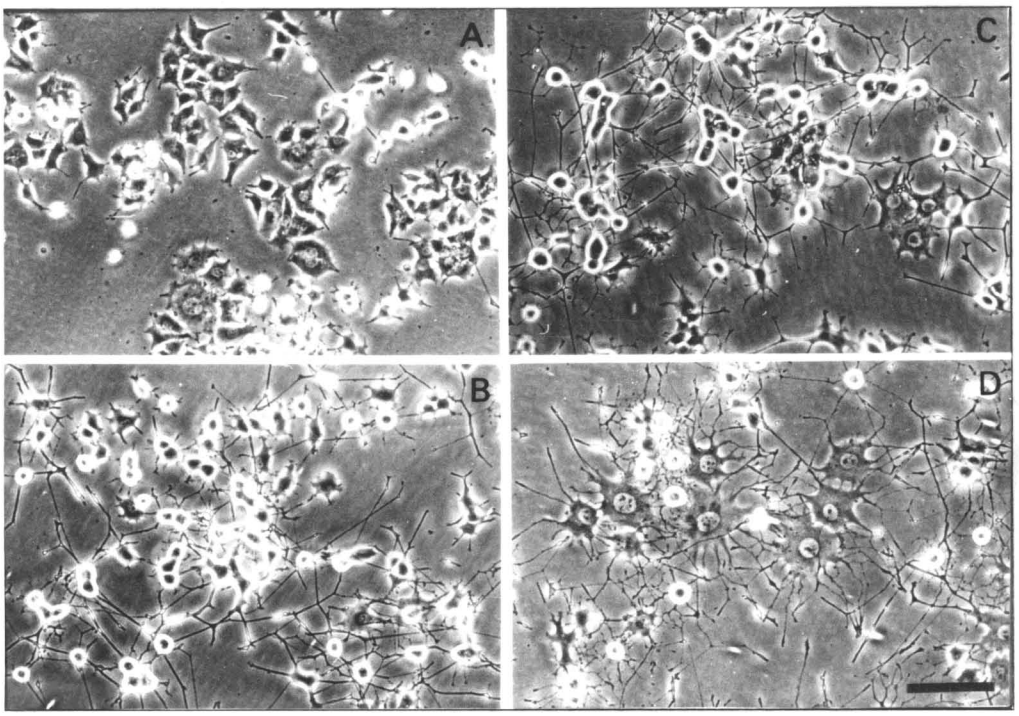

Fig. 4. Neurite outgrowth of PC12h-R cells induced by cAMP. PC12h-R cells were seeded on collagen-coated plates and cultured in DMEM containing 5% (v/v) precolostrum newborn calf serum and 5% (v/v) heat-inactivated horse serum. The cells were cultured without (A) or with 50 ng/ml EGF (B), 50 ng/ml NGF (C) and 100 μM CPTcAMP (D) for 5 days. Bar represents 100 μm.

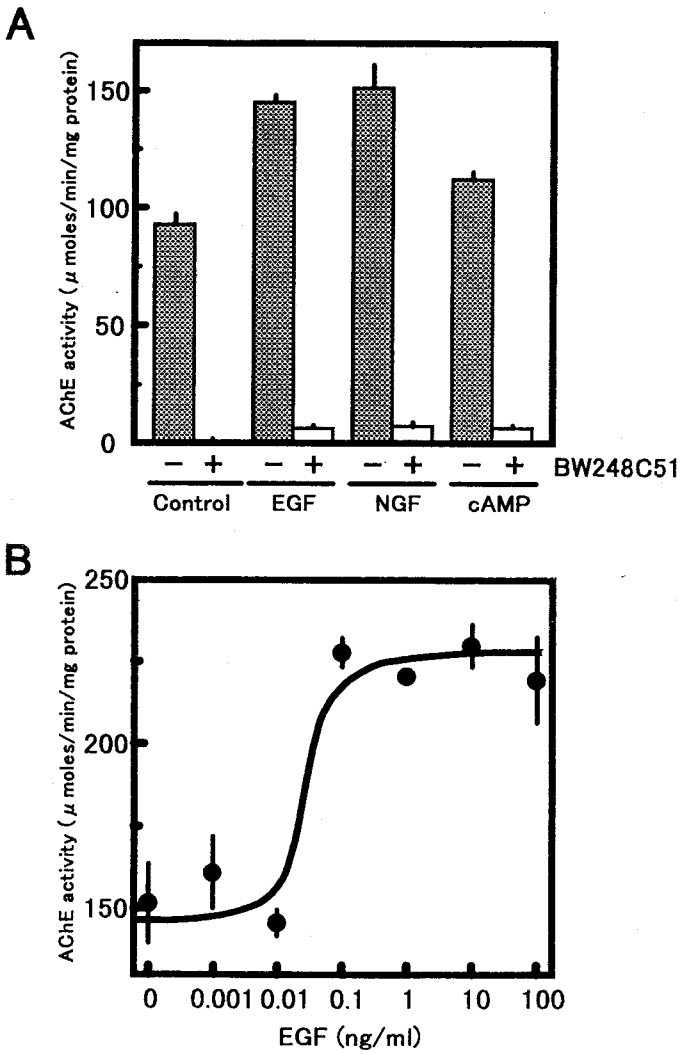

Fig. 5. EGF-, NGF-, and cAMP-induced increase of acetylcholinesterase activity in PC12h-R cells. (A) PC12h-R cells were seeded on collagen-coated plates and cultured in DMEM containing 5% (v/v) precolostrum newborn calf serum and 5% (v/v) heat-inactivated horse serum. AChE activities in the cells cultured without (Control) or with 50 ng/ml EGF (EGF), 50 ng/ml NGF (NGF) and 100 μM CPTcAMP (cAMP) for 5 days were determined in the absence (-) or presence (+) of 10 μM BW248C51 as described under "Materials and Methods". The values represent the means ± S.D. of three individual cultures. (B) PC12h-R cells were seeded on collagen-coated plates and cultured in DMEM containing 5 μg/ml human transferrin, 5 μg/ml bovine insulin and 20 nM progesterone (TIP/DMEM). The cells were cultured for 5 days with 0, 0.001, 0.01, 0.1, 1, 10 and 100 ng/ml EGF. AChE activities were determined as described under "Materials and Methods". The values represent the means ± S.D. of four individual cultures.

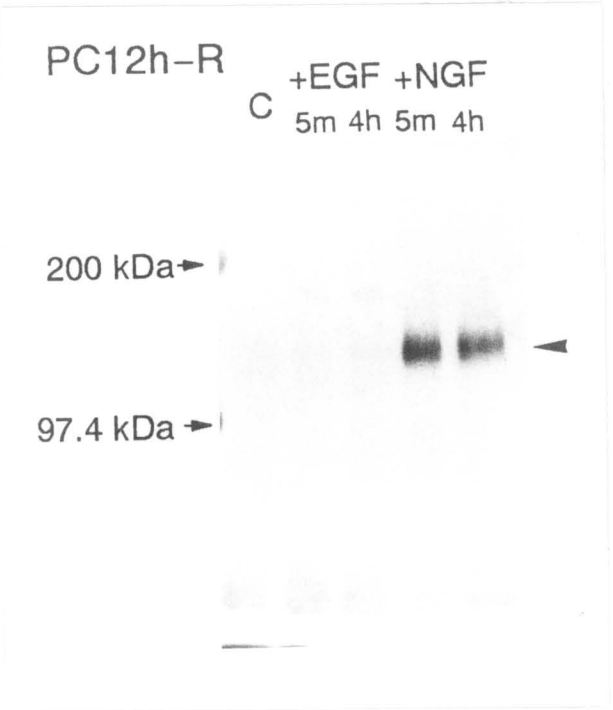

Fig. 6. Tyrosine phosphorylation of p140^{trkA} in PC12h-R cells treated with EGF and NGF. PC12h-R cells were not exposed (C), or incubated with 100 ng/ml EGF (+EGF) and 100 ng/ml NGF (+NGF) for 5 min (5m) and 4 hr (4h), and lysed. The p140^{trkA} was immunoprecipitated with the anti-p140^{trkA} antibody, and analyzed by Western blotting with the anti-phosphotyrosine antibody (4G10). The position of p140^{trkA} is indicated by the arrowhead. Molecular weights are shown on the left of the immunoblot.

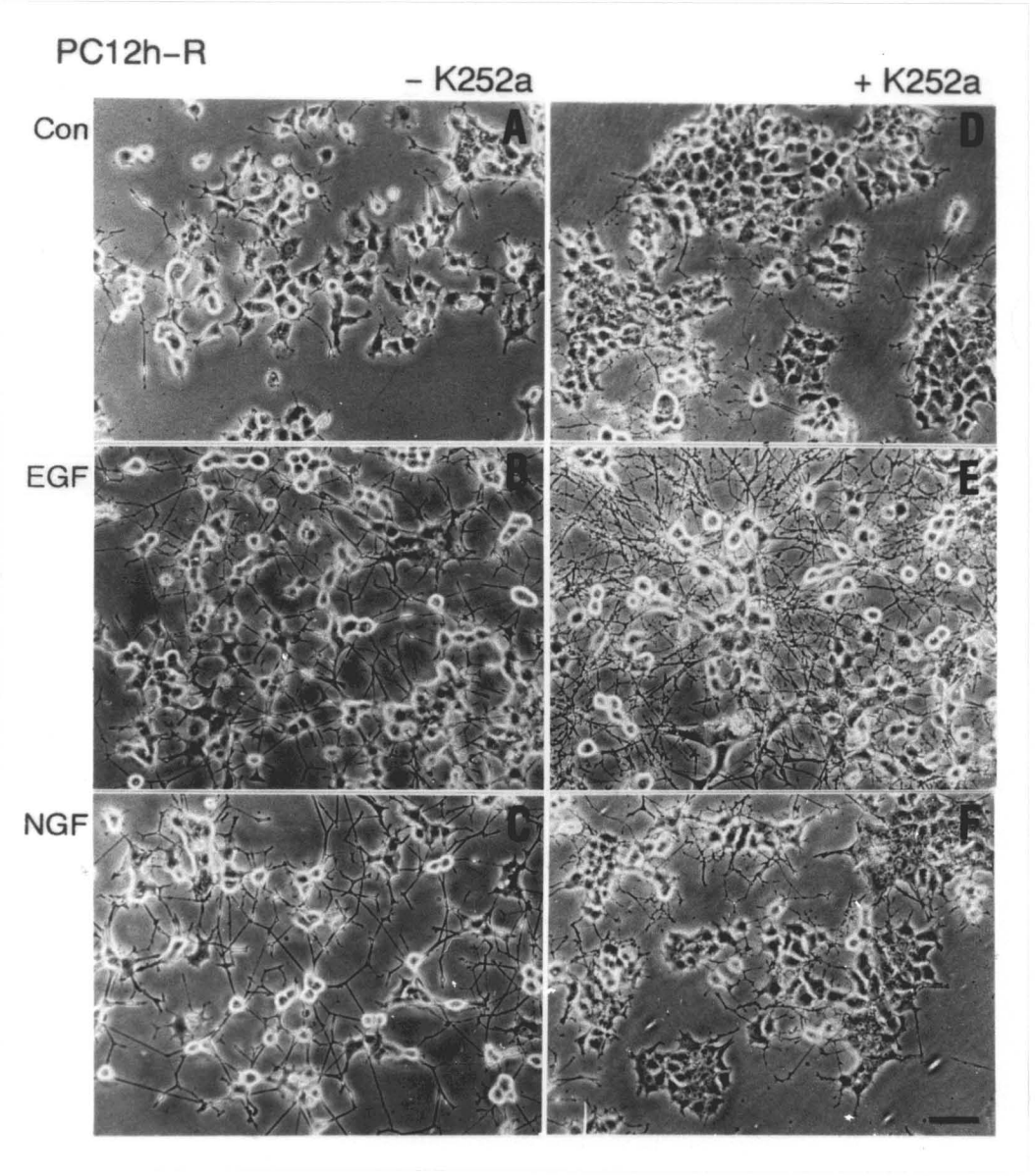

Fig. 7. Effect of K252a on the EGF- and NGF-induced neurite outgrowth of PC12h-R cells. PC12h-R cells were seeded on the collagen-coated plates and cultured in DMEM containing with 5% (v/v) precolostrum newborn calf serum and 5% (v/v) heat-inactivated horse serum. The cells were cultured without (A, D) or with 50 ng/ml EGF (B, E) and 50 ng/ml NGF (C, F) in the absence (A, B, C) or presence of 200 nM K252a (D, E, F) for 6 days. Bar represents 50 μm.

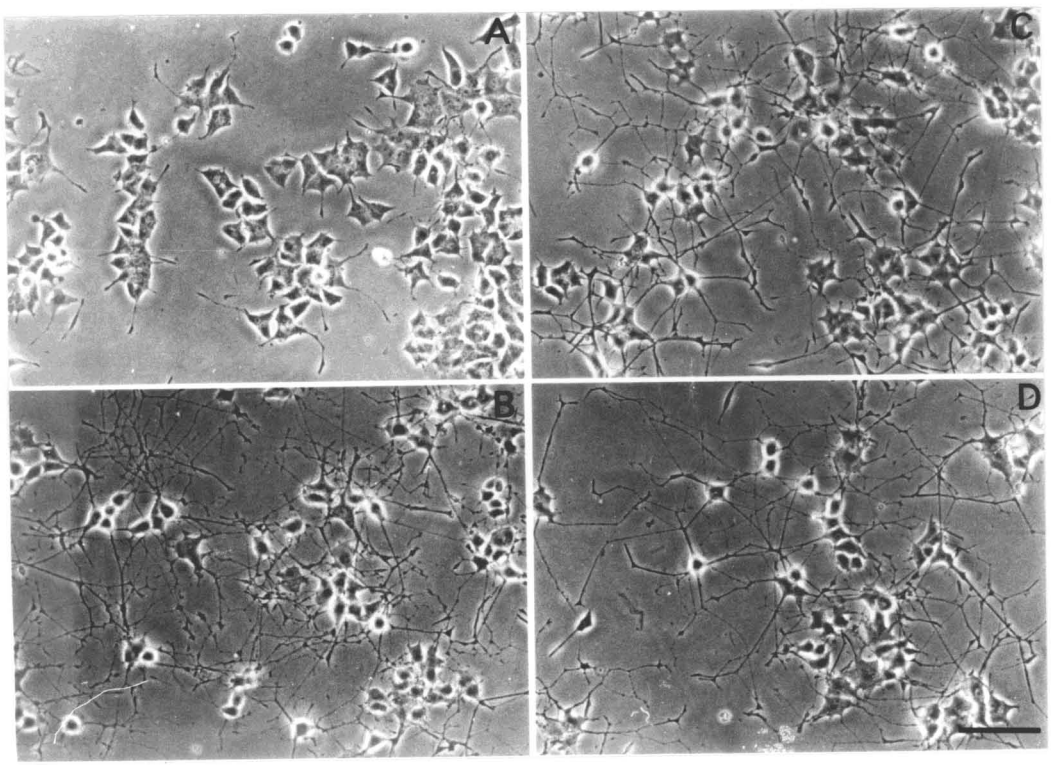

Fig. 8. TGFα-induced neurite outgrowth in PC12h-R cells. PC12h-R cells were plated on the collagen-coated dishes and cultured in DMEM supplemented with 5 μg/ml human transferrin, 5 μg/ml bovine insulin and 20 nM progesterone (TIP/DMEM). PC12h-R cells were cultured for 5 days in the absence (A) or presence of 50 ng/ml NGF (B), 50 ng/ml EGF (C), and 25 ng/ml TGFα **(D)**. Bar represents 100 μm.

