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Epidermal growth factor and transforming growth factor-α can induce neuronal differentiation of rat pheochromocytoma PC12 cells under particular culture conditions

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In rat pheochromocytoma PC12 cells, NGF induces neuronal differentiation. Upon stimulation with NGF, Ras is activated to a GTP-bound form, and the activated Ras can induce neuronal differentiation. Recently, we and others observed that epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) can also activate Ras in PC12 cells. This is puzzling since previous reports indicated that EGF stimulates proliferation rather than differentiation in PC12 cells. In this paper, we re-examined the biological effect of EGF and TGF-α, and found that these factors can also induce neuronal differentiation under particular culture conditions. Not only the outgrowth of long neurites, but the induction of neurofilament proteins and the metalloprotease transin was also observed in the EGF- and TGF-α-stimulated cells. These data clearly indicate that in addition to NGF, EGF and TGF-α can also induce the differentiation of PC12 cells under particular conditions.

Differentiation; Neuronal cell; Epidermal growth factor; Transforming growth factor-α; Ras; PC12 cell

1. INTRODUCTION

It has been established that the proliferation and differentiation of neuronal cells are controlled by a variety of peptide factors. Primary cultures and established cell lines from neuronal origins have greatly contributed to the exploration of the biological effects of defined factors in detail in vitro. For example, nerve growth factor (NGF) supports differentiation and neurite outgrowth of the primary culture of sympathetic ganglia [1] and embryonic rat brain [2], as well as several cell lines, including rat PC12 cells and human SH-SY5Y cells [1,3]. Recent studies have shown that epidermal growth factor (EGF), which was originally discovered as a potent mitogen during the study of NGF, promotes survival and differentiation of primary cultures of neonatal rat [4] and chicken [5] brain.

Rat pheochromocytoma PC12 cells have been widely used as an in vitro model of neuronal differentiation since the cells undergo differentiation to sympathetic neuron-like cells in response to NGF [6]. Fibroblast growth factor (FGF) [7–9] and interleukin (IL)-6 [10] can also induce differentiation of PC12 cells. It was previously reported that EGF acts on PC12 cells [11–13], but in these studies, EGF stimulated proliferation rather than differentiation.

Recently, we and others found that differentiation factors, including NGF, FGF, and IL-6 can induce the activation of Ras in PC12 cells [14–17]. This is consistent with previous studies which demonstrated that activated Ras can induce neuronal differentiation in PC12 cells [18,19]. Furthermore, dominant-negative Ras (Ras^{Asn17}) blocked the neuronal differentiation induced by NGF and FGF [20]. These observations indicate that the NGF and FGF signals to induce differentiation are transmitted through Ras. However, it has also been observed that EGF [14,15,17] and TGF-α [our unpublished observation], which did not induce differentiation in the previous studies, can activate Ras in PC12 cells. In view of these results, we have re-examined the biological effect of EGF and TGF-α on PC12 cells, and found that these factors can induce neuronal differentiation of PC12 cells when the cells are cultured on a plate with a chemically modified surface called Primaria (Becton Dickinson) in a low-serum medium. In this paper, we present evidence that EGF and TGF-α can induce neuronal phenotypes in PC12 cells, including neurite outgrowth; survival in a low-serum medium; and the induction of two markers of differentiation, neurofilament proteins and the metalloprotease transin.

2. MATERIALS AND METHODS

A PC12 cell line was kindly provided by Dr. T. Amano, Mitsubishi

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Abbreviations: EGF, epidermal growth factor; NGF, nerve growth factor; FGF, fibroblast growth factor; TGF-α, transforming growth factor-α; IL, interleukin; DMEM, Dulbecco's modified Eagle's medium; NF, neurofilament; CAT, chloramphenicol acetyltransferase.

Life Science Institute. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high (4.5 g/liter) glucose supplemented with 10% fetal bovine serum (JRH Biosciences), 5% horse serum (Gibco), and 100 units of penicillin and 100 mg of streptomycin per ml. To examine the effect of various factors, the cells were seeded at densities of 2–4 × 10^3 cells/cm^2 on Primaria plates (Becton Dickinson, NJ) (unless otherwise noted), and maintained in a low-serum medium containing DMEM supplemented with 0.5% fetal bovine serum, penicillin, and streptomycin as described above. The factor-containing medium was refreshed every 3 days.

