A Novel Differentiation Factor for PC12 Cells from Culture Supernatant of Mouse Hepatocyte Cell Line MLE-15A2

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We have found a factor that induces neurite outgrowth of rat PC12 cells in the culture supernatant of the cell line MLE-15A2. This factor was designated as MDDF. The factor was sensitive to protease, dithiothreitol, and high-temperature treatments. The apparent molecular mass was 80 kDa on Superdex 200 gel filtration. No significant tyrosine phosphorylation was detected after MDDF stimulation in Western blotting analysis with anti-phosphotyrosine antibody, suggesting that the signal transduction may not be mediated by a tyrosine kinase cascade that is involved in signaling of most of the known factors. Activation of MAP kinase was very weak and was seen only 5 min after stimulation, suggesting that prolonged activation of MAP kinase was not required for neurite outgrowth induced by MDDF. Because the biochemical characteristics of MDDF are different from those of any known peptide factors that induce neurite outgrowth of PC12 cells, MDDF may be a novel differentiation factor for PC12 cells.

Key words: PC12 cells; differentiation factor; signal transduction; neurite

Rat pheochromocytoma PC12 cells have been used in a number of laboratories as a good in vitro model system of neuronal differentiation. After stimulation with nerve growth factor (NGF), these cells differentiate to extend neurites and to develop the characteristics of sympathetic neurons.11 NGF stimulates its specific receptor tyrosine kinase to generate various second messengers. Ras, MAP kinases, phospholipase C, and phosphatidylinositol (PI)-3 kinase are the well-studied proteins that are activated after stimulation with NGF.2,7 Epidermal growth factor (EGF), on the other hand, does not induce neurite outgrowth, but induces cell growth.8 The paradox is that signal transduction of EGF and NGF are very similar, and yet the cell responses are completely different. Overexpression of EGF receptors enables EGF to induce differentiation of PC12 cells, indicating that the difference between signal transduction by EGF and NGF might be quantitative and not qualitative. Intense examination of signal transduction by these factors found that the activation period of MAP kinase was longer after NGF or fibroblast growth factor (FGF) stimulation than after EGF stimulation.9-11 This leads to the idea that MAP kinase might play a central role in deciding the fate of the cells. Indeed, constitutive activation of MAP kinase results in differentiation of PC12 cells.12 However, a recent study with a mutant NGF receptor lacking a juxtapembrane sequence indicated that the duration of MAP kinase activation is not sufficient for differentiation, suggesting that some additional signals may be required for complete neurite outgrowth.13 In addition, induction of neurite outgrowth by a combination of cAMP and EGF was not accompanied by prolonged activity of MAP kinase.14 There are still many arguments for and against the involvement of MAP kinase in neurite outgrowth of PC12 cells. PI-3 kinase is also involved in neurite outgrowth of these cells. PI-3 kinase is activated within a few minutes to elevate the PIP3 level.4 However, this immediate activation of PI-3 kinase after stimulation by the differentiation factors is not required for neurite outgrowth.15 Instead, inhibition of PI-3 kinase at the time of neurite outgrowth results in blockage of elongation of neurites. These results suggest that PI-3 kinase may be multifunctional in PC12 cells, and that PI-3 kinase is required only at the time of neurite outgrowth in PC12 cells.

It is important that precise connections of neuronal cells be formed during development for acquisition of the highly organized nervous system. It is known that an extracellular matrix or the cell surface molecules are involved in this process. Cell adhesion molecules and the members of ligands for the EGF and MAP kinase family are examples.16 On the other hand, involvement of diffusible factors in the process has been expected. There is increasing evidence that supports the idea that these factors are indeed important.17 Neurotrophins such as NGF and neurotrophin 3 (NT3), which are well known for their trophic effects,18,19 have been shown to stimulate regeneration of neuronal connections.20,21 Recent studies have demonstrated the additional role of neurotrophin in regulation of the branching of axons, which decides the patterns of neuronal connections in the target tissues.22