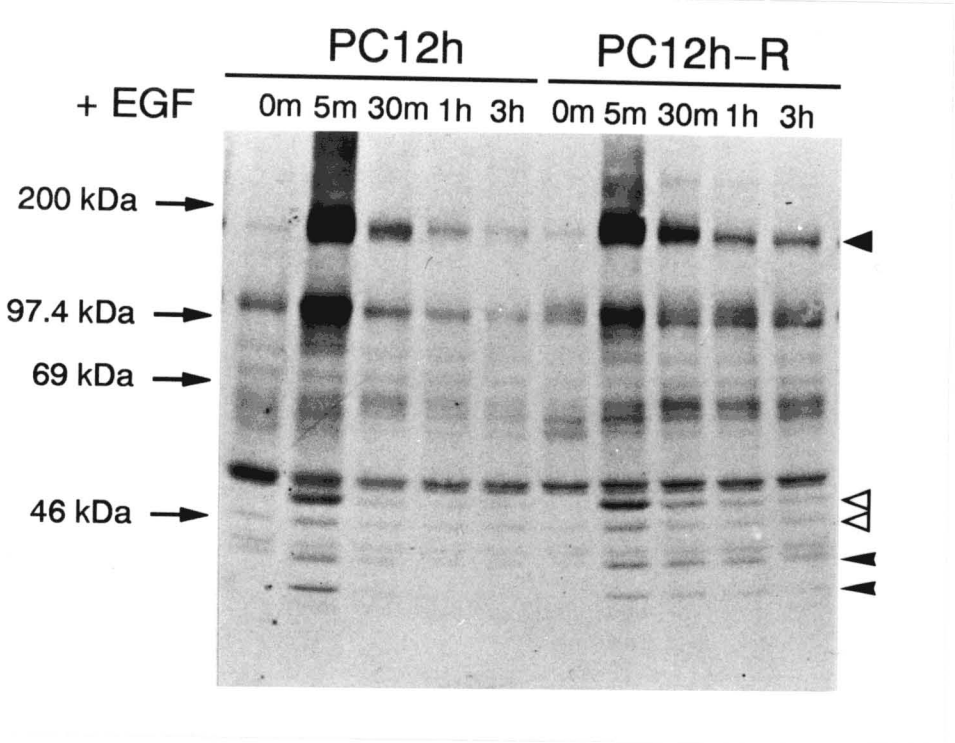

Fig. 9. EGF-induced tyrosine phosphorylation of cellular proteins in PC12h and PC12h-R cells. Lysates were prepared from cells incubated or not (0m) with 100 ng/ml EGF for 5 min (5m), 30 min (30m), 1 hr (1h) and 3 hr (3h), and were analyzed by Western blotting with the anti-phosphotyrosine antibody (4G10). The positions of EGF receptor (◀), Shc proteins (◁) and MAP kinases (◀) are indicated on the right of the immunoblot. Molecular weights are shown on the left.

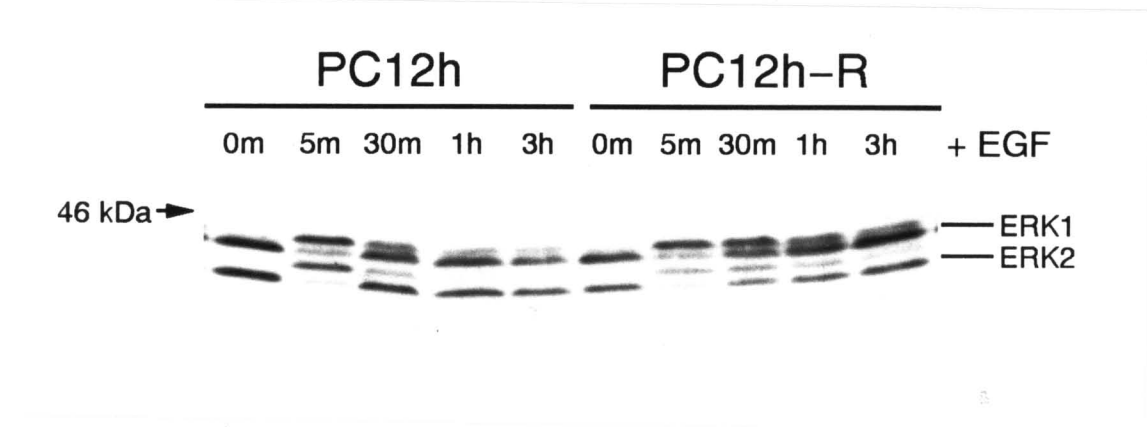

Fig. 10. EGF-induced mobility sifts of MAP kinases. Lysates were prepared from PC12h and PC12h-R cells incubated or not (0m) with 100 ng/ml EGF for 5 min (5m), 30 min (30m), 1 hr (1h) and 3 hr (3h), and were analyzed by Western blotting using the anti-MAP kinase antibody. The positions of ERK1 and ERK2 (MAP kinases) are indicated on the right of the immunoblot. Molecular weight is shown on the left.

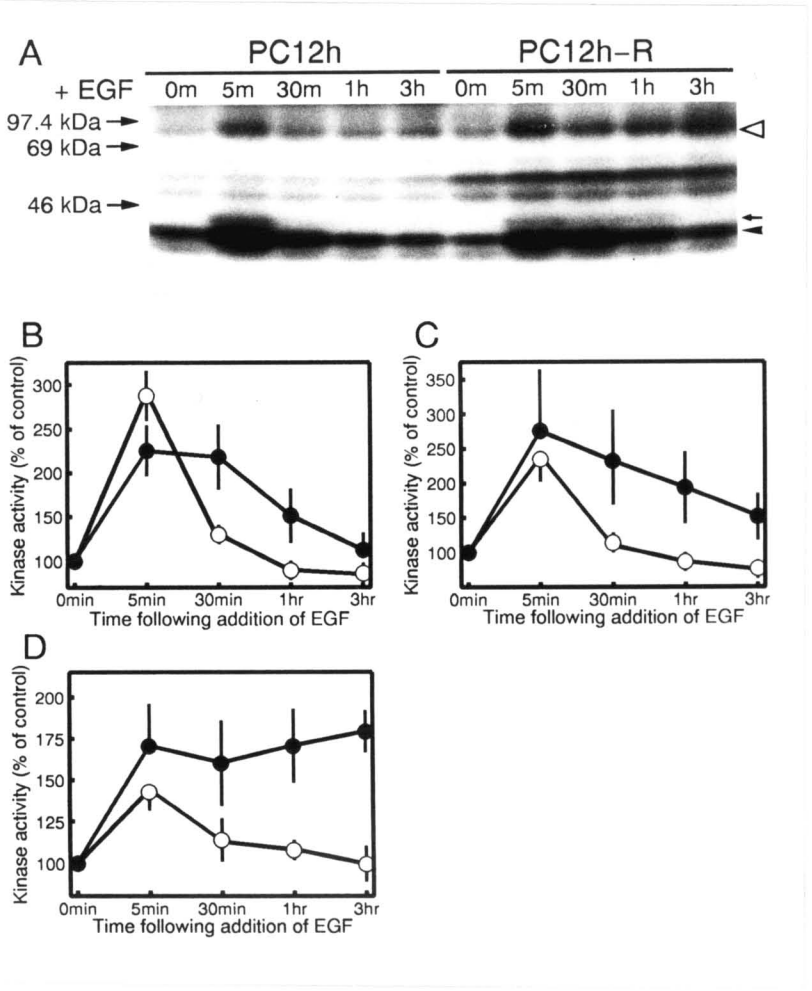

Fig. 11. EGF-induced activation of MAP kinases in PC12h and PC12h-R cells. The lysates were prepared from cells treated or not (0m) with 100 ng/ml EGF for 5 min (5m), 30 min (30m), 1 hr (1h) and 3 hr (3h), and analyzed by the renaturation gel assay using myelin basic protein (MBP) as a substrate (A). The positions of 42 kDa (closed arrow head) and 44 kDa (arrow) MAP kinases and 85 kDa kinase (open arrow head) are indicated on the right of the gel. Molecular weights are shown on the left. The activities of 42 kDa (B) and 44 kDa (C) MAP kinases and 85 kDa kinase (D) in PC12h (○) and PC12h-R (●) cells, were quantified by FUJIX Bio-imaging Analyzer. The activities are represented as the percentage of the activity in untreated cells (0 min). The values represent the means ± S.D. of four determinations.

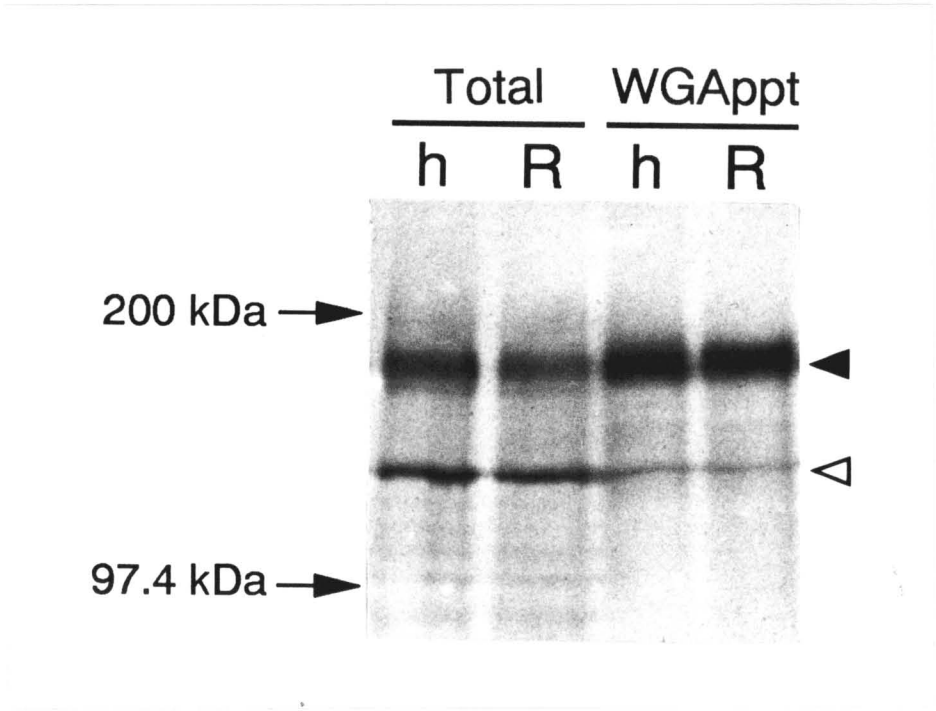

Fig. 12. Level of EGF receptor protein in PC12h and PC12h-R cells. The lysates (Total) and WGA precipitates (WGAppt) prepared from PC12h (h) and PC12h-R (R) cells, were analyzed by Western blotting with the anti-EGF receptor antibody. The positions of mature (closed arrow head) and precursor (open arrow head) proteins of the EGF receptor are indicated on the right of the immunoblot. Molecular weights are shown on the left.

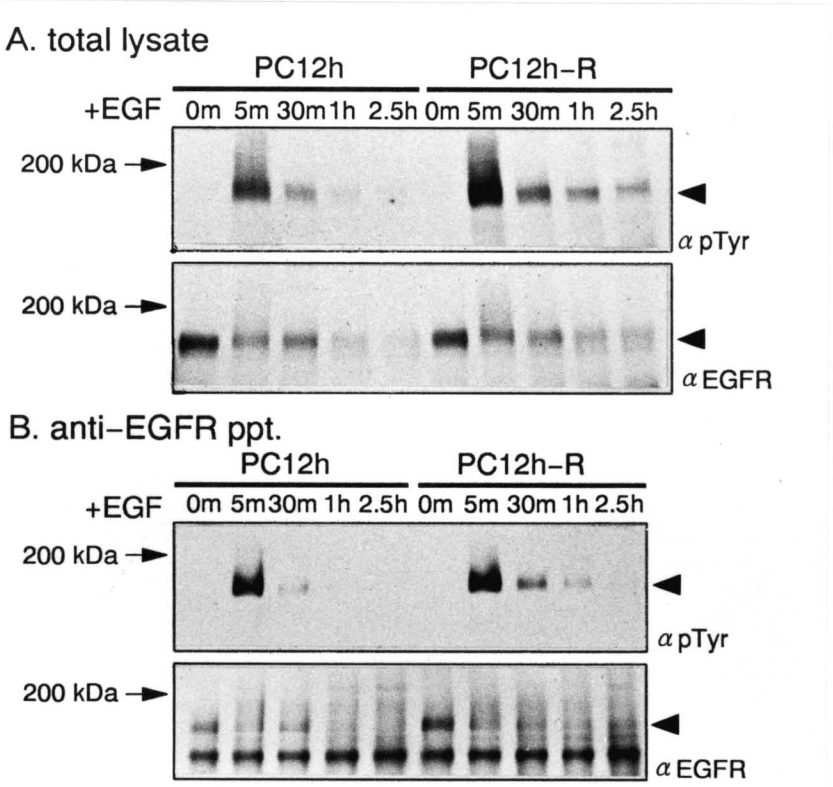

Fig. 13. EGF-induced tyrosine phosphorylation of the EGF receptor in PC12h-R and PC12h cells. (A) Lysates were prepared from cells incubated without (0m) or with 100 ng/ml EGF for 5 min (5m), 30 min (30m), 1 hr (1h) and 2.5 hr (2.5h), and Western blotted with the anti-phosphotyrosine antibody (4G10) (upper panel) and the anti-EGF receptor antibody (lower panel). The position of the EGF receptor (filled arrowhead) is indicated on the right of the immunoblot. The molecular weight is shown on the left. (B) Lysates were prepared from PC12h and PC12h-R cells incubated without (0m) or with 100 ng/ml EGF for 5 min (5m), 30 min (30m), 1 hr (1h) and 2.5 hr (2.5h). The EGF receptors were immunoprecipitated with the anti-EGF receptor antibody, and were Western blotted using the anti-phosphotyrosine antibody (upper panel) and the anti-EGF receptor antibody (lower panel). The position of the EGF receptor (filled arrowhead) is indicated on the right of the immunoblot. The molecular weight is shown on the left.

