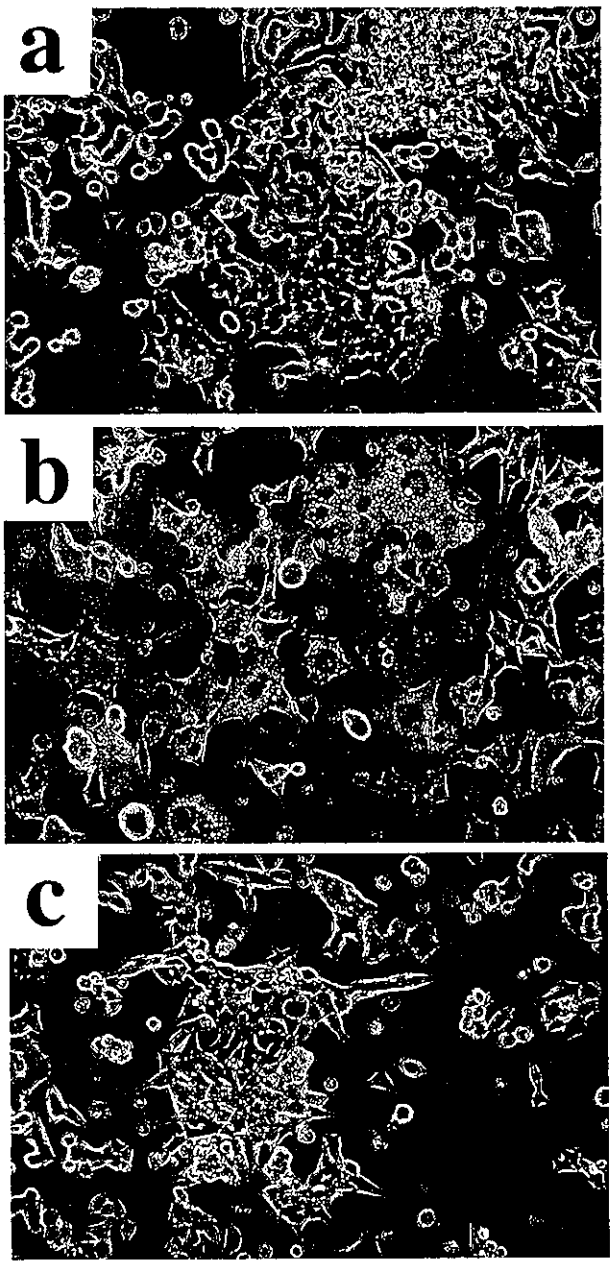

Fig. 1. Morphological changes in α7pCMV cells. (a) pCMV cells at 72 h after transfection. (b) α7pCMV cells at 72 h after transfection, showing marked neurite outgrowth, high migration capacity and adherence to the culture dish. (c) PC12 cells treated with nicotine for 72 h. Phase contrast photographs with original magnification of ×200.

pCMV cells (Fig. 1a) and PC12 cells+nicotine (Fig. 1c). The effects of α7 over-expression on the morphological changes in PC12 cells were not blocked by addition of 10 μM α-bungarotoxin (not shown).

3.2. Phosphorylation of ERKs and other MAPKs

Representative immunoblots for total ERKs (p44 and p42), phospho-ERKs (p44 and p42), FLAG (tag for α7 subunit protein) and actin (internal control) are shown in Fig. 2a. Expression of total ERKs did not change in any of the 3 groups after transfection or addition of nicotine to the medium. However, in α7pCMV cells, expression of phospho-ERKs and FLAG-tagged α7 subunit protein increased at 24 h after transfection, and these elevated levels were maintained from 24 to 72 h (Fig. 2a). Furthermore, α7 stably-expressing cells continued to exhibit phosphorylation of ERKs 7 days after transfection (Fig. 2a). These findings suggest that over-expression of α7 subunit protein immediately induced sustained ERK phosphorylation in PC12 cells. The effects of α7 over-expression on the sustained ERK phosphorylation were not blocked by addition of 10 μM α-bungarotoxin (not shown). However, whether more rapidly acting antagonists for nicotinic receptor than α-bungarotoxin such as mecamylamine could abolish these effects remains to be determined. In PC12 cells+nicotine, phospho-ERKs were transiently expressed at 48 h after addition of nicotine to the medium (Fig. 2a). At no time during the experiment was significant expression of phospho-ERKs detected in untreated PC12 cells or pCMV cells (Fig. 2a).