Other diffusible factors may also be involved in the early stage of neuronal development, when the direction of the axons is decided. It has been shown that some neurons direct their axons toward the diffusible factors secreted by the target tissues.23-26 Netrin was identified as one such factor; it attracts commissural axons to the floor plate.27 There may be a variety of other factors working on the formation of the complex nervous system by connecting each set of a neuron and a target tissue. For an understanding of the way in which the precise patterns of neuronal connection are decided, it may be necessary to identify a series of such factors. However, because of the
small amounts and the complexity of the factors in animals, it is a difficult task to identify them. To overcome this problem, we have screened for the factors that induce neurite outgrowth of PC12 cells, as a model system of neuronal cells, in culture supernatants of transformed cell lines. As a starting material, culture supernatants may be better than the tissues. The species of the protein may be much less, and the analysis and purification of the factors may be much easier in culture supernatants. The factors that have been identified as differentiation factors for PC12 thus far are neurotrophins and other growth factors, including NGF, FGF, NT3, IL3, VEGF, BMP2B, and activin. We report here on a factor capable of inducing differentiation in PC12 cells, the characteristics of which are distinct from those of the factors listed above.

**Materials and Methods**

*Cell culture, culture supernatant, and assay of neurite outgrowth.* A mouse hepatocyte cell line, MLE-15A2, was cultured in RPMI 1640 medium with 10% calf serum. The cells were washed with phosphate-buffered saline (PBS) and were exposed to serum-free RPMI 1640 medium. After 24 h, the culture supernatant was collected and used as a source of the differentiation factor. Rat pheochromocytoma PC12 cells were maintained in Dulbecco’s modified minimal essential medium (DMEM) supplemented with 10% calf serum and 5% horse serum. Twenty-four hours after replating to fresh dishes, the medium was replaced with serum-free DMEM, and the effects of the differentiation factor were assayed.

*Western blotting.* This was done as described previously. Briefly, the proteins were blotted onto a nylon filter and incubated in Tris buffered saline (TBS) containing 0.1% Tween 20 and 2% bovine serum albumin. After incubation with an anti-phosphotyrosine antibody, PV20, the filter was washed and the bands were made visible with an ECL system (Amersham), using goat anti-mouse IgG conjugated to horse radish peroxidase used as a second antibody.

*Immunocytochemistry.* Cells were grown on coverslips coated with collagen. After stimulation with MDDF (see Results section), the cells were fixed with 10% formalin for 10 min. After permeabilizing the cells with 0.05% Triton X-100 in PBS, we analyzed the distribution of the protein by immunofluorescence, using anti-GAP43 antibody (a gift from Dr. A. Beato Oesterhelt) as a first antibody and FITC-labeled anti-rabbit IgG as a second antibody.

*Column chromatography.* Phosphocellulose chromatography was done with P11 resin (Whatman). Culture supernatant (10 ml) was mixed with activated P11 resin (0.4 ml) and incubated for 1 h. The resin was packed in a column and washed with a buffer containing 5 mM Hepes, pH 7.4. The factor was eluted with a buffer containing 5 mM Hepes, pH 7.4, and 1 M NaCl, with a fraction size of 0.2 ml. The activity eluted slightly after the protein peak at fractions 3 and 4. For gel filtration, the culture supernatant was concentrated 20-fold in vacuo. The sample was put onto a Superdex 200 column (Pharmacia) and developed with a buffer containing 5 mM Hepes, pH 7.4, and 50 mM NaCl at a flow rate of 0.5 ml/min.

*Protease, diithiothreitol, acid, and heat treatments of the factor.* Proteinase K in 10 mM Tris HCl, pH 7.5, 150 mM NaCl, which was incubated for 2 h at 37°C to eliminate contaminating enzyme activity, was added to the MDDF fraction at a concentration of 10 μg/ml and incubated at 37°C for 1 h. For a mock experiment, buffer without proteinase K was added. Proteinase K was inactivated by incubation with 1 mM phenylmethylsulfonyl fluoride (PMSF) at 0°C for 2 h, and the factor was purified with a phosphocellulose column for removal of proteinase K.

*Diithiothreitol (DTT)* was added to the MDDF fraction at 1 mM and incubated at 0°C for 1 h. DTT was removed by phosphocellulose column chromatography. For acid treatment, HCl was added to the final concentrations of 100 mM and 10 mM at pH 1.0 and 2.0, respectively, and incubated at 0°C for 30 min. The samples were neutralized by addition of NaOH and assayed for the MDDF activity. For heat inactivation of the factor, the culture supernatant was treated at 70°C or 60°C for 30 min and quickly cooled on ice.