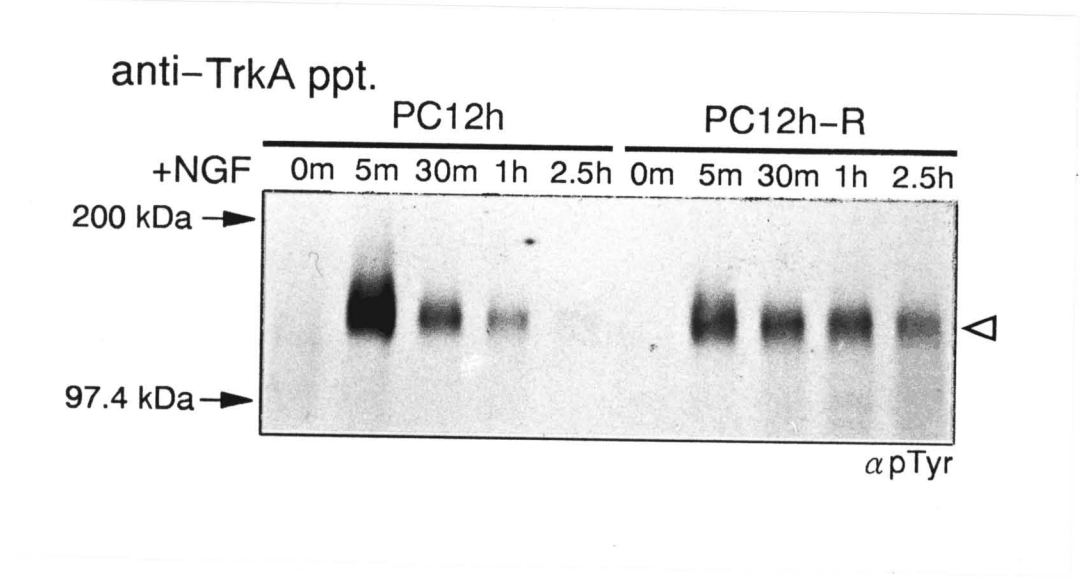

Fig. 14. NGF-induced tyrosine phosphorylation of p140^{trkA} in PC12h and PC12h-R cells. Cells were incubated without (0m) or with 100 ng/ml NGF for 5 min (5m), 30 min (30m), 1 hr (1h), and 2.5 hr (2.5h), and lysed. The p140^{trkA} was immunoprecipitated with the anti-p140^{trkA} antiserum and Western blotted with the anti-phosphotyrosine antibody. The position of p140^{trkA} (open arrowhead) is indicated on the right of the immunoblot. The molecular weights are shown on the left.

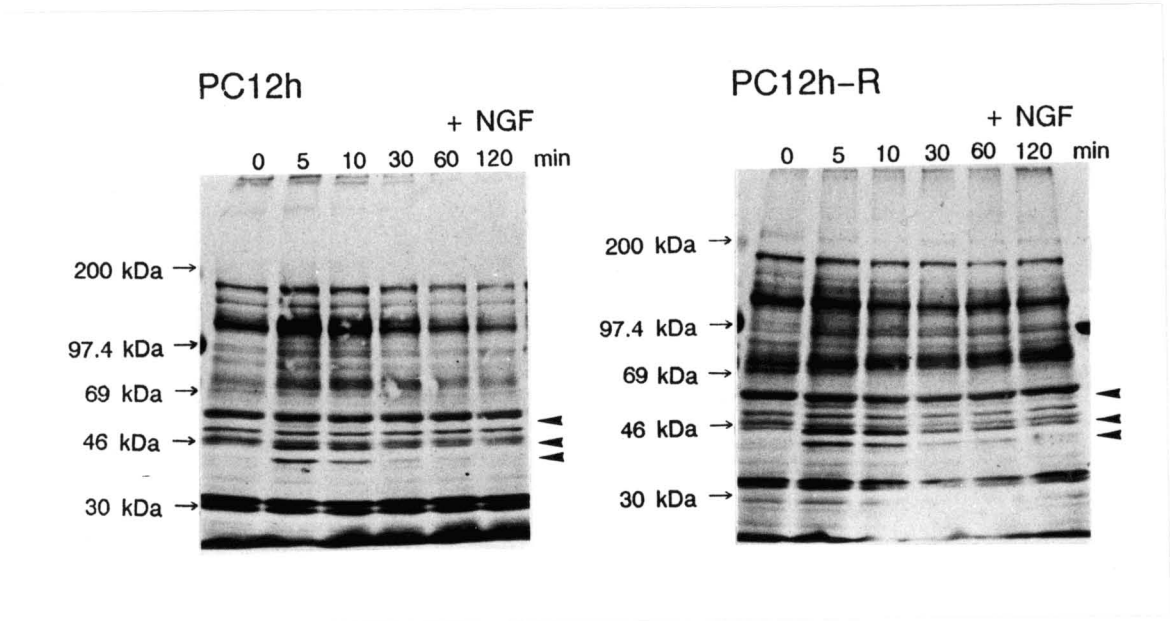

Fig. 15. NGF-induced tyrosine phosphorylation of cellular proteins in PC12h and PC12h-R cells. Lysates were prepared from cells treated with 100 ng of NGF per ml for 5, 10, 30, 60, and 120 min, as indicated at the top of each lane. The lysates were analyzed by Western blotting with anti-phosphotyrosine antibody (4G10). Arrowheads indicate tyrosine phosphorylated proteins of 54, 44, and 42 kDa. Molecular weights are shown at the left of each immunoblot.

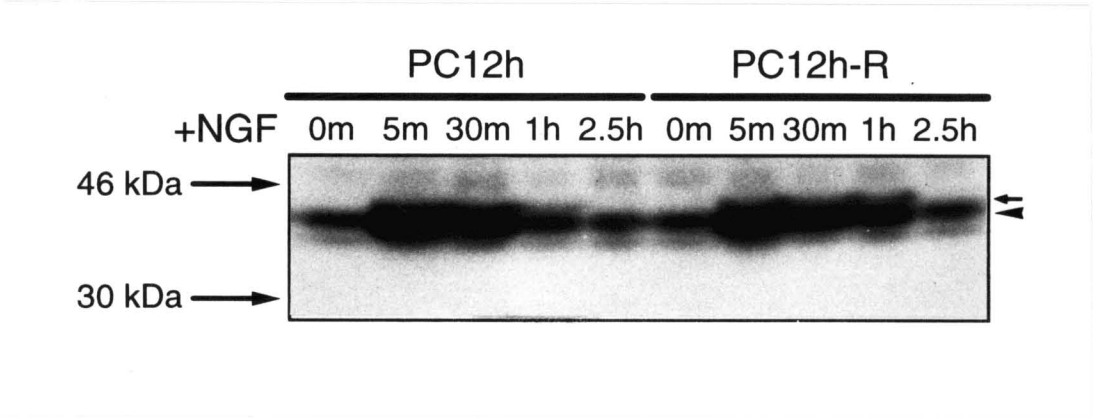

Fig. 16. NGF-induced activation of MAP kinases in PC12h and PC12h-R cells. The lysates were prepared from cells treated or not (0m) with 100 ng/ml EGF for 5 min (5m), 30 min (30m), 1 hr (1h) and 2.5 hr (2.5h), and analyzed by the renaturation gel assay using myelin basic protein (MBP) as a substrate. The positions of 42 kDa (closed arrow head) and 44 kDa (arrow) MAP kinases are indicated on the right of the gel. Molecular weights are shown on the left.

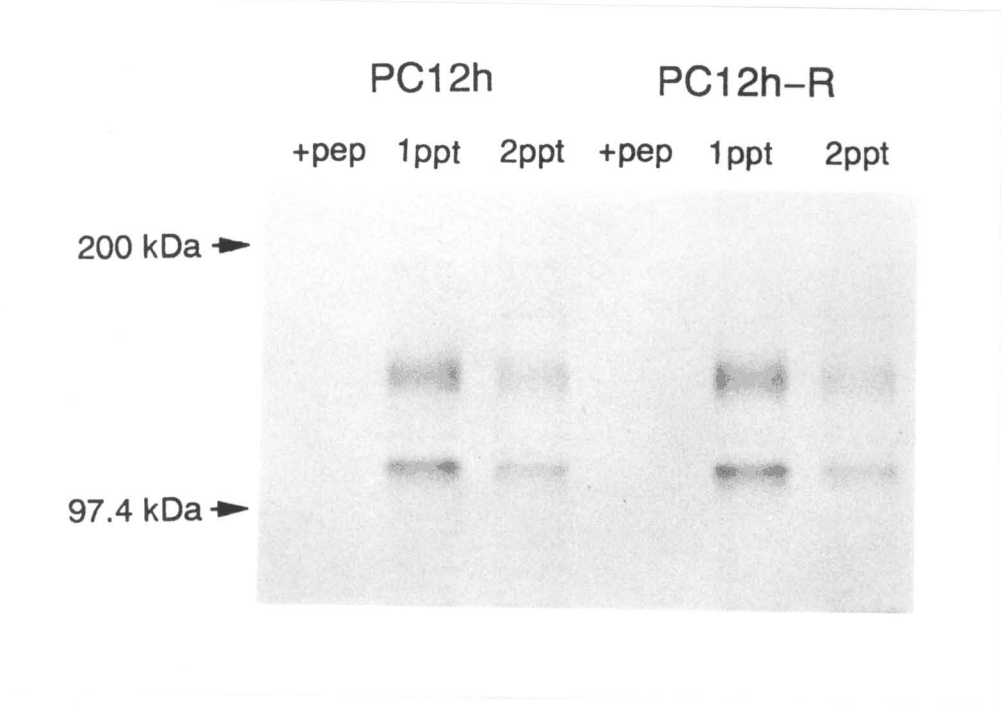

Fig. 17. Amounts of p140^{trkA} protein in PC12h and PC12h-R cells. Cells (PC12h-R) were labeled with [^{35}S] methionine and lysed. p140^{trkA} was immunoprecipitated with anti-p140^{trkA} antibodies in the presence (+pep) or absence (1ppt) of the immunizing peptides. Immunoprecipitation was carried out further from the supernatant of the first immunoprecipitation (2ppt). The immunoprecipitates were analyzed on 7.5% SDS-polyacrylamide gel. The positions of molecular weight markers are indicated on the left.

Part 2:

Epidermal Growth Factor Prevents Oxygen-triggered Apoptosis and Induces Sustained Signalling in Cultured Rat Cerebral Cortical Neurons

Abstract

Epidermal growth factor (EGF), a conventional mitogenic factor, acts as a neurotrophic factor on several types of neurons in the central nervous system. I found that EGF prevented the death of rat cerebral cortical neurons cultured in a 50% oxygen atmosphere. This high oxygen-induced cell death showed features of apoptotic cell death, which displayed the chromatin condensation and was blocked by inhibitors of RNA or protein synthesis. The oxygen-induced death was protected by antioxidants, vitamin E and N-acetyl-L-cysteine. EGF prevented the oxygen-induced death of the cultured cortical neurons in a dose-dependent manner. Basic fibroblast growth factor (bFGF) also prevented this cell death, although there was no apparent additive effect of EGF and bFGF. Among the cultured cortical neurons, I observed neurons possessing the EGF receptor, and cells expressing c-Fos protein in response to EGF. The cortical neurons were cultured in the presence of cytosine arabinoside, and the number of glial fibrillary acidic protein (GFAP)-positive astroglial cells were below 0.5% of that of the corresponding MAP2-positive neurons. Therefore, the effect of EGF on the cultured cortical neurons is thought to be due to a direct action.

I also examined EGF-induced signalling in the cultured cortical neurons. I found that EGF induced the sustained tyrosine phosphorylation of the EGF receptor and the sustained activation of mitogen-activated protein (MAP) kinase in the cultured cortical neurons. These results suggest that EGF exerts the survival effect through the prolonged activation of the EGF signalling.

Introduction

Epidermal growth factor (EGF) is a polypeptide growth factor which promotes cell proliferation of various types of cells including epithelial and fibroblast cells (Carpenter and Cohen, 1979). The action of EGF is initiated by binding to its receptor, a 180 kDa membrane-spanning glycoprotein possessing an intracellular domain containing a tyrosine kinase, which then becomes activated (Schlessinger and Ullrich, 1992). Thereafter, the EGF receptor is autophosphorylated on tyrosine residues, then it activates several enzymes and effectors including phospholipase C γ (PLC γ), phosphatidylinositol-3 (PI-3) kinase, $p21^{ras}$ and mitogen-activated protein (MAP) kinases or extracellular signal-regulated kinases (ERKs) (Schlessinger and Ullrich, 1992; Schlessinger, 1993), causing the expression of early-response genes such as *c-fos* and *c-myc* (Müller *et al.*, 1984).

In the central nervous system (CNS), the EGF receptor is expressed in several neurons of the cerebral cortex, cerebellum, and hippocampus (Adamson and Meek, 1984; Gómez-Pinilla *et al.*, 1988; Tucker *et al.*, 1993). the EGF receptor exists in mouse brain as early as embryonic day 15 and increases in number throughout the late embryonic development. In addition, EGF and transforming growth factor α (TGF α), both of which bind to the EGF receptor (Derynck, 1988), are expressed in various regions of the CNS, including cerebral cortex, hippocampus, cerebellum, and striatum (Lazar and Blum, 1992). The expression of both growth factors is detectable as early as embryonic day 14 and continues to be detected in the postnatal period in mouse brain. It has been

considered that EGF can act on postmitotic neurons as well as on mitotic cells. In fact, EGF acts as a neurotrophic factor on cultured cerebral cortical, subneocortical telencephalic, and cerebellar neurons, enhancing neurite outgrowth and cell survival (Morrison *et al.*, 1987; Morrison *et al.*, 1988; Kornblum *et al.*, 1990). In hippocampal neurons, EGF shows neuromodulatory effects (Abe and Saito, 1992) but not survival effects (Walicke and Baird, 1988).

Neurotrophic factors rescue cell death in a variety of neuronal degenerative circumstances (Barde, 1989). Apoptosis (or programmed cell death) is the most studied type of cell death, and it is mediated by an intrinsic death program (Ellis *et al.*,1991). It is blocked by protein or RNA synthesis inhibitors (Martin *et al.*, 1988; Oppenheim *et al.*, 1990), and is characterized by the chromatin condensation and by the fragmentation of chromosomal DNA into multimers of about 180 base pairs (Wyllie *et al.*, 1984). Apoptosis is observed in naturally occurring cell death which happens at specific stages during the development of embryonic neurons in sympathetic ganglion, retina, spinal cord, hippocampus, cerebral cortex and so on (Cowan *et al.*, 1984; Clarke, 1990; Oppenheim, 1991). In addition, apoptosis may be related to the neuronal degeneration that occurs during Alzheimer's and Parkinson's diseases, and Down's syndrome or aging (Dipasquale *et al.*, 1991; Loo *et al.*, 1993; Hartley *et al.*, 1994; Busciglio and Yankner, 1995). It is important to study the mechanisms of neurotrophic factors that block apoptosis of neurons.

Oxidative stress causes various types of neuronal degeneration in cerebrovascular injury, neuropathology and aging (Cao *et al.*, 1988; Ames *et al.*,

1992; Satoh *et al.*, in press). As a system for studying neuronal cell death induced by oxidative stress *in vitro*, Hatanaka and his colleagues cultured CNS neurons in a high oxygen atmosphere (Kushima *et al.*, 1990; Enokido *et al.*, 1992). It has been reported that the cell death induced by oxygen toxicity in cultured hippocampal neurons shows the features of apoptosis and is prevented by basic fibroblast growth factor (bFGF) (Enokido *et al.*1992; Enokido and Hatanaka, 1993). In this study, I found that EGF prevented the apoptotic cell death of cultured cerebral cortical neurons induced by oxygen toxicity. Furthermore, to elucidate the mechanisms of EGF action on cerebral cortical neurons, I examined EGF-induced signalling in the cultured cortical neurons.