NGF (mouse, 2.5 S form), FGF (from bovine pituitary gland), EGF (mouse), and recombinant TGF-α (human), were all purchased from Boehringer-Mannheim. Anti-NGF antibody (rabbit) was from Collaborative Research. Monoclonal antibodies against 160 kDa and 200 kDa neurofilament proteins (clone NN18, and N52, respectively) were obtained from Sigma. The recombinant plasmid p750TRCAT was a generous gift from Dr. L. Matrisian, Vanderbilt University, and is described in detail in [21].

Western blot analysis of neurofilament proteins in the cytoskeletal fraction was performed as described in [22]. Immunoblotting was performed with a mixture of two monoclonal antibodies which react with 160 kDa and 200 kDa neurofilament subunits, and [^{125}I]protein A (Amersham Corp, IM.144). Quantitation of immunoreactive bands was performed with PhosphorImager (Molecular Dynamics).

Transfection of p750TRCAT plasmid, and the measurement of chloramphenicol acetyltransferase (CAT) activity were carried out essentially as described in [23]. PC12 cells were seeded at a density of 3 × 10^4 cells/cm^2 on 60 mm Primaria dishes, and 10 mg of p750TRCAT DNA was transfected into the cells by the calcium phosphate precipitation method. After transfection, the cells were maintained in a medium containing DMEM supplemented with 0.5% fetal bovine serum, and stimulated with 50 ng/ml of various factors for 48 h. Subsequently, cell extracts were prepared, and CAT activity was measured as described in [24].

3. RESULTS

Upon stimulation with NGF, FGF, and IL-6, PC12 cells change their morphology, the most prominent of which is the outgrowth of long neurites. However, in PC12 cells used in the present study, the effect of FGF and IL-6 on this neurite formation was much weaker than that of NGF. As shown in Table I, the appearance of neurite-bearing cells by FGF was significantly delayed compared with that by NGF, and only about 50% of the cells showed differentiated morphology after a culture of two weeks or longer (Fig. 1B, and Table I). As we have reported previously [10], IL-6 induces the neurite extension of PC12 cells only in low (0.5% or less)-serum media (Fig. 1E), and the onset of the morphological change is even slower than that of FGF. To detect the possibly weak effect of EGF on the morphology of PC12 cells, the cells were seeded at a low density (4 × 10^3 cells/cm^2), and cultured in the presence of 50 ng/ml of EGF in a medium containing 0.5% serum. In the initial experiments in which the cells were cultured on uncoated regular plates, we observed that EGF could slightly increase the number of the cells bearing neurite-like processes, but the effect was much weaker than those of NGF and FGF (data not shown). Weak stimulation of neurite outgrowth by EGF had also been reported in PC12D cells, a subline of PC12 cells [25], but not in PC12 cells [11–13]. We noticed that EGF significantly promoted the survival of PC12 cells in the low-serum medium as described for NGF and FGF [8] (see below). Thus, we next compared several types of coated plates, including collagen (type IV)-, fibronectin-, laminin-, and poly-L-lysine-coated plates, and a plate with a chemically modified surface named Primaria (Becton Dickinson) since many recent studies have reported that coated culture substrates play an important role in neurite outgrowth [26,27]. We found that the process formation in response to EGF was greatly enhanced when the cells were cultured on Primaria dishes (Fig. 1C). Collagen- and fibronectin-coated plates were also found to slightly potentiate the process formation, while laminin and poly-L-lysine had little effect (data not shown). Under these conditions, the kinetics of the induction of neurite-bearing cells as well as the morphology of the differentiated cells were examined as shown in Fig. 1C and Table I. The EGF-stimulated cells started extending processes within the initial 3–5 days, and after 10 days culture, the cells which possessed long neurite-like structures were obvious under microscope (Fig. 1C). However, only 30–40% of the EGF-stimulated cells finally extended long neurites even after a culture of two weeks or longer, although more than 80% of the cells were flattened, and bore short processes. This was in a sharp contrast to the case of NGF in which more than 90% of the cells showed differentiated morphology (Table I). Furthermore, as shown in Fig. 2, EGF could significantly promote the survival of PC12 cells in the low (0.5%)-serum medium. This effect of EGF was slightly weaker than that of NGF, but significantly stronger than that of FGF under the conditions used in this experiment.