Because the mechanisms of activation of MAPK pathways are all somewhat similar [18], we also investigated phosphorylation of other members of the MAPK family (c-Jun-N-terminal kinase: JNK and p38). Representative immunoblots for JNK and p38, total and phosphorylated, are shown in Fig. 2b. Neither phospho-JNK nor phospho-p38 increased in α7pCMV or pCMV cells at any time after transfection (Fig. 2b). These observations were confirmed in three independent experiments.

3.3. Expression of N-cadherin

Representative histograms of surface *N*-cadherin expression for each cell line after transfection or addition of nicotine to the medium are shown in Fig. 3. Histograms for α7pCMV cells at 72 h after transfection and α7 stably-expressing cells treated with G418 both showed a cell population with elevated *N*-cadherin expression (Fig. 3, arrowhead), indicating that over-expression of α7 nAChR upregulated *N*-cadherin expression at a relatively late phase, 48 h after upregulation of phospho-ERK (Fig. 2a), and at the same time as onset of differentiation-like transformation (Fig. 1b). Neither pCMV cells nor PC12 cells+nicotine showed changes in *N*-cadherin expression at any time during the experiments.

4. Discussion

PC12 cells treated with NGF or bFGF have been widely used as a model for investigating the molecular mechanisms underlying neuronal differentiation and axon outgrowth [10,18]. Receptor tyrosine kinases activated by NGF or bFGF initiate a cascade of ras, raf-isoforms (Raf-