Materials and Methods

Cell culture

Primary cultures of dissociated cerebral cortical neurons were prepared from the brains of embryonic day E20-21 rats (Wistar ST, both sexes) as described previously (Enokido, *et al.*, 1992). The cells were cultured in a medium consisting of 5% precolostrum newborn calf serum (PNCS, Mitsubishi Kasei), 5% heat-inactivated (56℃, 30 min) horse serum (HS, Gibco) and 90% of a 1:1 mixture of Dulbecco's modified Eagle's (DME, Gibco) and Ham's F12 media (Gibco) containing 15 mM HEPES buffer, pH 7.4, 30 nM selenium, and 1.9 mg/ml sodium bicarbonate, at a final cell density of 5×10^5 cells/cm^2 on a polyethyleneimine-coated surface in Costar 24-well plates (2 cm^2 of culture surface area). After overnight incubation, the medium was changed to minimum essential medium (MEM, Gibco) containing 5 μg/ml human transferrin, 5 μg/ml bovine insulin (Colab. Res.), 20 nM progesterone, 30 nM selenium, 2.2 mg/ml glucose, 0.5 mg/ml sodium bicarbonate, and 1 μM cytosine arabinoside (Sigma). Culture plates were transferred to chambers under a 20 or 50% (v/v) O_2 and a constant 5% (v/v) CO_2 atmosphere in a N_2-O_2-CO_2 gas incubator (TABAI BNP-110M) 8 hr after the medium change. The oxygen tension in the 50% O_2 culture medium increased rapidly and reached a plateau level of about 300 mmHg within 2-3 hr. The tension value of CO_2 and the pH value were stable during the entire culture period in both the 20 and 50% O_2 cultures, as described previously (Enokido, *et al.*, 1992). EGF or bFGF was added to the MEM just after the medium change. Cycloheximide or actinomycin D (both Sigma) was added just before the culture plates were transferred.

For the immunostaining with anti-EGF receptor and anti-c-Fos antibodies, the assay of MAP kinase and to detect tyrosine phosphorylation of the EGF receptor,

the cerebral cortical neurons were seeded in polyethyleneimine-coated Costar 24-well plates (to immunostain with the anti-EGF receptor and anti-c-Fos antibodies and to assay the MAP kinase activity) and polyethyleneimine-coated Costar 6-well plates (9.5 cm^2 of culture surface area; to detect tyrosine phosphorylation of the EGF receptor), as descrived above. After overnight incubation, the medium was changed to MEM as described above, and the cells were cultured in a 20% O_2 atmosphere. The assays were performed 2 days after the medium change.

PC12h cells were maintained in 75 cm^2 flasks (Costar) in a mixture of 90% DME medium, 5% PNCS and 5% heat-inactivated HS, as described in Part 1. The cells were cultured in 60-mm collagen-coated Falcon dishes (21 cm^2 of culture surface area) to detect tyrosine phosphorylation of the EGF receptor and to assay the MAP kinase activity. The medium was changed to that without serum 2 hr before exposure to EGF.

Antibodies, factors, and reagents

Anti-phosphotyrosine monoclonal (4G10) and anti-EGF receptor antibodies were purchased from Upstate Biotechnology Inc. Anti-c-Fos antibody was purchased from Oncogene Sci. Inc.. The anti-microtubule-associated protein 2 (MAP2) antiserum was a gift from Dr. Murofushi (University of Tokyo). EGF was purchased from Toyobo Co., Ltd.. Human recombinant bFGF was a gift from Takeda Pharmaceutical Co. The anti-glial fibrillary acidic protein (GFAP) monoclonal antibody was purchased from Amersham. Vitamin E and N-acetyl-L-cysteine were purchased from Sigma.

Immunocytochemistry

The cells were fixed in 4% paraformaldehyde at room temperature for 20 min. For immunostaining with the anti-EGF receptor and the anti-c-Fos

antibodies, the cells were blocked with 5% (w/v) nonfat dry milk in phosphate-buffered saline containing 5% (v/v) normal goat serum and 0.4% (v/v) Triton X-100 overnight at 4℃, then incubated with the anti-EGF receptor antibody diluted 1:150 or the anti-c-Fos antibody diluted to 1:200 with the blocking buffer without the nonfat dry milk, overnight at 4℃. For immunostaining with the anti-MAP2 antiserum, the cells were blocked with phosphate-buffered saline (PBS) containing 5% (v/v) normal goat serum and 0.1% (v/v) Triton X-100 over 3 hr at 4℃, then incubated with the anti-MAP2 antiserum diluted to 1:5,000 with the blocking buffer, overnight at 4℃. For immunostaining with the anti-GFAP antibody, the cells were fixed in 100% methanol, and were blocked with PBS containing 5% (v/v) normal goat serum and 0.3% (v/v) Triton X-100 over 3 hr at 4℃, then incubated with the anti-GFAP monoclonal antibody diluted to 1:10,000 with the blocking buffer, overnight at 4℃. Staining was achieved using the Vectastain ABC kit (Vector Labo.) and 0.02% (w/v) 3,3'-diaminobenzidine 4-HCl and 0.1% (w/v) $(NH_4)_2Ni(SO_4)_2$ dissolved in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.01% (v/v) H_2O_2.

The number of immunoreactive cells was determined by microscopic observation and by examining microphotographs. The number of immunoreactive cells in more than two randomly selected microscopic fields per well were counted and averaged.

MTT assay

The MTT assay was performed in according to a modification (Hansen *et al.*, 1989; Kubo *et al.*, 1995) of the original procedure (Mosmann, 1983). Briefly, the tetrazolium salt MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) was added to the cultures at a final concentration of 1 mg/ml. After incubation at 37℃ for 2 hr, the assay was stopped by adding lysis buffer

consisting of 20% (w/v) SDS and 50% (v/v) N,N-dimethyl formamide, pH 4.7. The absorbance was measured photometrically at 570 nm after incubation at 37℃ overnight.

Hoechst 33,258 staining

For chromatin staining of the nucleus, the cerebral cortical neurons were plated on glass coverslips coated with polyethyleneimine. Neurons were fixed with 4% paraformaldehyde for 20 min, then stained for 15 min with 1 μg/ml Hoechst 33,258 (Wako) in PBS at room temperature. Neurons were then washed twice with PBS and viewed under UV illumination using a Nikon microphoto fluorescence microscope.

Detection of tyrosine phosphorylation of the EGF receptor

Cells were washed once with ice-cold Tris-buffered saline, TBS, and lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 20 mM Tris-HCl, pH 7.5, 1 mM PMSF, and 5 μg/ml aprotinin. Lysates were centrifuged at 10,000 × g at 4℃ for 30 min, then the protein concentration of the clarified lysate was determined by the BCA protein assay. The anti-EGF receptor antibody (5 μg) was added and incubated at 4℃ overnight. Protein G-Sepharose (4 μl) was then added, and rotated at 4℃ for 30 min. The immune complexes were pelleted by centrifugation at 10,000 × g at 4℃ for 1 min, then washed 4 times with the lysis buffer. The immune complexes were eluted with sample buffer (0.125 M Tris-HCl, pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS, and 10% (v/v) β-mercaptoethanol), boiled for 3 min, then recovered by centrifugation for 5 min in a microfuge. The eluates were resolved by electrophoresis on a 7% SDS-polyacrylamide gel (Laemmli, 1970). Immunoblotting with anti-phosphotyrosine antibody 4G10 was performed as

described in Part 1.

Assay of the MAP kinase activity in renatured Gels

Cells were washed once with ice-cold TBS, lysed in a buffer containing 1% SDS, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM Tris-HCl, pH 7.5, and 1 mM PMSF, and boiled for 5 min. The protein concentration was determined using the BCA protein assay, then the MAP kinase activity of the lysate was analyzed by the kinase renaturation gel assay according to Gotoh *et al.*(1990) with some modifications, as described in Part 1.

Results

Apoptotic cell death induced by oxygen toxicity in cerebral cortical neurons

It has been reported that hippocampal neurons cultured in a high oxygen atmosphere show apoptotic cell death which is prevented by protein or RNA synthesis inhibitors and displays the nuclear DNA fragmentation (Enokido and Hatanaka, 1993). To determine whether the oxygen-induced death of cultured cerebral cortical neurons is also apoptotic, I examined the effects of protein or RNA synthesis inhibitors. Neurons from embryonic rat (embryonic day 20-21) cerebral cortex were cultured in 20% and 50 % oxygen atmospheres in the absence or presence of cycloheximide or actinomycin D for 2.5 days. The neurons were immunostained with anti-MAP2 antiserum, and survival of neurons was quantified by counting the number of MAP2-positive neurons and by the MTT assay (Fig. 1, 2A and 2C). Both cycloheximide and actinomycin D prevented neuronal cell death caused by oxygen toxicity. The survival percentage of the cells cultured with cycloheximide or actinomycin D was over 90%, but that without them was 26% in the MTT assay (Fig. 2B and 2D). The survival percentage determined by counting the number of MAP2-positive neurons was similar to that in the MTT assay.

One of the characters of apototic cell death is the chromatin condensation (Wyllie *et al.*, 1984). To examined whether or not the high oxygen induces the chromatin condensation in the cultured cortical neurons, nuclei of the neurons cultured in 20% and 50% oxygen atomospheres for 2 days were stained with

Hoechst 33,258 (Fig. 3). Most of the neurons cultured in a 50% oxygen atmosphere showed the condensed chromatin stained brightly, although most of those in a 20% oxygen atmosphere displayed large nuclei stained sparsely. These results indicate that the high oxygen induces apoptotic cell death in the cultured cortical neurons.

Effects of antioxydants on the oxygen-induced cell death of cerebral cortical neurons

Reactive oxygen species are involved in the neuronal death in Alzheimer's diseases, Down's syndrome and naturally occurring cell death (Behl *et al.*, 1994; Busciglio and Yankner, 1995; Greenlund *et al.*, 1995). I consider that reactive oxygen species are major contributory factors to the high oxygen-induced death of the cultured cortical neurons. To examine the role of reactive oxygen species in the high oxygen-induced neuronal death, the neurons were cultured in 20% and 50 % oxygen atmospheres in the absence or presence of antioxidants for 2.5 days (Table 1). Survival of neurons was quantified by the MTT assay. The free radical scavenger vitamin E and the glutathione precursor/radical scavenger N-acetyl-L-cysteine prevented the oxygen-induced death of the cortical neurons. Vitamin E was much more effective than N-acetyl-L-cysteine. N-acetyl-L-cysteine might be effective at a higher concentration than that in this experiment, but it was toxic to the cultured cortical neurons at more than 100 μM. These results suggest that the high oxygen-induced death of the cortical neurons is induced through the production of reactive oxygen species.

The effect of EGF on the oxygen-induced cell death of cerebral cortical neurons

I determined whether EGF prevents the apoptotic death induced by oxygen toxicity in the cerebral cortical neurons. The neurons were cultured in 20% and 50% oxygen atmospheres with or without EGF for 2.5 days and immunostained using the anti-MAP2 antiserum (Fig. 1). The survival of neurons was quantified by counting the number of MAP2-positive cells and by means of the MTT assay (Fig. 2). The survival ratios of the cultured neurons in the presence of EGF are 81% and 63% according to the MTT assay and the MAP2-positive cell counting, respectively, whereas those in the absence of EGF are 26% and 11%. In addition, EGF prevented the chromatin condensation in the cortical neurons cultured in the high oxygen atmosphere (Fig. 3). These results indicate that EGF rescues the high oxygen-induced apoptosis of the cortical neurons.

To examine the dose dependence of this effect of EGF, I measured the survival percentage of the neurons cultured with various concentrations of EGF for 2.5 days in 20% and 50% oxygen atmospheres, by means of the MTT assay (Fig. 4). EGF protected the oxygen-induced neuronal cell death in a concentration-dependent manner. The maximal effect was exerted at an EGF concentration of 10 ng/ml, and the half maximal effect occurred at 0.3 ng/ml.

The time-dependent death of the cortical neurons cultured in a 50% oxygen atmosphere is shown in Figure 5A. Death occurred after 2 days under these culture conditions. The measured value of the cultured neurons in a 50% oxygen

atmosphere for 3 days was only 13% of that of the initial culture with the MTT assay. EGF prevented and delayed this cell death. Little cell death was observed after 2 days when cultured with EGF. The measured value of the cultured neurons with EGF in a 50% oxygen atmosphere for 3 days was 39% of that of the initial culture.

To examine the commitment time of the EGF effect on the oxygen-induced death of the cortical neurons, EGF was added to the culture at various time points. I measured the survival percentage of these cultured cells by means of the MTT assay (Fig. 5B). The later EGF was added to the culture, the less effective it was on the death induced by oxygen toxicity. When added just before, or 1 day after transfer to a 50% oxygen atmosphere, EGF effectively prevented the oxygen-induced cell death. On the other hand, the survival percentage of the neurons exposed to EGF 2 days after transfer to a 50% oxygen atmosphere was similar to that in the absence of EGF.

It has been reported that bFGF acts on cerebral cortical neurons as a neurotrophic factor (Morrison *et al.*, 1986). I investigated the effect of bFGF on the oxygen-induced death of the cortical neurons. bFGF also prevented the neuronal cell death induced by oxygen toxicity (Fig. 6 and 7). In order to investigate whether EGF and bFGF act on the same population of the cortical neurons, I examined the additive effect of these two factors at their maximum doses on the oxygen-triggered death of the cortical neurons, and found that there was no additive effect (Fig. 6 and 7).

EGF promotes not only the survival of neuronal cells but also the proliferation

and differentiation of non-neuronal cells, such as astroglial cells. To examine whether the survival effect of EGF on the neurons was direct or indirect, the cultured cells were immunostained using the anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (Fig. 8). Under culture conditions mentioned above, there were few GFAP-positive astroglial cells. I also found that there were less astroglial cells in cultures with EGF than in those without EGF. That is to say, viable GFAP-positive astroglial cells among the cultured cells for 2 days in a 20% oxygen atmosphere without and with EGF were 0.437 ± 0.095% (means ± S.D., n = 4) and 0.083 ± 0.025% (means ± S.D., n = 4) of the corresponding MAP2-positive neurons, respectively. EGF might enhance the effect of cytosine arabinoside added to the culture.

EGF receptor- and Fos-positive cells among cultured cerebral cortical neurons

EGF binds to the EGF receptor which induces intracellular signalling, thus exerting its various actions (Schlessinger and Ullrich, 1992). I determined whether the EGF receptor is expressed in cultured cerebral cortical neurons by immunostaining them with the anti-EGF receptor antibody (Fig. 9A). EGF receptor-positive cells in neurons cultured for 2 days in a 20% oxygen atmosphere accounted for 3.68 ± 0.53% (means ± S.D., n = 4) of the corresponding MAP2-positive neurons. Most of the EGF receptor-positive cells showed neuronal morphology. In addition, EGF receptor-positive cells in the cultured neurons with EGF were less immunostained than those without EGF (data not shown). This

finding may have arisen from the EGF-induced down-regulation of the EGF receptor in the cultured neurons.

In EGF-responsive cells, EGF induces the expression of immediate-early genes such as the *c-fos* gene (Müller *et al.*, 1984). To examine whether the cultured neurons express c-Fos protein in response to EGF, neurons exposed or not to EGF 2 hr were immunostained using the anti-c-Fos antibody (Fig. 9B and 9C). Fos-positive cells in cultures treated with and without EGF accounted for 6.08 ± 1.65% (means ± S.D., n = 4) and 1.30 ± 0.43% (means ± S.D., n = 4) of the corresponding MAP2-positive neurons, respectively. Therefore, the percentage of EGF-responsive cells in the cultured cortical neurons was estimated to be 4.78 ± 1.71% of the MAP2-positive neurons.

