Immunosuppressant FK506 Induces Sustained Activation of MAP Kinase and Promotes Neurite Outgrowth in PC12 Mutant Cells Incapable of Differentiating

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ABSTRACT. During the continuous culturing of neural PC12 cells, a drug hypersensitive PC12 mutant cell line (PC12m3) was obtained, which demonstrated high neurite outgrowth when stimulated by various drugs. When the immunosuppressant drug FK506 and nerve growth factor (NGF) were introduced to the PC12m3 cells, the frequency of neurite outgrowth increased approximately 40-fold for NGF alone. However, the effect of FK506 on neuritogenesis in PC12 parental and drug insensitive PC12m1 mutant cells was much lower than in PC12m3 cells. The sustained activation of mitogen-activated protein (MAP) kinase plays an important role in neurite outgrowth of PC12 cells. Interestingly, the drug hypersensitive PC12m3 cells exhibited the sustained activation of MAP kinase with FK506 in comparison to low or no activities in PC12 parental or drug insensitive PC12m1 cells. These results indicate that PC12m3 cells have a novel FK506-induced MAP kinase pathway for neuritogenesis.

Key words: PC12 mutant cells/nerve growth factor/FK506/MAP kinase

The immunosuppressant drug FK506 is a macrocyclic lactone that was isolated from the Streptomyces tsukubaensis bacterium in 1984 (Kino et al., 1987). FK506 is now being used as for primary immunosuppressant therapy because of its fewer levels of side effects. FK506 increases the rate of axonal regeneration in the sciatic nerve (Wang et al., 1997). Furthermore, FK506 enhances neurite outgrowth in neuronal PC12 cells by increasing their sensitivity to NGF (Lyons et al., 1994). However, the precise mechanisms leading to the enhancement of neurite outgrowth in PC12 cells are still unknown.

Rat pheochromocytoma cells, PC12 cells, have been useful model system for examining neuronal differentiation and signal transduction. PC12 cells differentiate in response to several growth factors including NGF and FGF (Greene and Tischler, 1976; Togari et al., 1983). Due to the widespread use of PC12 cells in various culture conditions, spontaneous variants are often encountered (Greene et al., 1991). PC12 clonal variants have been useful in the study of specific cellular phenomena in the past (Van Buskirk et al., 1985; Shoji-Kasai et al., 1992; Teng et al., 1993; Corradi et al., 1996; Larsen et al., 1998). In a variant PC12 cell line, NGF induced normal activation of intermediate early genes, whereas the activation of some delayed response genes as well as neurite outgrowth was impaired. Furthermore, in these mutant cells the activation of NGF-induced extracellular signal-regulated kinase (ERK also known as MAP kinase) was transient, not sustained (York et al., 1998). The authors developed novel PC12 mutant cells which shows poor neurite outgrowth in spite of normal sustained activation of MAP kinase by NGF treatment.

MAP kinase is a group of serine/threonine kinase that is activated by extracellular stimuli (Cobb et al., 1991; Nishida and Gotoh, 1993). MAP kinase activity is necessary for growth factor induced PC12 cell differentiation (Cowley et al., 1994). The duration of MAP kinase stimulation by NGF and EGF may account for the lack of neurite induction.
by EGF. NGF and EGF both stimulate rapid increases in MAP kinase activity but NGF stimulation is more persistent than EGF (Gotot et al., 1990; Traverse et al., 1992).

In this study, the effect of FK506 on neurite outgrowth of the drug hypersensitive PC12 mutant cells (PC12m3) is described. The mutant cell was developed in our laboratory (Kano et al., 2001). Under NGF treatment, the mutant cell exhibited high outgrowth of neurite in response to stimulants such as FK506, cAMP and calcimycin. The frequency of neurite outgrowth stimulated by FK506 was approximately 40-fold greater than NGF alone. Furthermore, this PC12 mutant cell exhibit sustained activation of MAP kinase by FK506 in comparison to the low or no activity in PC12 parental or drug insensitive PC12 mutant cells.

**Materials and Methods**

**Reagents**

NGF (2.5S) was purchased from Takara (Osaka, Japan). EGF was purchased from Sigma (St. Louis, MO). FK506 was purchased from Fujisawa (Osaka, Japan). U0126 was purchased from Promega (Madison, WI).

**Cell Cultures**

PC12 and PC12 mutant cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.35% glucose, 10% horse serum, 5% fetal bovine serum (FBS), and 100 units/ml kanamycin. All cells were grown at 37°C with 5% CO₂.

**Determination of neurite outgrowth**

A single-cell suspension of PC12 and PC12 mutant cells was obtained by trituration in DMEM. For culture experiments, cells were plated onto 25 cm² flasks at a density of 2 × 10⁶ cells per dish of serum-containing DMEM, then cells were immediately treated with NGF and/or various drugs. After 5 to 7 days of incubation, the frequency of neuritogenesis was determined by measuring the neurite length and neurite numbers. Cells with one or more neurites of a length 1.5 times greater than the diameter of the cell body were measured as previously described (Yao and Osada, 1997). Each value is the mean ± S.D. for 100–200 cells sampled from three independent experiments.