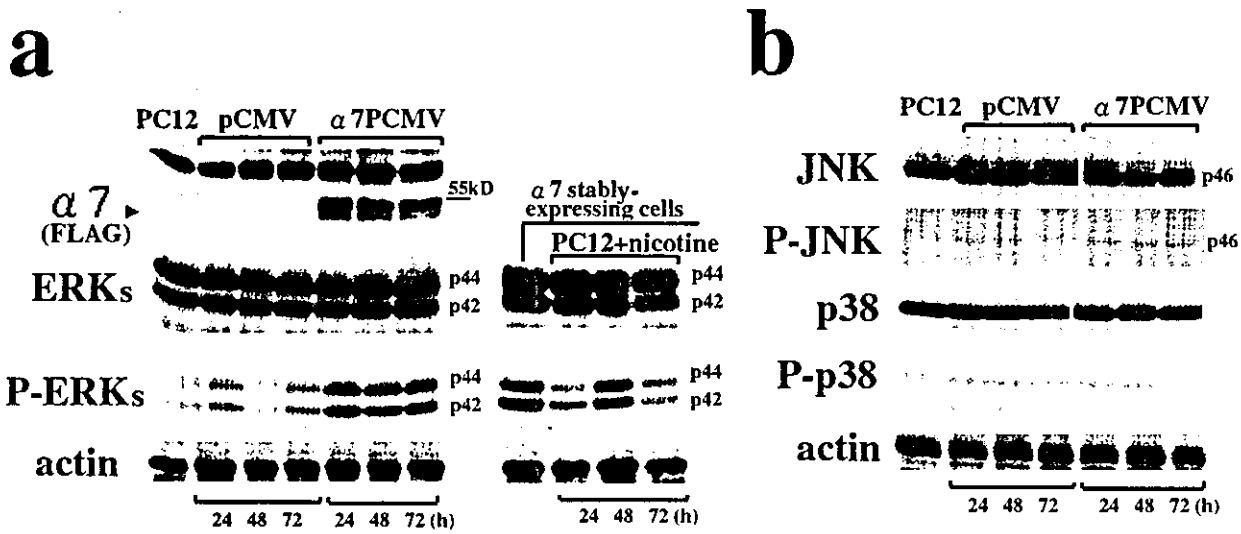

Fig. 2. (a) Representative immunoblots for total ERKs, phospho-ERKs, FLAG (tag for α7 subunit protein) and actin. Changes in expression of total ERKs (p42 and p44) are not evident in any of the 3 cell groups (pCMVcells, α7pCMV cells and PC12+nicotine cells) after transfection or addition of nicotine to the medium, or in α7 stably-expressing cells. In α7pCMV cells, phospho-ERKs (p42 and p44) and FLAG-tagged α7 subunit protein are clearly evident at 24 to 72 h after transfection. α7 stably-expressing cells selected from α7pCMV cells by treating with G418 for 3 days and maintaining for an additional 4 days also exhibited phosphorylation of ERKs. In PC12 cells+nicotine, phospho-ERKs are transiently expressed with at 48 h after addition of nicotine to the medium. Neither untreated PC12 cells nor pCMV cells showed significant expression of phospho-ERKs. (b) Representative immunoblots for both total and phosphorylated JNK and p38. There was no change in phospho-JNK or phospho-p38 in α7pCMV or pCMV cells.

1, B-Raf) and ERK, inducing neurite outgrowth and differentiation [30–32]. cAMP analogues activate protein kinase A (PKA), and this also leads to neurite outgrowth [30–32]. Although ERK signaling is reportedly independent of the cAMP/PKA cascade, depending on neuronal cell type [30], current hypotheses favor a final common pathway in which ras-dependent and cAMP/PKA-mediated signaling converge on the ERK pathway [31,32]. The sustained activation of ERK is crucial for its nuclear translocation and consequential activation of key transcription factors [18], and for induction of differentiation and neurite outgrowth by NGF in PC12 cells [18,21,31]. In the present study, the differentiation-like transformation of PC12 cells over-expressing α7 nAChR was probably induced by sustained activation of ERK. Although the present study did not address the mechanism of sustained ERK phosphorylation in the α7pCMV cells, it may probably be related to the finding that the activation of ERK started as immediately as expression of α7 subunit protein in the α7pCMV cells. Previous studies have reported a relationship between ERK activation and cytoskeletal reorganization [16,22] which may have some links to the cytoskeletal machinery necessary for sufficient expression of α7 nAChR consisting of α7 subunit proteins [3,8,24]. ERK signaling reportedly influences cyclin-dependent kinases [16,22] and is required for stress-induced G2-phase delay of the cell cycle [20], which is also induced by α7 nAChR over-expression [27]. Given that the effects of α7 over-expression on ERK phosphorylation and neurite outgrowth were not blocked by addition of α-bungarotoxin, and that nicotine only induced transient expression of phospho-ERKs after some delay (48 h after administration) and did not induce neurite outgrowth in the present study, the sustained ERK phosphorylation that occurred shortly (24 h) after α7 transfection in α7pCMV cells was probably independent of agonistic stimulation. Although the FLAG at the C-terminal of α7 subunits is small (8 amino acids), the possibility that the tagging peptides contributed to the results obtained for α7pCMV cells can not be completely ruled out. Activation of α7 nAChR may promotes quick Ca^{2+} influx [4,29], which is also a common component of many pathways promoting axonal growth [10,18]. However, α7 nAChR should quickly be desensitized after agonist binding [7,29]. Furthermore, naive PC12 cells are not competent to mount a neurite outgrowth response to Ca^{2+} influx [10], a conclusion consistent with the present finding that nicotine alone did not induce neurite outgrowth. However, we can not rule out the possible role of Ca^{2+} in ERK activation and axonal growth in PC12 cells. Although the mechanism and significance of delayed effects of nicotine on transient ERK activation were not investigated in the present study, transient ERK activation possibly is not sufficient enough to induce *N*-cadherin expression and neurite outgrowth in PC12 cells.