**MAP kinase assay.** This was done according to the method described by Gotot et al. 9

**Results**

*Culture supernatant of MLE-15A2 cells induces neurite outgrowth of PC12 cells*

PC12 cells were stimulated with the culture supernatant of MLE-15A2 cells. A neurite-like process was seen 12 h after stimulation with MDDF (Fig. 1A). We analyzed the signal transduction induced by the culture supernatant and found that some proteins were phosphorylated on tyrosine within a few minutes after stimulation (data not shown). To find whether or not this tyrosine phosphorylation was due to the factor that induced process formation, we partially purified the factor by Superdex 200 gel filtration chromatography. The activity eluted at 22 to 24 min, which corresponded to a molecular size of 80 kDa (Fig. 2). When we used the active fractions to stimulate the cells, no significant tyrosine phosphorylation was detected, suggesting that the factor that induced tyrosine phosphorylation of the proteins had been removed from the sample. It is likely that the cell line MLE-15A2 secretes multiple factors into the culture medium and that the factor that induces the tyrosine-phosphorylation of the proteins does not induce neurite formation in PC12 cells. The factor found here was named MDDF (MLE-15A2 cells-derived differentiation factor). We measured the activity by the limiting dilution method and defined 1 unit as the activity that can give the minimum effects on PC12 cells in 96-well microplates (Corning Co., Ltd., Corning, NY, U.S.A.). According to this definition, the recovery was about 10% and the activity was purified by about 5-fold in gel filtration. The length of the neurites was in parallel with the dose of MDDF. The activity reach to the saturation at about 10 units. More than 10 units of MDDF purified through gel filtration was used in the further study.

We found that MDDF could bind to Q-Sepharose, phosphocellulose, and reverse-phase resins, but not to heparin Sepharose, blue Sepharose, or hydroxypatite resins (data not shown). Although the recovery from Q-Sepharose and reverse-phase resins was poor, the activity eluted in single peaks in any chromatography suggesting that the activity is due to a single factor. Since phosphocellulose gave the best recovery among various resins, it was useful for purification of the factor.

**Course of differentiation of PC12 cells after MDDF stimulation**

The course of morphological changes in PC12 cells after MDDF treatment is shown in Fig. 1. The cells changed their morphology by extending the tips of the processes within 2 h after stimulation (Fig. 1A, G), then stopped extending them for several hours. After this, the elongation of the processes started again, and the cells continued to extend them, but attenuation of the growth of the processes was seen after incubation with MDDF for one day (Fig. 1A, J and K). To understand this course of process extension quantitatively, we counted the number of cells the processes of which were longer than the cell body. As shown in Fig. 1B, the time required for 80% of MDDF-treated cells to extend the processes was much shorter than that for NGF-treated cells. The neurite of NGF-treated cells was
A. PC12 cells were incubated with NGF (10 ng/ml) (A to C), the culture supernatant of MLF-15A2 cells (D to F), or MDDF (G to K, M, N), and the course of morphological changes was observed. Incubation times were: A, 12 h; B, 24 h; C, 36 h; D, 12 h; E, 24 h; F, 36 h; G, 2 h; H, 6 h; I, 12 h; J, 24 h; and K, 30 h. In M and N, a second dose of MDDF was added at 24 h after the first stimulation, and the cells were incubated for an additional 6 h (M) or 10 h (N). L, control PC12 cells.

B. PC12 cells were treated with MDDF or NGF (10 ng/ml), and the cells the processes of which were longer than the cell bodies were scored. The percentages of cells with long processes at 12 h, 36 h, and 60 h after the stimulation and plotted. Closed circles, MDDF treatment; open circles, NGF treatment.

Fig. 1. Induction of Differentiation in PC12 Cells by MDDF.
not seen until about 12 h (Fig. 1A, A), when the processes of either unpurified or purified MDDF-treated cells became longer than the cell body (Fig. 1A, B). When a second dose of MDDF was added after incubation for 24 h, attenuation of process elongation was prevented (Fig. 1A, M and N). These data indicate that MDDF is degraded rapidly in cultures of PC12 cells. Indeed, MDDF activity was almost lost after incubation with PC12 cells for 24 h (data not shown).

To characterize the neurite-like processes, we stained the cells with anti-GAP43 antibody. GAP43 is one of the well-characterized antigens found in the growth cones of the neurites. As shown in Fig. 3, the GAP43 antigen was found at the tips of the processes induced by MDDF.