**Detection of activated p44/42 MAP kinase**

MAP kinase activities were determined as modified previously described (Sakai et al., 1999). PC12 and PC12 mutant cells were plated at a density of 1 × 10⁶ cells / 25 cm² in a flask of serum-containing medium and cultured for three days. Cultures were then replaced by 0.5% FBS-containing medium for 48 h. Before the NGF treatment, the cells were incubated for 2 h in a serum-free medium. Cells were stimulated for 10, 30, or 120 min with the addition of NGF (30 ng/ml), EGF (10 ng/ml) or FK506 (40 µM). MAP kinase activities were then assayed in the cell lysates. Cells were lysed in lysing buffer (20 mM Tris, pH 7.6, 1 mM EDTA, 150 mM NaCl, 1% Triton x-100, 1 mM NaVO₃, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin). Aliquots of the lysates (10–15 µg) from each sample were fractionated on SDS-10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (0.45-µm pore size Immobilon-P, Millipore). The blots were probed with the phospho-p44/42 MAP Kinase antibody (New England Biolabs) at a dilution of 1:1000 in blocking buffer (5% nonfat dry milk) for 12 h in 4°C. The blots were probed with secondary antibody, horseradish peroxidase-linked anti-rabbit IgG, at a dilution of 1: 2000 in blocking buffer for 60 min at room temperature. The blots were stained for 1 min using the nucleic acid chemiluminescence reagent (LumiGLO chemiluminescent reagent, Kirkegaard and Perry Laboratories, Gaithersburg, MD) and exposed to x-ray film.

**Results**

**Isolation and morphological characterization of PC12 mutant cells**

During continuous culturing PC12 cells are subject to spontaneous mutations that lead to the new generation of PC12 variants. We obtained a variant cell line which showed spontaneous impairment in NGF-induce neurite outgrowth in our laboratory. When these culture cells were cultivated for 2 weeks under the acidic conditions of Cl−, several surviving clones appeared. Using the ring isolation procedure, ten colonies were selected and propagated in mass culture, and they were termed PC12m-1, PC12m-2, PC12m-3 and so on. Then, these PC12 mutant cells were examined for their sensitivities to NGF and various drugs. Two clones (PC12m-1 and m-4) showed the impaired NGF-induced neurite outgrowth and their morphology was small and round like a parental PC12 cells. The other eight clones (PC12m-2, m-3, m-5 to m-10) showed poor neurite outgrowth in reaction to the NGF and these cells were morphologically distinct from their parental PC12 cells. They had a large, flattened morphology and showed small cytoplasmic extensions. Among these PC12 mutant clones, PC12m1 and PC12m3 cells were used for experiments. PC12m1 cells are typically round, phase-bright cells like PC12 parental cells under non-stimulus control conditions (Fig. 1-A, B). The naive PC12m3 cells are more flattened and more polygonal in shape than PC12 parental and PC12m1 cells. Many of the PC12m3 cells also exhibit short cytoplasmic extensions or spick which are very rarely observed on naive PC12 parental and PC12m1 cells under non-stimulus conditions (Fig.1-C).

**FK506 induce neurite outgrowth of PC12 mutant cells**

We have tested the FK506 for its ability to induce neurite outgrowth on PC12 parental and mutant cells. FK506 treatment on PC12m3 cells or PC12m1 cells produces distinc-
Sustained Activation of MAPK by FK506

tive responses in each. It was observed that some cells were induced to extend neurites by FK506 in PC12m3 cells. In contrast, there is no induction of neurites extension even after 7 days of continuous FK506 treatment in PC12m1 cells (Fig. 1). Less than 15% of total cells were neurite bearing among the FK506 treated PC12m3 cells. However, this frequency was much higher than FK506 treated PC12 parental cells (Fig. 2). NGF, in contrast, stimulates the elaboration of an extensive neurite network in PC12 parental cells. However, NGF induced only minor neurite outgrowth in cell adhesion in PC12m3 cells. Interestingly, FK506 combined with NGF produced much greater enhancement of neurite outgrowth than NGF alone in PC12m3 cells, whereas FK506 had little effect on neuritogenesis in PC12 parental and PC12m1 cells (Fig. 1). The frequency of neurite outgrowth of PC12m3 cells stimulated by NGF+FK506 was approximately 40-fold greater than NGF alone, showing almost the same frequency as NGF treated PC12 parental cells (Fig. 2).