EGF-induced sustained signalling in the cultured cerebral cortical neurons

EGF induces the tyrosine phosphorylation and activation of the EGF receptor and MAP kinases, which are key molecules in the signalling pathway induced by EGF (Schlessinger, 1993). The sustained signalling is correlated with the response of neuronal differentiation in PC12 cells (Marshall, 1995). To elucidate the mechanism of EGF action on cerebral cortical neurons, I examined the duration of EGF-induced intracellular signalling in the cultured cortical neurons.

I compared the time course of the EGF-induced tyrosine phosphorylation of the EGF receptor in the cultured cerebral cortical neurons with that in PC12h cells, a subclone of PC12 cells (Hatanaka, 1981). In PC12h cells, EGF acts as a weak mitogen and induces the transient signalling in the same way as it dose

in PC12 cells. In both the cultured cortical neurons and PC12h cells, the tyrosine phosphorylation level of the EGF receptor reached the maximum within 5 min of exposure to EGF (Fig. 10). In the cortical neurons, this tyrosine phosphorylation was remarkably sustained, and remained detectable even 3 hr after EGF addition. On the other hand, the tyrosine phosphorylation of the EGF receptor was transient in PC12h cells, and declined to the basal level within 1 hr of EGF exposure.

I examined the duration of EGF-induced activation of MAP kinase by means of the kinase renaturation gel assay using myelin basic protein (MBP) as a substrate, in the cultured cortical neurons and PC12h cells (Fig. 11A and 11C). EGF induced the activation of the 42 kDa MAP kinase (ERK2), in both cultures. EGF also induced activation of the 44 kDa MAP kinase (ERK1), but at a quite low level. When I used a gel without MBP in the kinase assay, the activation of 42 kDa MAP kinase was not observed (data not shown). I quantified the activity of the 42 kDa MAP kinase using the FUJIX Bio-imaging analyzer (Fig. 11B and 11D). In both cultures, the activity of the 42 kDa MAP kinase reached the maximum within at least 5 min of EGF exposure. In PC12h cells, this kinase activity rapidly declined to the basal level within 1 hr of EGF exposure. However, in the cultured neurons, the 42 kDa MAP kinase activity was sustained at the high level for at least 30 min, then it gradually declined to the basal level 3 hr after EGF addition. These results indicate that EGF induces the sustained tyrosine phosphorylation of the EGF receptor and the prolonged activation of MAP kinase in the cultured cortical neurons.

Discussion

Neuronal death induced by oxidative stress resulting in excessive production of reactive oxygen species, is related to a variety of neuronal degenerative disorders caused by cerebrovascular injury, neuropathology and aging (Cao *et al.*, 1988; Ames *et al.*, 1992; Satoh *et al.*, in press). Oxidative damage is closely associated with Parkinson's, Alzheimer's and familial amyotrophic lateral sclerosis (ALS) diseases, and Down's syndrome (Dyrks *et al.*, 1992; Przedborski *et al.*, 1992; Rosen *et al.*, 1993; Behl *et al.*, 1994; Busciglio and Yankner, 1995). Recently, it has been reported that reactive oxygen species are related to naturally occurring cell death (Greenlund *et al.*, 1995). Therefore, studies concerning neuronal cell death induced by oxidative stress and its prevention are thought to be important.

To examine the neuronal death caused by oxidative stress *in vitro*, Hatanaka and his colleagues cultured CNS neurons in an atmosphere of high oxygen (50% O_2) (Kushima *et al.*, 1990; Enokido *et al.*, 1992). It has been reported that embryonic rat hippocampal neurons cultured in a 50% oxygen atmosphere died. This death closely resembled apoptosis, a type of cell death that plays an important role in early development and the subsequent maturation of immune and neuronal cells (Clarke, 1990; Ellis *et al.*, 1991; Oppenheim, 1991). The hippocampal neurons cultured in a 50% oxygen atmosphere showed nucleosomal DNA fragmentation, nuclear condensation and cytoplasmic compaction (Enokido and Hatanaka, 1993; Enokido and Hatanaka, 1995). In addition, the oxy-

gen-induced death of cultured hippocampal neurons was blocked by RNA or protein synthesis inhibitors. In this study, I showed that the death of the cultured cerebral cortical neurons in a 50% oxygen atmosphere displayed apoptotic features. The cultured cortical neurons in a 50% oxygen atmosphere showed the chromatin condensation and the oxygen-induced death of the cultured cortical neurons was prevented by RNA or protein synthesis inhibitors, in the same manner as that of the cultured hippocampal neurons. These results suggest that the oxygen toxicity activates an intracellular death cascade and induces apoptotic cell death in the cultured cortical neurons. Cultured embryonic cortical neurons show typical apoptosis, when they incubated with homocysteate, which causes oxidative stress (Ratan *et al.*, 1994). It remains unclear and intriguing how oxidative stress induces the apoptotic neuronal cell death.

EGF is a potent polypeptide mitogen in a variety of cell types (Carpenter and Cohen, 1979). In the CNS, EGF acts as a neurotrophic factor, enhancing neurite outgrowth and survival of the cultured subneocortical telencephalic, cerebellar and cerebral cortical neurons (Morrison *et al.*, 1987; Morrison *et al.*, 1988; Kornblum *et al.*, 1990). Here, I showed that EGF prevented the apoptosis caused by oxidative stress in a dose-dependent manner. The EC_{50} value of EGF was about 5×10^{-11} M, which was close to the Kd value of the EGF receptor, 10^{-9} to 10^{-10} M (Carpenter, 1987). In addition, bFGF, which is a neurotrophic factor for cerebral cortical neurons (Morrison *et al.*, 1986), prevented this apoptosis in the same way as EGF did. However, the additive effect of EGF and bFGF was little, if any. These results suggest that EGF and bFGF affect a largely overlapping population

of the cortical neurons. This suggestion agrees with an other report regarding the effects of EGF and bFGF on survival and neurite outgrowth of cortical neurons (Kornblum *et al.*, 1990).

I considere that the protective effect of EGF on the cultured cortical neurons in a 50% oxygen atmosphere results from its direct action on the neurons, rather than from an indirect action through glial cells, and not from a mitogenic effect on progenitor cells of neurons. I conclude this because the cortical neurons were cultured without serum in the presence of 1 μM cytosine arabinoside (AraC), and the number of GFAP-positive astroglial cells was below 0.5% of that of the MAP2-positive neurons. In addition, EGF exerted the effect even in the presence of 10 μM AraC (data not shown).

Kushima *et al.* (1990) reported that CNS neurons acquire tolerance to the oxidative damage induced by oxygen toxicity during maturation. In addition, the death of PC12 cells in a high oxygen atmosphere is prevented by NGF or bFGF, which induces the neuronal differentiation of PC12 cells (Enokido and Hatanaka, 1990). Therefore, the rescue effect of EGF on the cultured cortical neurons in a high oxygen atmosphere may result from the EGF-induced differentiation and maturation of these neurons.

It remains unclear how EGF can prevent the neuronal cell death induced by oxidative stress. One possibility is that EGF induces the production of some molecules that scavenge reactive oxygen species. Brain-derived neurotrophic factor (BDNF) increases the activity of glutathione peroxidase in SH-SY5Y cells (Spina *et al.*, 1992). The protooncogene *bcl-2*, the product of which has been

identified primarily in the outer mitochondrial membrane, as well as in membranes of the nucleus and endoplasmic reticulum, suppresses the apoptosis of lymphocytes caused by oxidative stress (Hockenbery *et al.*, 1993). The protective effect of EGF may be mediated by the regulation of Bcl-2 protein and its relatives, including Bax (Oltvai *et al.*, 1993) and a splice variant of Bcl-x, Bcl-x_s (Boise *et al.*, 1993). EGF must bind to the EGF receptor to exert its action. The EGF receptor is expressed in cerebral cortical neurons (layers IV and V) (Gómez-Pinilla *et al.*, 1988). I also found that some cells were immunostained with the anti-EGF receptor antibody in the cultured cerebral cortical neurons. Because most of the EGF receptor-positive cells had a neuronal morphology, and because the percentage of the EGF receptor-positive cells was over 8 times as large as that of GFAP-positive astroglial cells, most of cells expressing the EGF receptor in the cultured cortical neurons are thought to be neurons.

The EGF-responsive cells express c-Fos protein, an immediate-early gene product, in response to EGF (Müller *et al.*, 1984). When the cultured cortical neurons exposed or not to EGF were immunostained with the anti-c-Fos protein antibody, the EGF-induced expression of the Fos protein was detected. Since the percentage of these cells expressing c-Fos in response to EGF nearly coincided with that of the EGF receptor-positive cells, and scince the percentage was over 10 times as large as that of the GFAP-positive astroglial cells in the culture, most of these cells expressing c-Fos in response to EGF were thought to be the EGF-responsive neurons. I considere that EGF affects these EGF-responsive neurons, causing a neurotrophic effect in cultured cortical neurons.

Although EGF prevented the oxygen-induced death of the cultured cortical neurons, the percentage of the cells that possessed the EGF receptor or expressed the c-Fos protein in response to EGF was small. Kinoshita, *et al.* (1990) have reported that about 70% of cultured cortical neurons were immunostained by anti-EGF receptor antibody. In addition, I found that the percentage of cells expressing c-Fos in response to bFGF was similar to that in response to EGF (data not shown). The apparently low percentage of these cells may be due to the low sensitivity of immunostaining for the EGF receptor and c-Fos. However, the possibility that the percentage of the EGF-responsive cells is indeed small can not be excluded. Because the protective effect of EGF on the apoptosis of the cultured cortical neurons is weeker than those of RNA and protein synthesis inhibitors, which are thought to affect the total population, the effect of EGF may result from its action on a small population. The effects of EGF on the oxidative stress-induced apoptosis may arise from secondary effects of EGF through the EGF-responsive neurons; for example, by the release of some neurotrophic factors from the EGF-responsive neurons.

Although studies of the effects of EGF on the neurons of the CNS are expanding, relatively little is known about the mechanisms of its action. I investigated the EGF-induced signalling in cultured cerebral cortical neurons, especially the tyrosine phosphorylation of the EGF receptor and the activation of MAP kinase, which are essential for the signal transduction pathway induced by EGF (Schlessinger, 1993). EGF induced the tyrosine phosphorylation of the EGF receptor and the activation of a 42 kDa MAP kinase. The activation of the MAP

kinase in the cultured cortical neurons was less striking than that in PC12h cells. This may be due to the high basal activity of the MAP kinase or to the low number of the EGF-responsive neurons in the culture. I found that the EGF-induced signalling in the cultured cortical neurons was sustained in comparison with that in PC12h cells, in which EGF acts as a mitogen. This sustained signalling may be a general feature of the neurotrophic action of EGF.

Rat pheochromocytoma PC12 cells are used as a model system in which to elucidate the mechanisms of the neurotrophic actions (Greene and Tischler, 1976). PC12 cells differentiate into neuron-like cells in response to nerve growth factor (NGF), but show an increase in cell proliferation in response to EGF (Huff *et al.*, 1981). The NGF-induced signalling in PC12 cells including the activation of $p21^{ras}$ and MAP kinases is reported to be sustained in comparison with the EGF-induced signalling (Marshall, 1995). In addition, I found that PC12h-R cell, a subclone of PC12 cells, which differentiated to neuron-like cells in response to EGF as well as to NGF, showed the sustained EGF-induced signalling, such as the tyrosine phosphorylation of the EGF receptor, Shc proteins and MAP kinases, and the activation of MAP kinases, as described in Part 1. Here, I reported that the cultured cortical neurons displayed the sustained EGF-induced signalling. I suggest that the sustained signalling is a general feature of the neurotrophic actions. EGF might exert distinct effects such as mitogenic, survival, differentiative and neuromodulatory effects, in different subpopulations of neurons or even in the same population at different stages of development, through different mechanisms including different durations of the EGF-induced signalling.

It remains unclear as to how the EGF-induced signalling is sustained in the cultured cerebral cortical neurons. A possible mechanism of the sustained signalling is a change in the protein tyrosine phosphatase (Sun and Tonks, 1994) that dephosphorylates the EGF receptor and the MAP kinase. The sustained signalling is thought to be due to a neuron specific down-regulation or lack of the protein tyrosine phosphatase. The other possibility is that the EGF-induced sustained signalling is resulted from the decreased rate of down-regulation of the EGF receptor as PC12h-R cells, as described in Part 1. PC12h-R cells sustain the EGF-induced signalling in the same manner as cultured cerebral cortical neurons do, therefore PC12h-R cells may be a good model system with which to study the mechanisms of this EGF-induced process in cerebral cortical neurons.

References

Abe, K. and Saito, H. (1992) Epidermal growth factor selectively enhances NMDA receptor-mediated increase of intracellular Ca^{2+} concentration in rat hippocampal neurons. *Brain Res.*, **587**, 102-108.

Adamson, E. D. and Meek, J. (1984) The ontogeny of epidermal growth factor receptors during mouse development. *Dev. Biol.*, **103**, 62-70.

Ames, B. N., Shigenaga, M. K. and Hogen, T. M., (1992) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA.*, **90**, 7915-7922.

Barde, Y.-A. (1989) Trophic factors and neuronal survival. *Neuron*, **2**, 1525-1534.

Behl, C., Davis, J. B., Lesley, R. and Schubert, D. (1994) Hydrogen peroxide mediates amyloid β protein toxicity. *Cell*, **77**, 817-827.

Boise, L. H., González-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nuñez, G. and Thompson, C. B. (1993) *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*, **74**, 597-608.

Busciglio, J. and Yankner, B. A. (1995) Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons *in vitro*. *Nature*, **378**, 776-779.

Cao, W., Carney, J. M., Duchon, A., Floyd, R. A. and Chevion, M. (1988) Oxygen free radical involvement in ischemia and reperfusion injury to brain. *Neurosci. Lett.*, **88**, 233-238.

Carpenter, G. and Cohen, S. (1979) Epidermal growth factor. *Annu. Rev. Biochem.*, **48**, 193-216.

Carpenter, G. (1987) Receptor for epidermal growth factor and other polypeptide mitogens. *Ann. Rev. Biochem.*, **56**, 881-914.

Clarke, P. G. H. (1990) Developmental cell death: Morphological diversity and

multiple mechanisms. *Anat. Embryol.*, **181**, 195-213.

Cowan, W. M., Fawcett, J. W., O'Leary, D. D. M. and Stanfield, B. B. (1984) Regressive events in neurogenesis. *Science*, **225**, 1258-1265.

Derynck, R. (1988) Transforming growth factor α. *Cell*, **54**, 593-595.

Dipasquale, B., Marini, A. M. and Youle, R. J. (1991) Apoptosis and DNA degeneration induced by 1-methyl-4-phenylpyridinium in neurons. *Biochem. Biophys. Res. Commun.*, **181**, 1442-1448.

Dyrks, T., Dyrks, E., Hartmann, T., Masters, C. and Beyreuther, K. (1992) Amyloidogenicity of βA4 and βA4-bearing amyloid protein precursor fragments by metal-catalyzed oxidation. *J. Biol. Chem.*, **267**, 18210-18217.

Ellis, R. E., Yuan, J. Y. and Horvitz, H. R. (1991) Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.*, **7**, 663-698.