The possibility that the observed effect of EGF may

Table I

Induction of neurite-bearing cells in response to various factors in PC12 cells

Factor	Neurite-bearing cells (%)					
	day 1	day 3	day 5	day 7	day 10	day 14
control	<0.1	<0.1	<0.2	<0.5	N.D.	N.D.
NGF	7.4	47.6	67.3	87.7	94.0	93.5
EGF	1.0	3.2	4.2	10.0	16.9	33.5
TGF-α	1.6	5.5	7.5	9.6	17.9	38.4
FGF	0.9	2.0	6.4	20.6	45.3	48.6

The cells were cultured on Primaria dishes in the presence of 50 ng/ml of either NGF, EGF, TGF-α, or FGF. At several time points (day 1 to day 14), the cultures were scored for the percentage of neurite-bearing cells, which was determined by counting the number of the cells at least twice the length of the cell body under the microscope. For each time point, at least 1000 cells and 20 fields were randomly selected and scored, except for the control and FGF-treated cultures in which the number of surviving cells was low after day 7 (see Fig. 2) and only 100–200 cells could be scored. The data are representatives of three independent experiments in which essentially similar results were obtained.

N.D. = not determined.

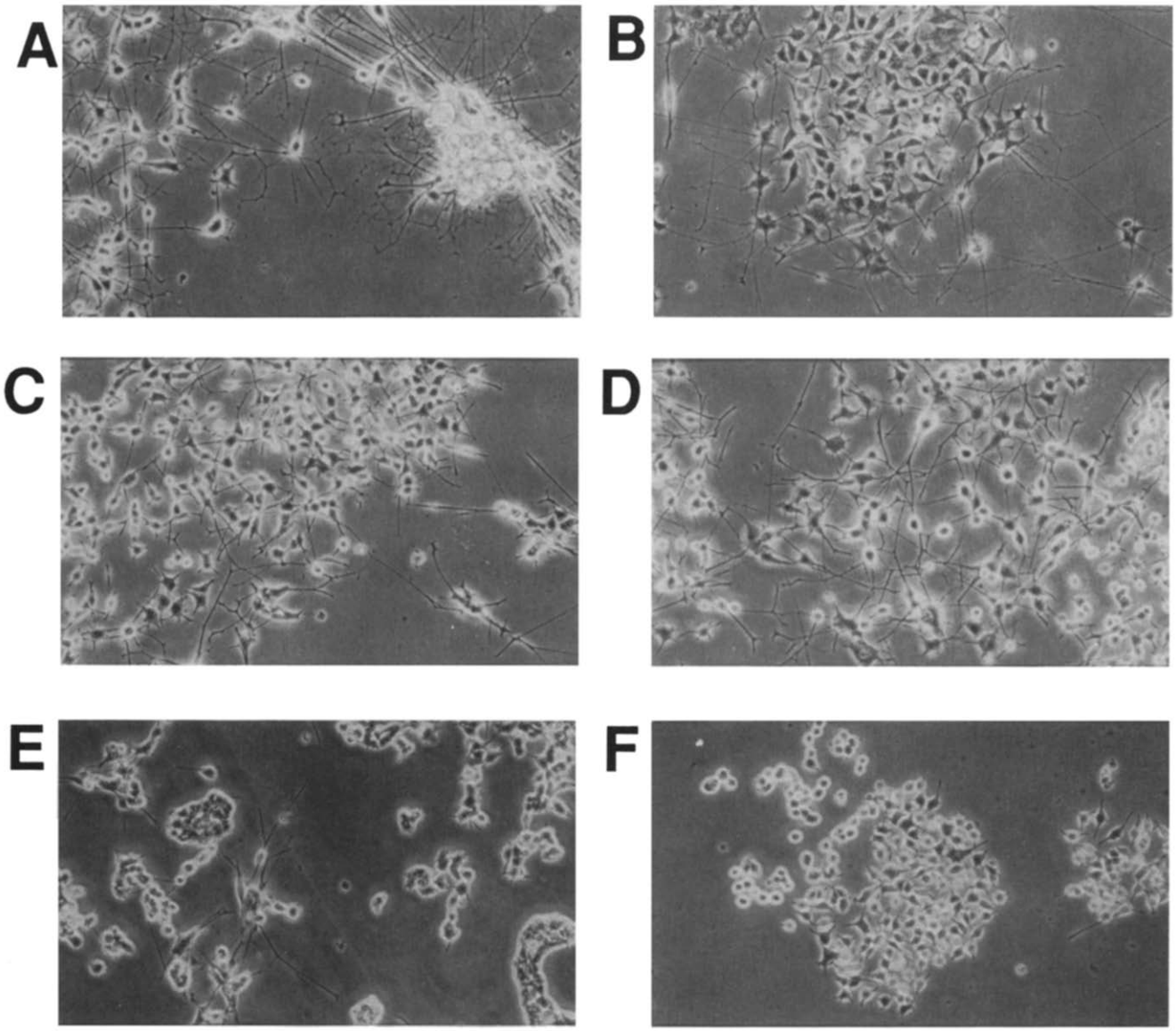

Fig. 1. Neurite outgrowth following treatment with various differentiation factors in PC12 cells. The cells were cultured on Primaria dishes as described in Materials and Methods, and the photographs were taken after two weeks (magnification: × 100). The factors used were as follows: (A) NGF; (B) FGF; (C) EGF; (D) TGF-α; (E) IL-6; (F) buffer alone as a control. The photograph of the control culture (F) was taken at day 3 since the cells did not survive for two weeks in the low-serum medium used in this study.

be due to some contaminants in the preparation of EGF is unlikely since recombinant TGF-α, which utilizes the same receptor for EGF [28], can also induce a similar phenotype (Fig. 1D, and Table I). Moreover, the addition of an anti-NGF neutralizing antibody, which was capable of blocking the effect of 5 ng/ml NGF, did not abolish the morphological changes induced by EGF (dada not shown). Thus, it is also unlikely that EGF stimulated the secretion of NGF from PC12 cells, although an autocrine mechanism through other neurotrophic factors cannot be ruled out at present.