Previous studies have reported that *N*-cadherin is essential for neurite outgrowth and differentiation [10,12,22,23,28], and that *N*-cadherin overexpression is sufficient to initiate neuronal differentiation in P19 cells [12]. *N*-cadherin is coprecipitated with fibroblast growth factor (FGF) receptor, and structural studies of the FGF

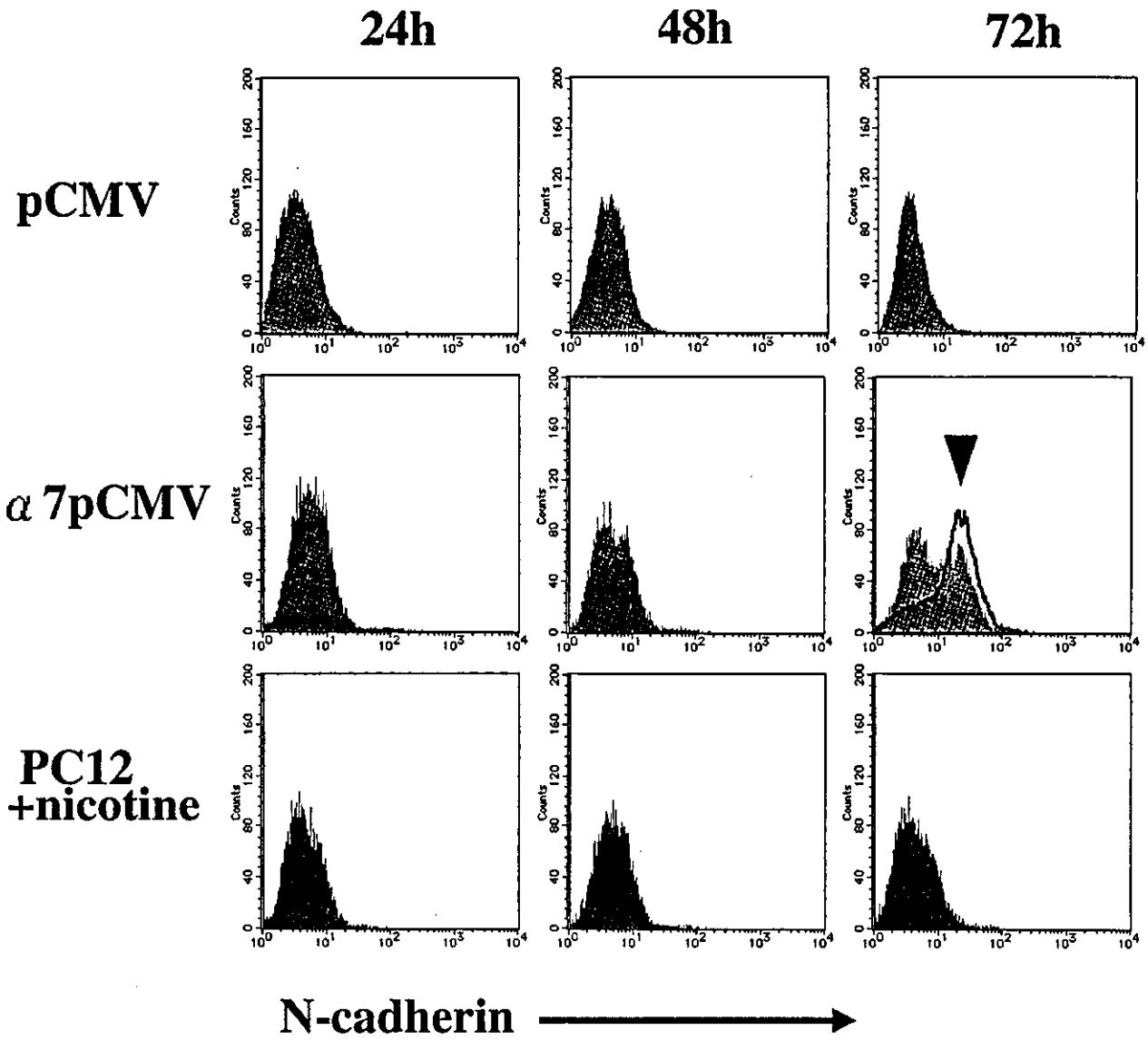

Fig. 3. Representative histograms of *N*-cadherin expression on cell surface in each cell line after transfection or nicotine treatment. The histogram of α7pCMV cells at 72 h after transfection shows 2 peaks of cell population with *N*-cadherin expression (filled histogram). The right peak (filled histogram, at arrowhead) shows a higher *N*-cadherin expression level that is almost identical to that of α7 stably-expressing cells treated with G418 (open thick-lined histogram, at arrowhead). Changes in *N*-cadherin expression are not evident on pCMV cells or PC12 cells+nicotine.