Enokido, Y. and Hatanaka, H. (1990) High oxygen atmosphere for neuronal cell culture with nerve growth factor. II. Survival and growth of clonal rat pheochromocytoma PC12h cells. *Brain Res.*, **536**, 23-29.

Enokido, Y., Akaneya, Y., Niinobe, M., Mikoshiba, K. and Hatanaka, H. (1992) Basic fibroblast growth factor rescues CNS neurons from cell death caused by high oxygen atmosphere. *Brain Res.*, **599**, 261-271.

Enokido, Y. and Hatanaka, H. (1993) Apoptotic cell death occurs in hippocampal neurons cultured in a high oxygen atmosphere. *Neuroscience*, **57**, 965-972.

Enokido, Y. and Hatanaka, H. (1995) Oxygen toxicity induces apoptotic neuronal death in cultured rat hippocampal neurons. In *Alzheimer's and Parkinson's Diseases: Recent Advances*, Hanin, I., Fisher, A., and Yoshida, M., eds by Plenum Pub. Co., New York.

Gómez-Pinilla, F., Knauer, D. J. and Nieto-Sampedro, M. (1988) Epidermal growth factor receptor immunoreactivity in rat brain. Development and cellular localization. *Brain Res.*, **438**, 385-390.

Gotoh, Y., Nishida, E., Yamashita, T., Kawakami, M. and Sakai, H. (1990)

Microtubule-associated protein (MAP) kinase activated by nerve growth factor and epidermal growth factor in PC12 cells. *Eur. J. Biochem.*, **193**, 661-669.

Greene, L. A. and Tischler, A. S. (1976) Establishment of noradrenergic clonal cell line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA*, **72**, 2424-2428.

Greenlund, L. J. S., Deckwerth, T. L. and Johnson, E. M., Jr. (1995) Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. *Neuron*, **14**, 303-315.

Hansen, M.B., Nielsen, S.E. and Berg, K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods*, **119**, 203-210.

Hartley, A., Stone, J. M., Herton, C., Cooper, J. M. and Schapira, A. H. V. (1994) Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: relevance to Parkinson's disease. *J. Neurochem.*, **63**, 1987-1990.

Hatanaka, H. (1981) Nerve growth factor-mediated stimulation of tyrosine hydroxylase activity in a clonal rat pheochromocytoma cell line. *Brain Res.*, **222**, 225-233.

Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Milliman, C. L. and Korsmeyer, S. J. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*, **75**, 241-251.

Huff, K., End, D. and Guroff, G. (1981) Nerve growth factor-induced alteration in the response of PC12 pheochromocytoma cells to epidermal growth factor. *J. Cell Biol*, **88**, 189-198.

Kinoshita, A., Yamada, K., Hayakawa, T., Kataoka, K., Mushiroi, T., Kohmura, E. and Mogami, H. (1990) Modification of anoxic neuronal injury by human recombinant epidermal growth factor and its possible mechanism. *J. Neurosci. Res.*, **25**, 324-330.

Kornblum, H. I., Raymon, H. K., Morrison, R. S., Cavanaugh, K. P., Bradshaw, R. A. and Leslie, F. M. (1990) Epidermal growth factor and basic fibroblast growth factor: effects on an overlapping population of neocortical neurons in vitro. *Brain Res.*, **535**, 255-263.

Kubo, T., Nonomura, T., Enokido, Y. and Hatanaka, H. (1995) Brain-derived neurotrophic factor (BDNF) can prevent apoptosis of rat cerebellar granule neurons in culture. *Dev. Brain Res.*, **85**, 249-258.

Kushima, Y., Tsukui, H., Enokido, Y., Nishio, C. and Hatanaka, H. (1990) High oxygen atmosphere for neuronal cell culture with nerve growth factor. I. Primary culture of basal forebrain cholinergic neurons from fetal and postnatal rats. *Brain Res.*, **536**, 16-22.

Lazar, L. M. and Blum, M. (1992) Regional distribution and developmental expression of epidermal growth factor and transforming growth factor-α mRNA in mouse brain by a quantitative nuclease protection assay. *J. Neurosci.*, **12**, 1688-1697.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Loo, D. T., Copani, A., Pike, C. J., Whittemore, E. R., Walencewicz, A. J. and Cotman, C. W. (1993) Apoptosis is induced by β-amyloid in cultured central nervous system neurons. *Proc. Natl. Acad. Sci. USA*, **90**, 7951-7955.

Marshall, C. J. (1995) Specificity of Receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. *Cell*, **80**, 179-185.

Martin, D. P., Schmidt, R. E., DiStefano, P. S., Lowry, O. H., Carter, J. G. and Johnson, E. M. Jr. (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.*, **106**, 829-844.

Morrison, R. S., Sharma, A., DeVellis, J. and Bradshow, R. A. (1986) Basic

fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. *Proc. Natl. Acad. Sci. USA*, **83**, 7537-7541.

Morrison, R. S., Kornblum, H. I., Leslie, F. M. and Bradshaw, R. A. (1987) Trophic stimulation of cultured neurons from neonatal rat brain by EGF. *Science*, **238**, 72-75.

Morrison, R. S., Keating, R. F. and Moskal, J. R. (1988) Basic fibroblast growth factor and epidermal growth factor exert differential trophic effects on CNS neurons. *J. Neurosci. Res.*, **21,** 71-79.

Mosmann, T. (1983) Rapid calorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55-63.

Müller, R., Bravo, R., Burckhardt, J. and Curran, T. (1984) Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. *Nature*, **312**, 716-720.

Oltvai, Z. N., Milliman, C. L. and Korsmeyer, S. J. (1993) Bcl-2 hetero-dimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609-619.

Oppenheim, R. W., Prevette, D., Tytell, M. and Homma, S. (1990) Naturally occurring and induced neuronal death in the chick embryo *in vivo* requires protein and RNA synthesis: Evidence for the role of cell death genes. *Dev. Biol.*, **138**, 104-113.

Oppenheim, R. W. (1991) Cell death during development of the nervous system. *Annu. Rev. Neurosci.*, **14**, 453-501.

Przedborski, S., Kostic, V., Jackson-Lewis, V., Naini, A. B., Simonetti, S., Fahn,S., Carlson, E., Epstein, C. J. and Cadet, J. L., (1992) Transgenic mice with increased Cu/Zn-Superoxide dismutase activity are resistant to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *J. Neurosci.*, **12**, 1658-1667.

Ratan, R. R., Murphy, T. H. and Baraban, J. M. (1994) Oxidative stress induces apoptosis in embryonic cortical neurons. *J. Neurochem.*, **62**, 376-379.

Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H.-X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S. M., Berger, R., Tanzi, R. E., Halperin, J. J., Herzfeldt, B., Van den Bergh, R., Hung, W.-Y., Bird, T., Deng, G., Mulder, D. W., Smyth, C., Laing, N. G., Soriano, E., Pericak-Vance, M. A., Haines, J., Rouleau, G. A., Gusella, J. S., Horvitz, H. R. and Brown, R. H. Jr. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, **362**, 59-62.

Schlessinger, J. and Ullrich, A. (1992) Growth factor signalling by receptor tyrosine kinases. *Neuron*, **9**, 383-391.

Schlessinger, J. (1993) How receptor tyrosine kinases activate Ras. *Trends Biochem. Sci.*, **18**, 273-275.

Spina, M. B., Squinto, S. P., Miller, J., Lindsay, R. M. and Hyman, C. (1992) Brain-derived neurotrophic factor protects dopaminergic neurons against 6-hydroxydopamine and N-methyl-4-phenylpyridinium ion toxicity: involvement of the glutathione system. *J. Neurochem.*, **59**, 99-106.

Sun, H. and Tonks, N. K. (1994) The coordinated action of protein tyrosine phosphatases and kinases in cell signaling. *Trens. Biochem. Sci.*, **19**, 480-485.

Tucker, M. S., Eves, E. M., Wainer, B. H. and Rosner, M. R. (1993) Activation of mitogen-activated protein kinase by epidermal growth factor in hippocampal neurons and neuronal cell lines. *J. Neurochem.*, **61**, 1376-1387.

Walicke, P. A. and Baird, A. (1988) Neurotrophic effects of basic and acidic fibroblast growth factors are not mediated through glial cells. *Dev. Brain Res.*, **40**, 71-79.

Wyllie, A. H., Morris, R. G., Smith, A. L. and Dunlop, D. (1984) Chromatin

cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.*, **142**, 67-77.

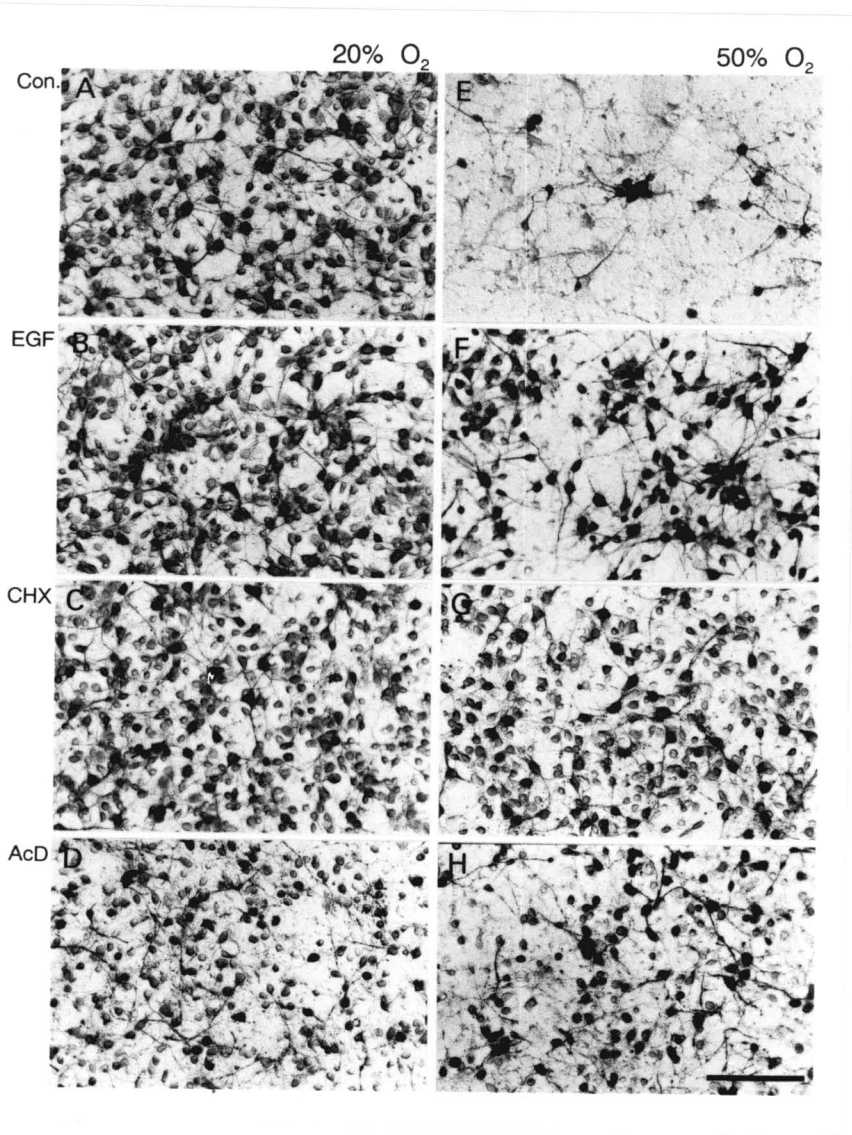

Fig. 1. Immunocytochemical staining of cultured cerebral cortical neurons with anti-MAP2 antibody in the presence or absence of EGF, cycloheximide, and actinomycin D. The cortical neurons were cultured in 20 (A, B, C, D) and 50% (E, F, G, H) oxygen atmospheres in the absence (A, E) or presence of 100 ng/ml EGF (B, F), 0.5 μM cycloheximide (C, G), or 0.1 μM actinomycin D (D, H) for 2.5 days. The neurons were immunostained with anti-MAP2 antiserum. Bar represents 100 μm.

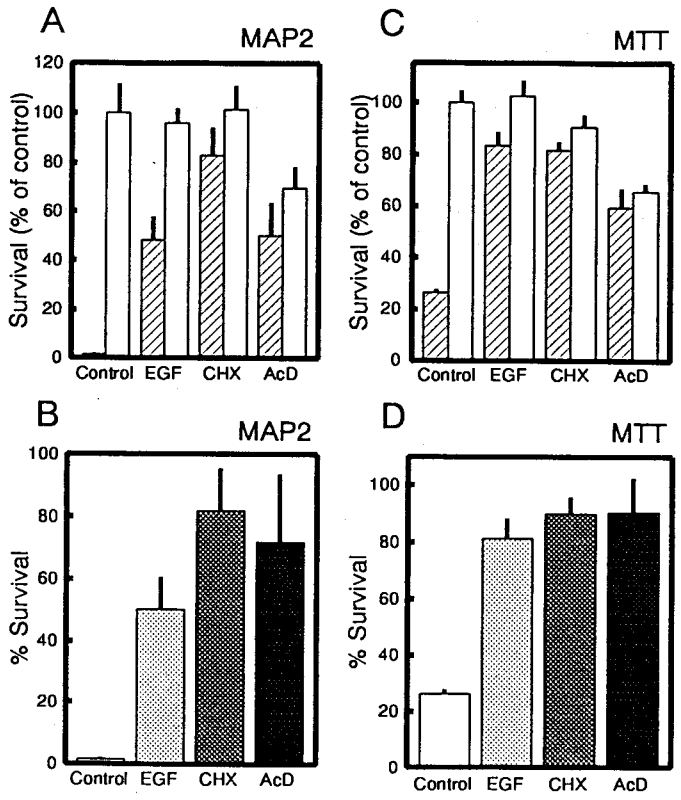

Fig. 2. Preventive effects of EGF, cycloheximide, and actinomycin D on the oxygen-induced death of the cultured cerebral cortical neurons. The cortical neurons were cultured in 20 (open bar in A, C) and 50% (hatched bar in A, C) oxygen atmospheres in the absence (Control) or presence of 100 ng/ml EGF (EGF), 0.5 μM cycloheximide (CHX), or 0.1 μM actinomycin-D (AcD) for 2.5 days. The viable cultured neurons were quantified by counting the number of neurons immunostained with anti-MAP2 antiserum (A, B), or by the MTT assay (C,D). The values are expressed as the percentage of the measured value in the culture without the reagents in a 20% oxygen atmosphere in (A) and (C). In (B) and (D), the values of the cultured neurons in a 50% oxygen atmosphere are expressed as the percentage of the measured value in the corresponding cultures in a 20% oxygen atmosphere. The values represent the means $\pm$ S.D. of four individual cultures.