To confirm that the processes observed in the EGF-stimulated cells are actually neurites, we examined the expression of neurofilament proteins. Neurofilament (NF) is a neuronal cell-specific cytoskeletal component present only in axon structure [22]. It consists of 3 subunits, including 200 kDa, 160 kDa, and 68 kDa NF proteins, and NGF is known to increase the level of all these NF proteins [22]. In this study, the effect of EGF and TGF-α on the levels of 160 kDa and 200 kDa NF proteins in the cytoskeletal fraction of PC12 cells was examined by Western blot analysis. The data shown in Fig. 3 demonstrated that the expression of 160 kDa NF protein, which was hardly detectable in the unstimulated PC12 cells, was strongly induced by treatment with EGF and TGF-α. Quantitative analysis estimated that both EGF and TGF-α induced the protein about 50-fold, whereas the increase by NGF was about 100-

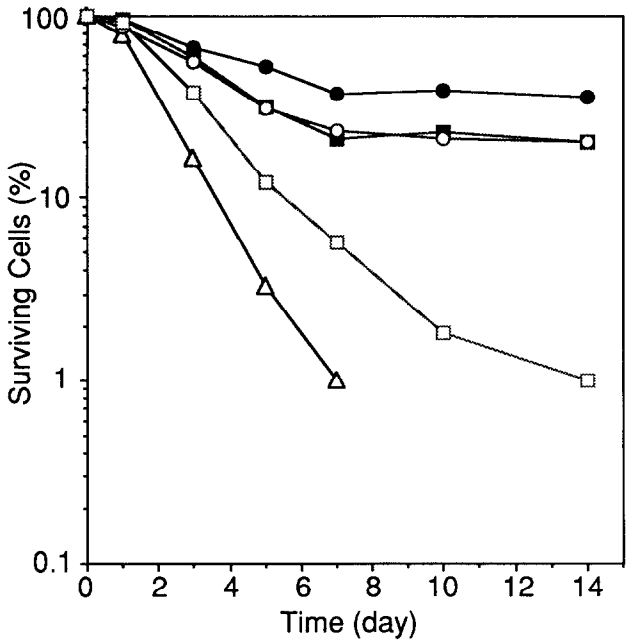

Fig. 2. Survival of PC12 cells in a low-serum medium in the presence of various differentiation factors. The cells were cultured in a medium containing DMEM and 0.5% fetal bovine serum and the number of the cells present in one visual field of the microscope (magnification. × 100) was counted as described in the legend of Table I. The percentage of the surviving cells in comparison with the number of the cells counted at day 0 was calculated and plotted against the length of the culture. Symbols used are: buffer alone as a control, △; NGF, ●; EGF, ○; TGF-α, ■; FGF, □.

fold. However, unstimulated PC12 cells expressed a certain level of 200 kDa NF subunit; NGF, and EGF and TGF-α increased its expression about 8-fold, and 3-fold, respectively (Fig. 3). This result clearly demonstrates the induction of NF proteins by EGF and TGF-α, concluding that PC12 cells can differentiate to neuronal cells in response to these factors.