**MDDF is sensitive to protease and heat treatment, but is stable at low pH**

Biochemical characterization of MDDF was done. To find whether or not the factor was a peptide factor, we tested the effects of protease treatment on the activity. MDDF was incubated with 10 μg/ml of proteinase K at 37°C for 30 min. After inactivation of the protease with 1 mM PMSF, MDDF was partially purified by phosphocellulose column chromatography for removal of protease. A marked decrease in activity was observed with proteinase K treatment (Fig. 4, A and B). Next, the sensitivity to dithiothreitol (DTT) was tested. After treatment with 1 mM DTT, MDDF lost its activity, suggesting that it may contain S-S bonds (Fig. 4, C and D).

Effects of heat treatment were also tested. MDDF was heat-labile, because treatment at 70°C for 30 min abolished the activity almost completely. Treatment at 60°C partially inactivated the factor (Fig. 4, E, F, and G). The above results suggest that MDDF is a peptide factor. In contrast, MDDF was fairly stable under acidic conditions (Fig. 4, H, I, and J). After exposure to pH 1.0 for 30 min, activity was still detected, although it appeared to be somewhat weaker. Treatment at pH 2.0 did not affect the activity at all.

**Course of appearance of phosphotyrosine-containing protein after MDDF stimulation**

To study the appearance of phosphotyrosine-containing protein after MDDF stimulation, we did Western blotting using anti-phosphotyrosine antibody. As seen in Fig. 5, no significant phosphorylation on tyrosine was observed immediately after the stimulation. At 5 min after stimula-

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**Fig. 2.** Molecular Mass of MDDF by Superdex 200 Gel Filtration.
Culture supernatant of MLE-15A2 cells was concentrated 20-fold in vacuo and put onto a Superdex 200 column. Each fraction was assayed for activity in media as in the legend to Fig. 1. The results of the assay were shown in the lower part of the figure. In another experiment, molecular standards (116 kDa, 68 kDa, and 45 kDa) were developed on the column, and the elution profile was analyzed by SDS polyacrylamide gel electrophoresis. The results are shown in the graphs.

**Fig. 3.** Staining of GAP43 Antigen in MDDF-Stimulated Cells.
PC12 cells grown on the coveships coated with collagen were stimulated with MDDF for 24 h. After fixation with formalin, cells were incubated with (A and B) or without (C) anti-GAP43 antibody and stained with FITC-labeled anti-rabbit IgG. A, MDDF-stimulated cells; B, unstimulated cells; C, MDDF-stimulated cells.
PC12 cells were stimulated with the MDDF treated with various conditions as described in Materials and Methods. The morphological change was observed at 18 h after stimulation. B, treated with proteinase K (10 μg/ml) at 37°C for 1 h; D, treated with 1 mM DTT for 30 min; F, incubated at 60°C for 30 min; I, incubated at pH 2 for 30 min. A, C, E, and H are for untreated controls.

Fig. 5. Phosphorylation of Proteins on Tyrosine after MDDF Treatment.
PC12 cells were treated with MDDF (A), NGF (10 ng/ml) (B), or EGF (10 ng/ml) (C), and incubated for various periods. After lysing the cells with the sample buffer,14 we analyzed phosphorylated proteins by Western blotting with anti-phosphotyrosine PY20. The positions of the Shc protein and MAP kinases are shown by arrowheads.

Fig. 4. Treatment of MDDF with Proteinase K, DTT, High Temperature, and Acid Conditions.
Two weak bands appeared at a molecular size of 42-44 kDa, which appeared to be MAP kinases. It has been shown that NGF induces activation of MAP kinases and that activation of MAP kinases is required for NGF-induced neurite outgrowth. To confirm that the bands were MAP kinases, we did an in vitro MAP kinase assay. As shown in Fig. 6, activation of MAP kinase was detected, corresponding to the appearance of 42-44 kDa bands. Judging from the molecular size, the band seen in the in vitro assay was assigned to the lower band in Fig. 5. Activation of MAP kinase was no longer seen at 10 min after stimulation. These results suggest that prolonged activation of MAP kinase is not required for neurite outgrowth induced by MDDF.

PI-3 kinase may not be required for neurite outgrowth induced by MDDF
PI-3 kinase may also be required for neurite outgrowth induced by NGF (14). We tested whether MDDF induced activation of PI-3 kinase. Elevation of the PIP3 level was readily detectable at 4 to 6 min after NGF stimulation. In the case of MDDF, we did not see elevation of the PIP3 level at any time (data not shown). The association of a 100 kDa protein phosphorylated on tyrosine with PI-3 kinase, which was detected after NGF stimulation, was not seen in MDDF-stimulated cells (data not shown).