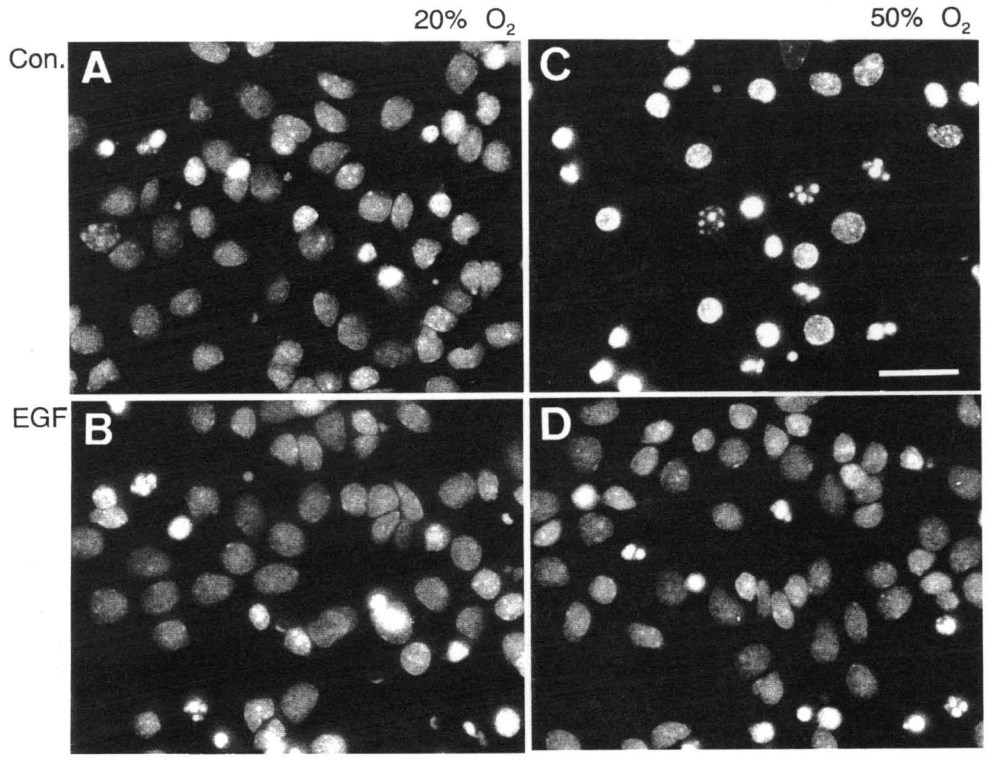

Fig. 3. The high oxygen-induced chromatin condensation of the cultured cerebral cortical neurons. The cortical neurons were cultured in 20 (A, B) and 50% (C, D) oxygen atmospheres in the absence (A, C) or presence of 100 ng/ml EGF (B, D) for 2 days. The neurons were stained with Hoechst 33258. Bar represents 25 μm.

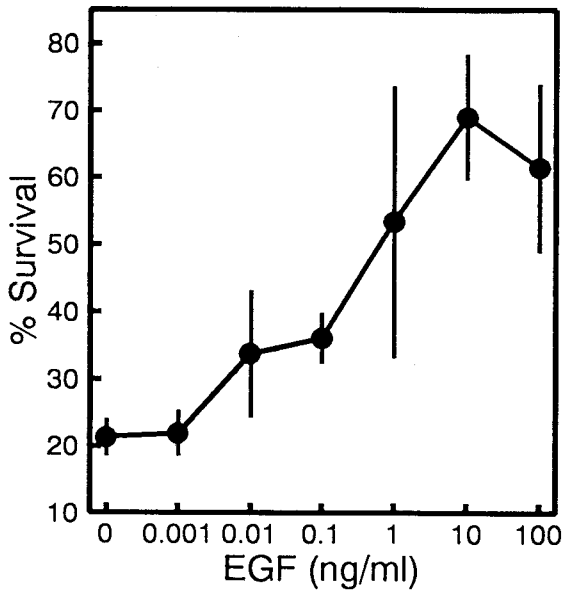

Fig. 4. EGF promotes the dose-dependent survival of cerebral cortical neurons cultured in a high oxygen atmosphere. The cortical neurons were cultured for 2.5 days with 0, 0.001, 0.01, 0.1, 1, 10, and 100 ng/ml EGF in 20 and 50% oxygen atmospheres. The viable cells were quantified by means of the MTT assay. The values of the cultured neurons in a 50% oxygen atmosphere are expressed as the percentage of the measured values in the corresponding culture in a 20% oxygen atmosphere. The values represent the means ± S.D. of four individual cultures.

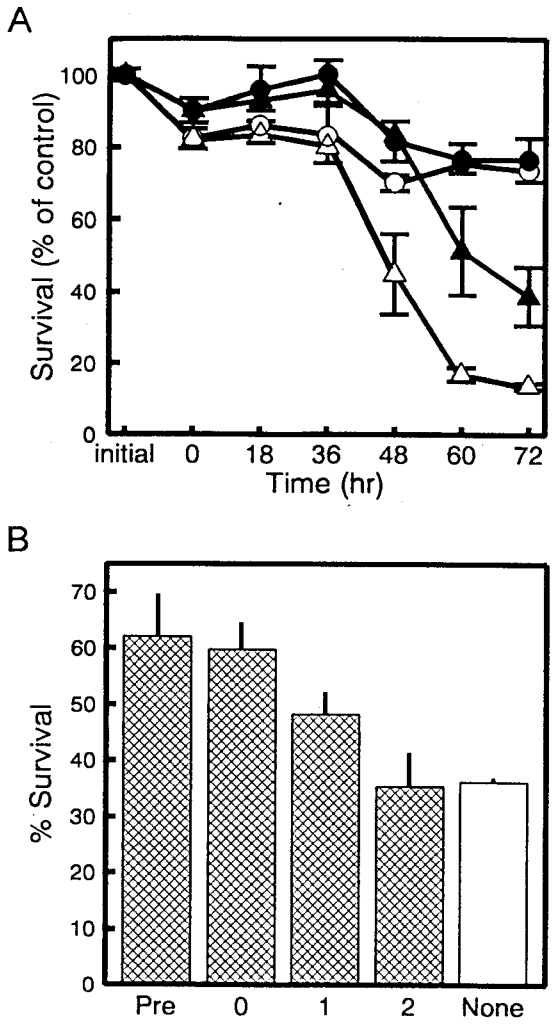

Fig. 5. Time course of the EGF-mediated survival of the cultured cerebral cortical neurons in a high-oxygen atmosphere. (A) The medium was changed on the day after the cortical neurons were seeded. No factor (○, △) or 100 ng/ml EGF (●, ▲) was added to the culture just after the medium change. The cultures were transferred into chambers with 20% (○, ●) and 50% (△, ▲) oxygen atmospheres 8 hr after the medium change. The MTT assay was performed immediately after the medium change (initial), and just before (0), and 18, 36, 48, 60 and 72 hr after the transfer of the cultures. The values are represented as the percentage of the measured value just after the medium change (initial), and show the means ± S.D. of four individual cultures. (B) No factor (None) or 100 ng/ml EGF was added to the culture just after the medium change (Pre), just before (0), and 1 and 2 days after the cultures were transferred. The cells were cultured for 2.5 days after the transfer to 20% and 50% oxygen atmospheres, then examined by the MTT assay. The values of the cultured neurons in a 50% oxygen atmosphere are expressed as the percentage of those measured in the correspon of four individual cuding culture in a 20% oxygen atmosphere. The values represent the means ± S.D.ltures.

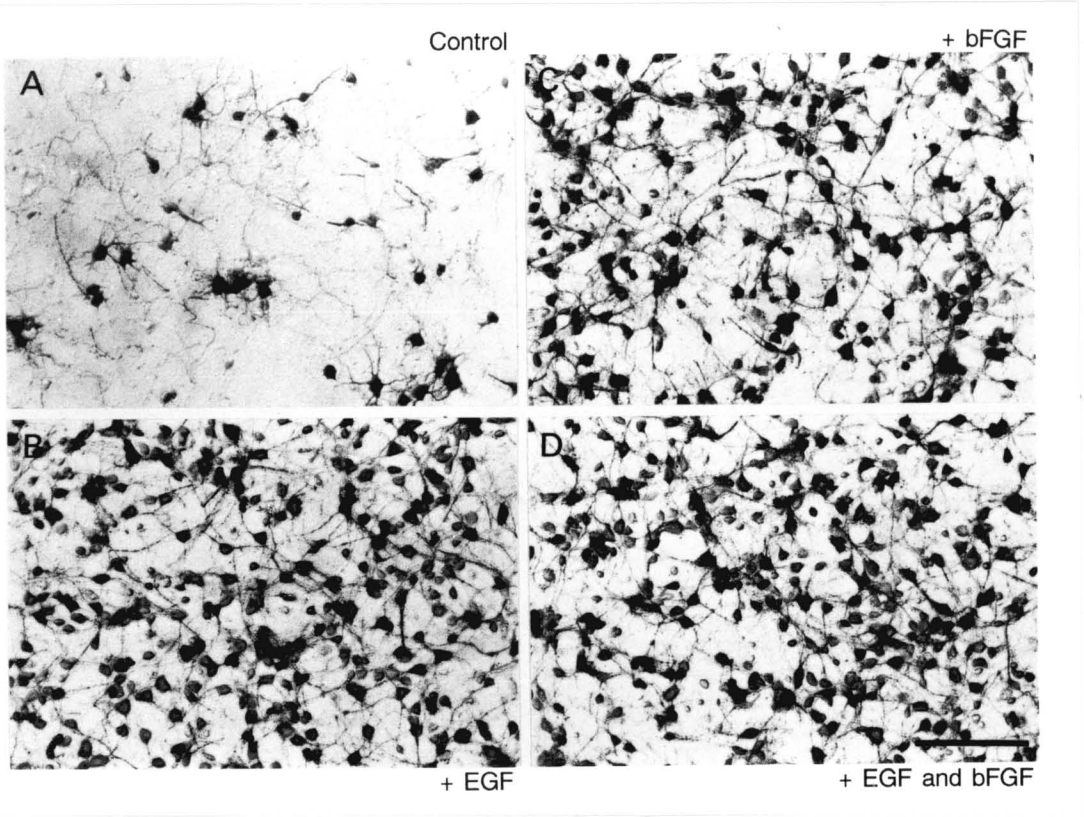

Fig. 6. Immunocytochemical staining of cultured cerebral cortical neurons with anti-MAP2 antibody in the presence or absence of EGF and bFGF. The cortical neurons were cultured in 50% oxygen atmosphere in the absence (A) or presence of 100 ng/ml EGF (B), 100 ng/ml bFGF (C), or both 100 ng/ml EGF and bFGF (D) for 2.5 days. The neurons were immunostained with anti-MAP2 antiserum. Bar represents 100 μm.

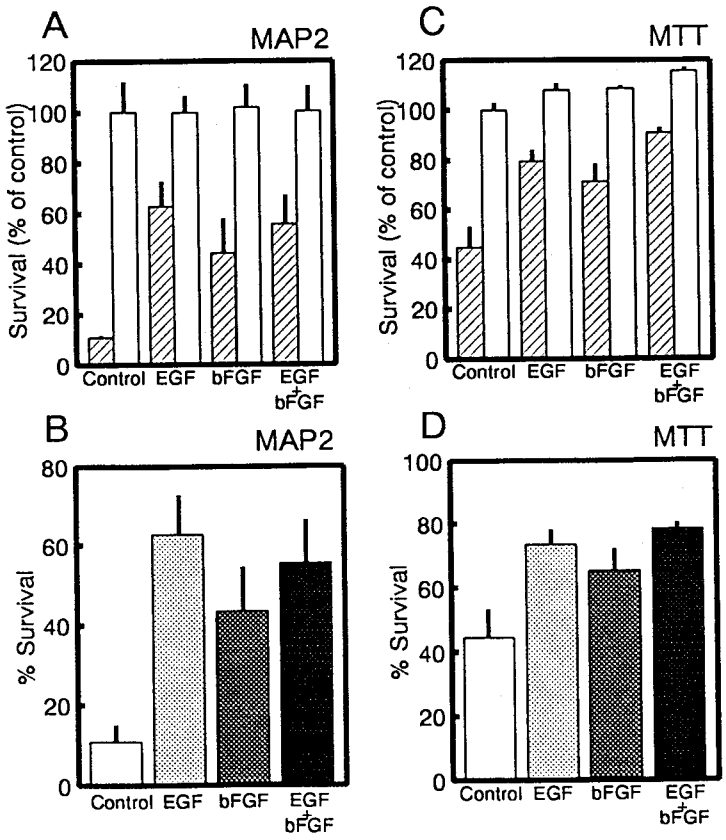

Fig. 7. Preventive effects of bFGF on the oxygen-induced death of the cultured cerebral cortical neurons. The cortical neurons were cultured in 20 (open bar in A, C) and 50% (hatched bar in A, C) oxygen atmospheres in the absence (Con) or presence of 100 ng/ml EGF (EGF), 100 ng/ml bFGF (bFGF), or both 100 ng/ml EGF and bFGF (EGF + bFGF) for 2.5 days. The viable cultured neurons were quantified by counting the number of neurons immunostained with anti-MAP2 antiserum (A, B), or by the MTT assay (C,D). The values are expressed as the percentage of the measured value in the culture without the factors in a 20% oxygen atmosphere in (A) and (C). In (B) and (D), the values of the cultured neurons in a 50% oxygen atmosphere are expressed as the percentage of the measured value in the corresponding cultures in a 20% oxygen atmosphere. The values represent the means ± S.D. of four individual cultures.

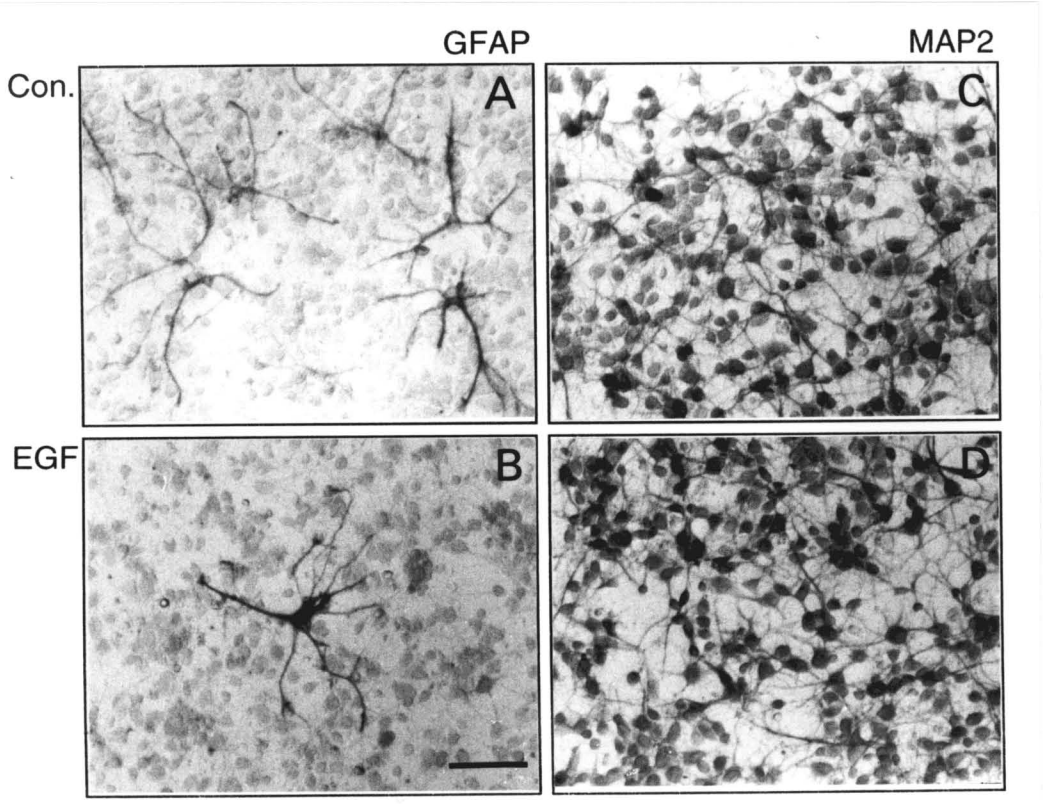

Fig. 8. Immunocytochemical staining of the cultured cerebral cortical neurons with anti-GFAP antibody. Cortical neurons were cultured in the absence (A, C) or 100 ng/ml EGF (B, D) for 2 days after the medium change on the day after the cells were seeded. The cells were immunostained using the anti-GFAP monocronal antibody (A, B) or the anti-MAP2 antiserum (C, D). Bars represent 50 μm.