As another differentiation-associated event in PC12 cells, we next studied the activation of the transin gene. This gene encodes a secreted metalloprotease which degrades collagen, laminin, and fibronectin, and is possibly involved in the reorganization of extracellular matrices during the process of axonal elongation [29]. In PC12 cells, the activation of the transin gene accompanied neurite outgrowth, and its induction was restricted to the differentiation signal from NGF and FGF [23,30]. Other signals including EGF, TGF-b, dibutyryl-cAMP, and phorbor ester had no effect on its expression [23,30]. Therefore, we asked whether EGF and TGF-α could activate the transin gene in PC12 cells under the conditions in which these factors could induce neuronal differentiation. It has already been demonstrated that the specific induction of the transin gene by NGF is due to the transcriptional activation through the 750 bp 5′ promoter region [21,23]. Thus, we transfected the recombinant plasmid p750TRCAT containing this 750 bp promoter fragment fused to the bacterial reporter gene, chloramphenicol acetyltransferase (CAT). Subsequently, the cells were cultured in the presence of various factors, and CAT activity in the cells was compared with that in the control cells (Table II). As demonstrated in the previous report [23], NGF-treatment of PC12 cells induced a dramatic increase of CAT activity derived from p750TRCAT plasmid. We found that EGF and TGF-α could also increase CAT activity about 4-fold, and this induction was even stronger than that by FGF. Hence, we concluded that the activation of the transin genes accompanies neuronal differentiation in response to EGF and TGF-α, as is the case for NGF.

4. DISCUSSION

Increasing evidence has demonstrated that EGF and NGF transduce many overlapping signals downstream of their receptors in PC12 cells [31]. Both of their receptors belong to a family of transmembrane tyrosine kinases, and elicit a variety of intracellular events through tyrosine-phosphorylation of proteins. A large number of molecules which are known to play an important role in signal transduction are commonly activated by the EGF and NGF receptor tyrosine kinases. They include phospholipase C-γ [32–34], phosphatidylinositol-3-kinase (PI3 kinase) [35,36], and Ras [14–17] in the plasma membrane, and a set of cytoplasmic serine/threonine kinases, including Raf-1 kinase, Mitogen-activated protein kinases (MAP kinases), and S6 kinases [37,38]. In addition, many transcription factors, such as c-Fos, c-Jun, and c-Myc, are also induced upon stimulation with both factors [39].

Consistent with these observations, EGF and NGF induce many common cellular responses in PC12 cells, such as an increase in 2-deoxyglucose uptake [12], ornithine decarboxylase activity [12], and even an increase in the density of Na^+ channel [40] which is closely associated with neuronal phenotypes. Both NGF- and

Table II

Activation of transin-CAT in PC12 cells

Stimulation	Relative CAT activity
control	1.0 ± 0.4
NGF	17.7 ± 2.4
EGF	3.9 ± 0.7
TGF-α	3.6 ± 0.8
FGF	1.9 ± 0.3

p750TRCAT plasmid was transfected into PC12 cells and subsequently the cells were stimulated with various factors for 48 h. CAT activity in cell extracts was measured as described in [24] and expressed as a relative value compared to the value of the control cells. Specific activity of CAT in the control cell extract was 9.5 ± 3.8% acetylation/mg protein/h at 37°C. The data are the mean ± SEM from three independent experiments.