Finally, we tested the effects of wortmannin, a specific inhibitor of PI-3 kinase. We have shown that treatment of PC12 cells with wortmannin blocks neurite elongation in NGF-stimulated cells. Wortmannin was effective at the late phase of differentiation after NGF stimulation, when the neurites grew out (Fig. 7, D and E). However, no inhibition of neurite outgrowth was observed in MDDF-stimulated cells (Fig. 7, B and C). Even if wortmannin was present over the early and late phase of differentiation, no inhibition of neurite outgrowth was observed (data not shown). These results suggest that PI-3 kinase is not involved in...
Fig. 6. Activation of MAP Kinase after MDDF Treatment.

Left panel: PC12 cells were treated with MDDF and incubated for various periods. After lysing of the cells, the proteins were separated on a polyacrylamide gel containing myelin basic protein. After denaturation of the proteins by guanidine HCl, we renatured the MAP kinase by diluting guanidine HCl, and the activity in the gel was assayed by incubation with γ-32P-ATP. Control, without any treatment; NGF 5m, a positive control made by stimulation of the cells with NGF for 5 min, others, incubated with MDDF for the periods indicated. The arrowhead marks the position of MAP kinase. Right panel: The intensity of the bands for MAP kinase activity was measured by densitometer and plotted.

Fig. 7. Wortmannin Did Not Inhibit Neurite Outgrowth Induced by MDDF.

PC12 cells were treated with MDDF (B and C) or NGF (10 ng/ml) (D and E). Cells were treated with (C and E) or without (B and D) wortmannin (100 nm) every 4 h starting at 12 h after differentiation factor stimulation. 12 h later, the morphological changes in PC12 cells were observed. A, control PC12 cells without any treatment.

MDDF-induced neurite outgrowth.

Discussion

A number of factors have been identified that induce differentiation of PC12 cells. We screened for factors that have not been identified as differentiation factors of PC12 cells. Culture supernatant of the cell line MLE-15A2 contained a factor that induces neurite outgrowth of PC12 cells. Immunostaining of MLE-15A2-stimulated PC12 cells with anti-GAP43 antibody suggested that their processes may be neurites. The factor appeared to be a peptide factor because it was relatively large, with a molecular mass of 80 kDa on gel filtration, and because it was sensitive to protease treatment. The possibility that MDDF is one of the factors that have been reported to be capable of inducing differentiation of PC12 cells was tested. The factor was not NGF or FGF, because it did not bind to heparin Sepharose (data not shown). Our PC12 cells were insensitive to authentic IL6, suggesting that MDDF was not IL6. Messenger RNA of NT3 was not detected in RT-
PCR (reverse transcriptase mediated polymerase chain reaction) analysis of mRNA from MLE-15A2 cells, suggesting that the factor was not NT3 (data not shown). BMP2B and Activin were shown to be effective only in the presence of serum but MDDF was clearly effective in the absence of serum. Together with the difference of the molecular sizes, MDDF was distinct from these factors. VIP consists of only 28 amino acids and it is far too small for MDDF. These data suggest that MDDF may be a novel differentiation factor.

No significant tyrosine phosphorylation was observed after stimulation by MDDF, although weak activation of MAP kinase was seen. This finding suggests that MDDF is novel type of the factor which does not use a tyrosine kinase cascade. IAP was not effective in blocking the signal transduction, suggesting that the receptor may not be coupled to Gi (data not shown). It has been proposed that prolonged activation of MAP kinase is responsible for the neurite outgrowth induced by differentiation factors such as NGF or FGF. MDDF did activate MAP kinase; however, the period was much shorter and the strength was much weaker than in the case of NGF or FGF, suggesting that differentiation of PC12 cells is not always accompanied by prolonged activation of MAP kinase and that mechanism of regulation of neurite outgrowth by MDDF is different from that by NGF or FGF. Indeed, PD98059, a specific inhibitor of MAP kinase kinase (MAPKK), did not inhibit neurite outgrowth induced by MDDF, suggesting that activation of MAP kinase was not required in this case (data not shown). In addition, neurite outgrowth induced by MDDF was resistant to wortmannin treatment, indicating that the cell response was independent of PI-3 kinase. In the case of NGF treatment, it is obvious that both MAP kinase and PI-3 kinase are required for neurite outgrowth. Taken together, our findings suggest that MDDF is a new factor that is capable of inducing neurite outgrowth in PC12 cells.

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