Neurite outgrowth actions of FK506 was mediated by Ras-MAP kinase signal transduction in PC12m3 cells

Since the activation of MAP kinase has been shown to play an important role in neuronal differentiation in PC12 cells, we were interested in finding out whether the inability of NGF to induce complete neurite outgrowth of PC12 mutant cells could be a reflection of its effect on MAP kinase activity. We therefore examined the NGF-induced MAP kinase activation in various PC12 mutant clones. Cells were treated with NGF, EGF or FK506 for up to 120 min and MAP kinase activities were detected by immunoblotting. Unexpectedly, every PC12 mutant cells had a strong NGF induced MAP kinase activity, the same as the PC12 parental cells. On the other hand, the EGF-induced MAP kinase activation was transient, i.e. the activity reached a peak after 10 min and then decreased rapidly in all PC12 cell. However, FK506-induced MAP kinase activity was detected only in drug hypersensitive PC12m3 cells. FK506 did not induce MAP kinase activity even in PC12 parental cells. The activ-

Fig. 1. Stimulation of PC12 cell neurite outgrowth by NGF and or FK506. PC12 parental (A), drug nonsensitive PC12m1 (B) or drug hypersensitive PC12m3 (C) cells were treated (+) or not (−) continuously with 30 ng/ml NGF and/or 40 µM FK506. Phase-contrast photomicrographs of PC12 cells were taken seven days after treatment. (×140)
ity of MAP kinase induced by FK506 was sustained and moderately increased at 120 min (Fig. 3). Interestingly, the effects of FK506 for MAP kinase activation were consistent with the phenomenon of neurite outgrowth.

Discussion

The MAP kinase pathway represents one of the major intracellular signaling mechanisms activated by polypeptide growth factors. In PC12 cells, evidence has accumulated to indicate that growth factors causing prolonged activation of the MAP kinase pathway such as NGF and FGF induce differentiation judged by significant neurite outgrowth (Cowley et al., 1994; Marshall, 1995), whereas EGF induced transient activation of the MAP kinase without inducing neurite outgrowth (Greene and Kaplan, 1995). However, recent studies suggested that the MAP kinase pathways might be essential but not sufficient for neurite outgrowth on PC12 cells (Vaillancourt et al., 1995). In PC12m3 cells, FK506-induced neurite outgrowth was accompanied by prolonged MAP kinase activation. These results are in agreement with the previously described linkage of PC12 differentiation and prolonged the MAP kinase activation (York et al., 1998). To verify the involvement of the MAP kinase pathway in FK506-induced neurite outgrowth of PC12m3 cells, we used the MAP kinase inhibitor U0126 which completely blocked the neurite outgrowth induced by FK506, indicating that prolonged the MAP kinase activation is indeed required for FK506-induced neurite outgrowth (data not shown).

On the other hand, in PC12m3 cells NGF did not induce neurite outgrowth in spite of sustained activation of MAP kinase. The precise nature of the defect in PC12 mutant cells that impairs the NGF-dependent induction of neurite outgrowth is not known. One possible explanation is that the defect which impairs NGF-promoted neuritogenesis in PC12m3 cells is in the downstream target of MAP kinase, and that this defect is overcome by the treatment of FK506. The second possibility is that PC12 mutant cells may have other signaling pathways mediated by various inducers such as FK506 and cAMP, beside the Ras-MAP Kinase pathway, that induce the multiplicity of neuronal differentiation response. A third possibility is that PC12m3 cells may be dominant in proliferation ability rather than differentiation, and FK506 leads cells to differentiation.

When PC12 cells are treated with NGF, they stop dividing and differentiate with process outgrowth. The correlation between cessation of proliferation and morphological differentiation suggests that the antimitotic activity of NGF may be required for neurite outgrowth (Burstein and
Fig. 3. Activation of ERK1 and ERK2 by various stimuli in PC12 cells. PC12 parental (A), drug nonsensitive PC12m1 (B) or drug hypersensitive PC12m3 (C) cells were serum-starved, and treated (+) or not (−) for 10, 30 or 120 minutes with NGF (30 ng/ml), EGF (10 ng/ml) or FK506 (40 µM). After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospho-ERK1/2 antibody. Arrows labeled phospho ERK1 and phospho ERK2 indicate the position of the phosphorylated forms of ERK1 and ERK2 proteins.

Greene, 1978). In fact, NGF may function as a progression factor with both mitogenic and antimitogenic activities that allow cells to reach G1 before they differentiate (Rudkin, 1989). On the other hand, EGF is a mitogen for PC12 cells and stimulates proliferation in these cells without affecting neurite outgrowth (Huff et al., 1981), but FK506 is antimitogenic. Therefore, FK506 may synergize with NGF by inhibiting cell proliferation and allowing differentiation to occur in PC12m3 cells.

The present study demonstrated that FK506 failed to induce phosphorylation of MAP kinase in PC12 parental cells. This result suggested that the NGF-induced neurite outgrowth was not mediated by the FK506 related pathway in PC12 parental cells. All of these findings indicate that
FK506 may induce neurite outgrowth by activating the downstream target of MAP kinase or by a novel mechanism which is distinct from the NGF-activated pathway in PC12m3 cell differentiation. What defects contribute to the impaired responsiveness of PC12 mutant cells to NGF remains to be clarified.

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References


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