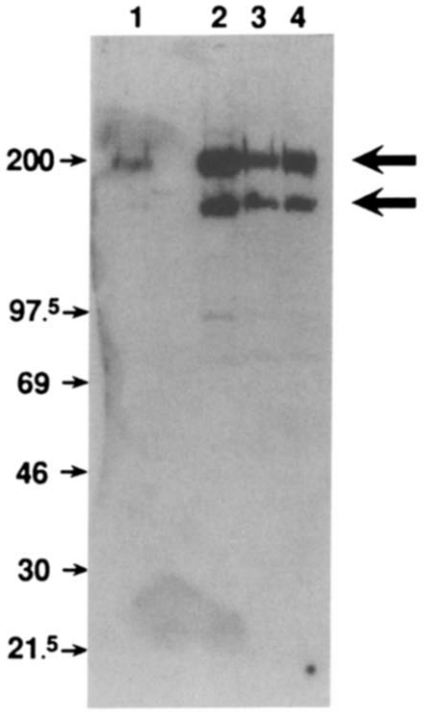

Fig. 3. Induction of 160 kDa and 200 kDa neurofilament proteins by differentiation factors. The cytoskeletal fractions were obtained from PC12 cells cultured in the presence of various factors and subjected to Western blot analysis by using monoclonal anti-neurofilament antibodies as described in Materials and Methods. Molecular weight markers are shown on the left side. The bands of 160 kDa and 200 kDa neurofilament proteins are indicated by arrows. Samples are from the cells stimulated with: lane 1, buffer alone as a control; lane 2, 50 ng/ml NGF; lane 3, 50 ng/ml EGF; lane 4, 50 ng/ml TGF-α.

EGF-treatment induce rapid membrane ruffling [13], flattening of the cells, and subsequent increase in cell adhesion [11,12], indicating that some of the morphological changes are also similar in both cases. However, despite these facts, it has been reported that NGF induces differentiation of PC12 cells, whereas EGF does not [11–13]. In this paper, we have demonstrated that EGF is also capable of inducing neuronal differentiation of PC12 cells under particular culture conditions. To unmask the weak effect of EGF, we seeded the cells at a low density, and cultured them for more than two weeks in a low-serum medium on the culture dishes called Primaria, which has a chemically modified unique surface. This coated plate has been known to enhance a variety of cellular activities, including activation, proliferation, and differentiation in many types of cells [41]. We observed that collagen (type IV)-, and fibronectin-coated plates also weakly support neurite formation by EGF, but on the regular uncoated plates, the effect of EGF was quite weak. Thus, in this case, the coated surface of culture dishes has a significant effect on the neurite extension of PC12 cells. This seems quite reasonable since many recent studies have revealed an important role of culture substrates including extracellular matrix proteins in the induction and extension of neurites [26,27]. It is not currently known why EGF-stimulated, but not NGF-stimulated, cells require the coated surface to extend neurites. We observed that neurite formation by NGF and FGF was also greatly enhanced when the cells were maintained on Primaria dishes, indicating that the effect of the coated plate is not limited to EGF and TGF-α.

In previous studies [11–13], the effect of EGF was examined under conditions in which PC12 cells were cultured on ordinary uncoated culture flasks at relatively higher densities (e.g. 1.6×10^4 cells/cm^2 in [12]), and maintained in the presence of a high concentration (15%) of serum. We suppose that under these conditions, the EGF-stimulated culture reaches confluence within a relatively short period of time, and hence morphological changes which are obvious only after a culture of 10 days or longer may be masked.

Under our experimental conditions, the differentiated cells induced by EGF and TGF-α were very similar in their morphologies to those induced by NGF and FGF (Fig. 1). Western blot analysis shown in Fig. 3 demonstrated that neurofilament proteins, which are specifically expressed in neuronal cells, are strongly induced, indicating that the processes extended from the EGF-stimulated cells are actually neurites. It was previously reported that the activation of the transin gene accompanied neuronal differentiation in PC12 cells [23,30], and the gene was induced only by NGF and FGF but not by EGF under the conditions in which the cells did not differentiate in response to EGF [23,30]. Our data shown in Table II demonstrated that the activation of transin also occurs in association with differentiation induced by EGF and TGF-α.

Taking all these observations together, we have shown that EGF and TGF-α have similar biological effects on PC12 cells to those of NGF, and both can induce differentiation to neuronal cells. Previous studies have demonstrated that EGF elicits many cellular responses overlapping with those elicited by NGF even under the conditions in which it cannot induce differentiation of PC12 cells [31]. Thus, we speculate that EGF and TGF-α may have a weaker effect than NGF on the induction of certain extracellular matrix proteins and/or their receptors which are quite important for neurite extension, and particular culture substrates such as Primaria and fibronectin-coated dishes may partially compensate for the weak effect of EGF and TGF-α. As shown in the previous studies [14–17], both EGF and NGF induce the activation of Ras in PC12 cells. It has been demonstrated that Ras plays a crucial role in the induction of differentiation in PC12 cells [18–20]. Thus, our observation is in accordance with the previous findings, and it can be concluded that in all cases, activation of Ras leads to the induction of neuronal differentiation in PC12 cells.

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