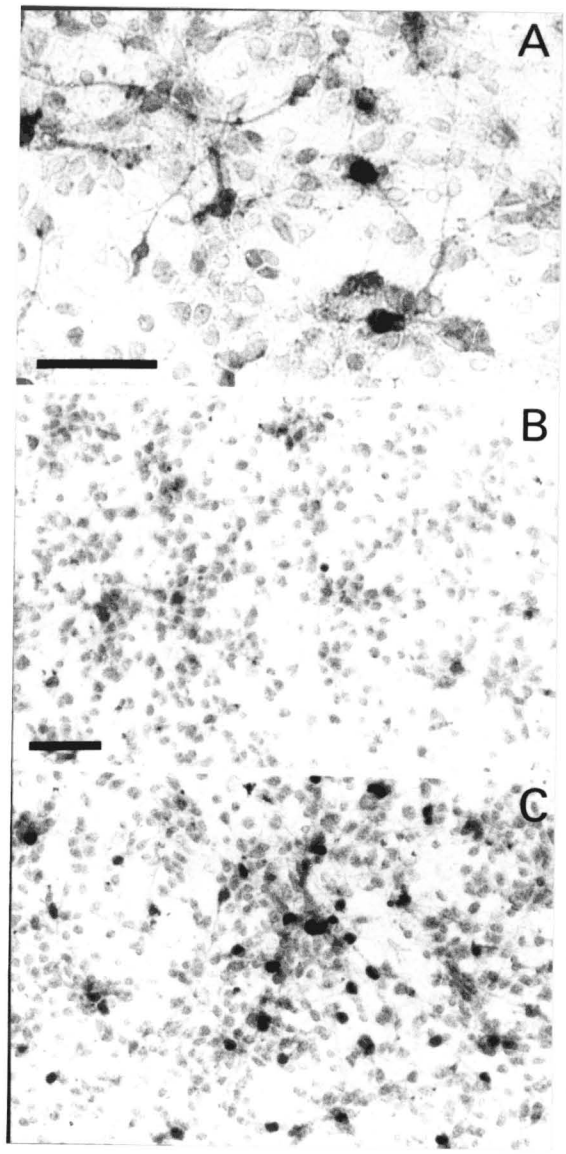

Fig. 9. Immunocytochemical staining of the cultured cerebral cortical neurons using anti-EGF receptor and the anti-c-Fos antibodies. Cortical neurons were cultured without factors for 2 days after the medium change on the day after the cells were seeded. The neurons were immunostained using the anti-EGF receptor antibody (A), or incubated without (B) or with 100 ng/ml EGF (C) for 2 hr, and immunostained using the anti-c-Fos antibody (B, C). Bars represent 50 μm.

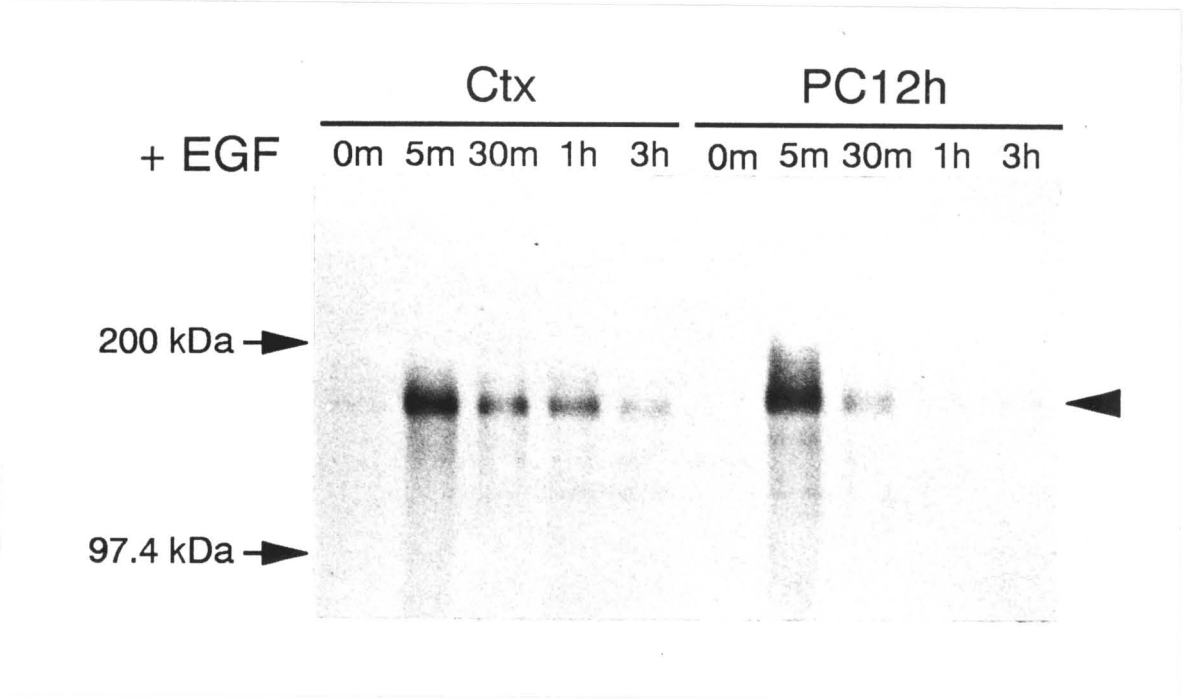

Fig. 10. EGF induced the tyrosine phosphorylation of the EGF receptor in the cultured cerebral cortical neurons. The cultured cortical neurons (Ctx) and PC12h cells (PC12h) were not incubated (0m) or incubated with 100 ng/ml EGF throughout for 5 min (5m), 30 min (30m), 1 hr (1h) or 3 hr (3h), then lysed. The EGF receptor was immunoprecipitated with the anti-EGF receptor antibody, then western blotted with the anti-phosphotyrosine antibody (4G10). The position of the EGF receptor is indicated by the arrowhead. Molecular weights are shown on the left.

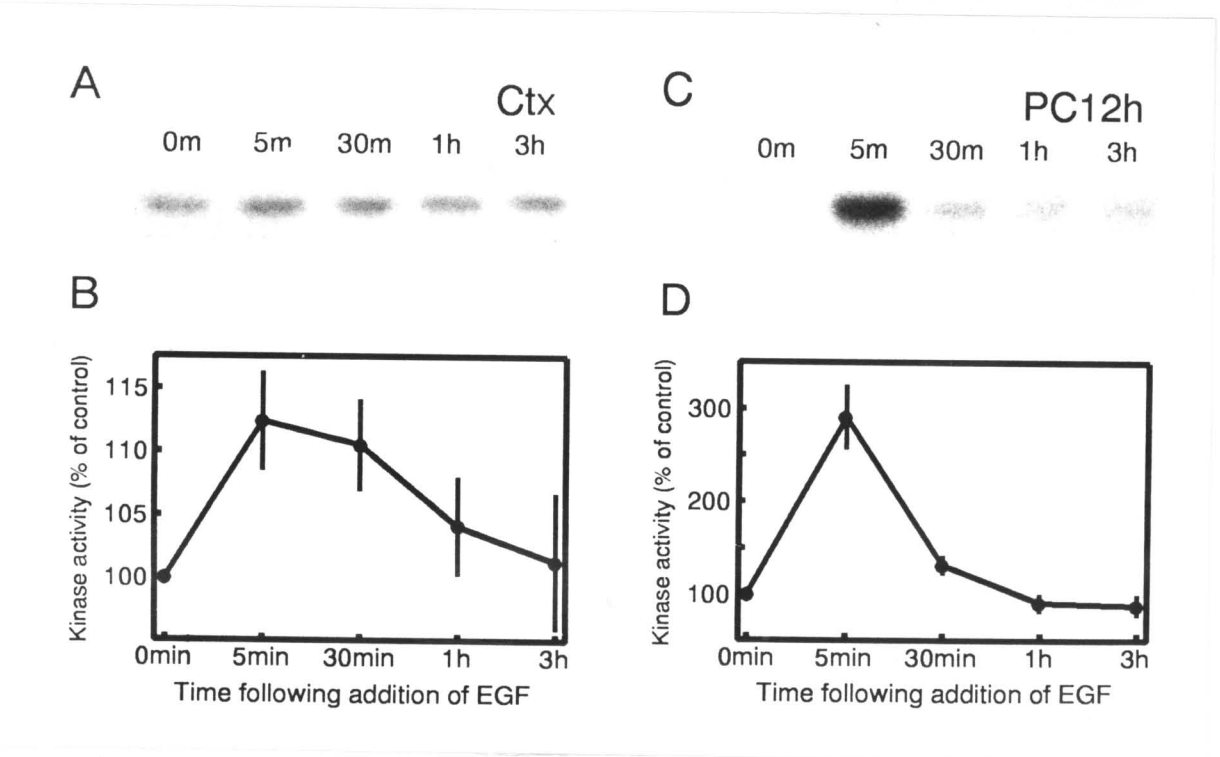

Fig. 11. EGF induced the activation of MAP kinase in the cultured cerebral cortical neurons. Lysates were prepared from the cultured cortical neurons (A, B) and PC12h cells (C, D) not treated (0m) or treated with 100 ng/ml EGF throughout for 5 min (5m), 30 min (30m), 1 hr (1h) or 3 hr (3h). The activities of the 42 kDa MAP kinase in the lysates were determined by the renaturation gel assay using myelin basic protein (MBP) as the substrate (A, C). The activities are quantified by the FUJIX Bio-imaging Analyzer (B, D). The values are represented as the percentage of the activity in the untreated cells (0min). The values represent the means ± S.D. of three determinations.

Table 1. Effects of Vitamin E and N-acetylcysteine on the Oxygen-induced Death of the Cultured Cerebral Cortical Neurons

Treatment	Concentration	% Survival
Control	—	46.4 ± 5.2
EGF	100 ng/ml	83.7 ± 7.1**
Vitamin E	0.3 μM	95.2 ± 3.2**
	1 μM	98.3 ± 4.6**
	3 μM	94.4 ± 3.4**
N-acetylcysteine	10 μM	55.7 ± 11.1
	30 μM	59.2 ± 3.2*
	100 μM	58.8 ± 3.0*

The cortical neurons were cultured in 20 and 50% oxygen atmospheres in the absence (Control) or presence of EGF, vitamine E or N-acetyl-L-cysteine at the indicated concentrations for 2.5 days. The viable cultured neurons were quantified by the MTT assay. The values of the cultured neurons in a 50% oxygen atmosphere are expressed as the percentage of the measured value in the corresponding cultures in a 20% oxygen atmosphere. The values represent the means ± S.D. of four individual cultures; *, $P<0.01$ and **, $P<0.001$ in comparison with the control culture (Student's t-test).

Bibliography

Publications:

1. **Yamada, M.**, Ikeuchi, T., Aimoto S., and Hatanaka, H. EGF-induced sustained tyrosine phosphorylation and decreased rate of EGF receptor in PC12h-R cells which show neuronal differentiation in response to EGF. *Neurochem. Res.*, in press.
2. **Yamada, M.**, Ikeuchi, T., Aimoto S., and Hatanaka, H. PC12h-R cell, a subclone of PC12 cells, shows EGF-induced neuronal differentiation and sustained signaling. *J. Neurosci. Res.*, in press.
3. **Yamada, M.**, Enokido, Y., Ikeuchi, T. and Hatanaka, H. (1995) Epidermal growth factor prevents oxygen-triggered apoptosis and induces sustained signalling in cultured rat cerebral cortical neurons. *Eur. J. Neurosci.*, **7**, 2130-2138.
4. **Yamada, M.**, Ikeuchi, T., Tukui, H., Aimoto, S. and Hatanaka, H. (1994) Sustained tyrosine phosphorylation of $p140^{trkA}$ in PC12h-R cells responding rapidly to NGF. *Brain Res.*, **661**, 137-146.
5. Tokunaga, F., **Yamada, M.**, Miyata, T., Ding, Y.-L., Hiranaga-Kawabata, M., Muta, T., Iwanaga, S., Ichinose, A. and Davie, E. W. (1993) Limulus hemocyte transglutaminase. *J. Biol. Chem.*, **268**, 252-261.
6. **Yamada, M.** and Hatanaka, H. The neurotrophic action and signalling of epidermal growth factor. *Prog. Neurobiol.*, in preparation.
7. **山田雅司**、畠中　寛 (1994) ニューロトロフィンとその作用機構 生体の科学 **45,** 164-169.

Oral presentations:

1. **Yamada, M.**, Enokido, Y., Ikeuchi, T. and Hatanaka, H. Epidermal Growth Factor Prevents Oxidative Stress-Induced Neuronal Apoptosis in Cultured Cerebral Cortical Neurons. *25th Annual Meeting Society for Neuroscience* (San Diego, USA) November, 1995.
2. **Yamada, M.**, Enokido, Y., Ikeuchi, T. and Hatanaka, H. Trophic Action of Epidermal Growth Factor on PC12h-R Cells and Cultured Cerebral Cortical Neurons. *15th International Society for Neurochemistry (ISN) Biennial meeting* (Kyoto, Japan) July, 1995.
3. **山田雅司**、榎戸　靖、池内俊彦、畠中　寛
   培養神経細胞における上皮増殖因子 (EGF) のリン酸化シグナル解析。
   第38回　日本神経化学会大会　(京都)、1995年7月。
4. **山田雅司**、榎戸　靖、池内俊彦、畠中　寛
   上皮増殖因子 (EGF) の PC12h-R 細胞および大脳皮質ニューロンに対する栄養因子作用。第37回　日本神経化学会大会　(松本)、1994年10月。
5. **山田雅司**、池内俊彦、畠中　寛
   上皮増殖因子 (EGF) の神経細胞への作用機構解析モデルとしての PC12h-R 細胞。第67回　日本生化学会大会　(大阪)、1994年9月。
6. **山田雅司**、池内俊彦、畠中　寛
   PC12h-R 細胞は上皮増殖因子 (EGF) に応答して神経細胞様に分化する。
   第36回　日本神経化学会大会 (大阪)、1993年10月。
7. **山田雅司**、小島正己、池内俊彦、畠中　寛
   PC12 細胞における $p140^{trkA}$ のチロシンリン酸化とその NGF 応答性の関係。第66回　日本生化学会大会 (東京)、1993年10月。
8. **山田雅司**、小島正己 、池内俊彦 、畠中　寛
   PC12h および PC12h-R 細胞での NGF による $gp140^{trkA}$ のチロシンリン酸化。第16回　日本神経科学学会 (大阪)、1992年12月。

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