receptor support the hypothesis of a direct *N*-cadherin-FGF receptor interaction [10,28]. *N*-cadherin can activate receptor tyrosine kinases and ERK, and induce neurite outgrowth [22,23]. However, in the present study, both differentiation-like transformation and up-regulation of cell-surface *N*-cadherin expression were observed relatively late after α7 transfection, and much later than the initiation of ERK phosphorylation. These findings suggest that sustained ERK activation may, in addition to differentiation-like transformation, induce surface *N*-cadherin expression in PC12 cells, which has yet to be confirmed.

ERK signaling systems are involved in essential elements of neuronal development such as the initiation of neurite outgrowth and rearrangement of neurites [25]. In fact, expression of α7 nAChR has been studied primarily in developing thalamocortical systems [2,5,6], in which α7 nAChR was transiently upregulated in cortical neurons associated with transient ingrowing of afferents [2,6]. α7 nAChR is assumed to modulate the plasticity of neuronal circuitry [2,5,6]. Although PC 12 cells differ somewhat from mature neurons in the brain, and the significance of sustained ERK activation may differ depending on cell type [10], the present findings provide the possibility that the linking between the expression machinery of α7 nAChR and ERK signaling can be a target for further study of the systems that modulate neuronal circuitry.

In conclusion, over-expression of α7 nAChR, independent of agonistic stimulation, induces sustained ERK phosphorylation which probably promotes differentiation-like transformation and *N*-cadherin expression in PC12 cells.

Acknowledgements

We wish to thank Doctor Taiju Utsugisawa for his technical advice, and Ms. Sakuyo Nagai for her technical assistance. This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Hypoxic condition interferes with phosphorylation of Akt at Thr^{308} in cultured rat pheochromocytoma-12 cells

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Received 26 July 2002; received in revised form 14 August 2002; accepted 19 August 2002

Abstract

Phosphorylation of Akt induced under hypoxic or ischemic conditions has been reported only for residue Ser^{473}. We examined whether Akt can be phosphorylated at Thr^{308}, another phosphorylation site on Akt, and can exhibit neuroprotective effects under conditions of hypoxia /reoxygenation, comparing pheochromocytoma-12 (PC12) cells transfected with constitutively active Akt (Myr-pCMV cells) and those transfected with pCMV vector only (pCMV cells). Expression levels of Akt phosphorylated at Ser^{473} were 2.1-fold higher in Myr-pCMV cells compared with pCMV cells, before the onset of hypoxia, which were increased transiently during hypoxia, and then decreased gradually during reoxygenation. In contrast, Akt phosphorylated at Thr^{308} was not detected in pCMV cells under any conditions but was expressed in Myr-pCMV cells prior to hypoxia, followed by an immediate decrease during hypoxia and a further decline during reoxygenation. However, G1-arrest of the cell cycle observed at 12 h after hypoxia in pCMV cells was prevented in Myr-pCMV cells. These findings suggest that hypoxia activates Akt by phosphorylation at Ser^{473} only, which is sufficient to elicit a neuroprotective function against hypoxic neuronal damage.

Keywords: Akt; Cell cycle; Cell death; G1-arrest; Hypoxia; Pheochromocytoma-12 cells; Phosphorylation; Myristoylation signal

Akt (protein kinase B:PKB) is a serine/threonine kinase functioning downstream of phosphatidylinositide 3-kinase (PI3-K), which targets and activates several proteins that act to prevent cell death. The PI3-K–Akt pathway is activated by various cellular stresses including hypoxia and ischemia as well as growth factor-mediated stimuli [6]. Expression levels of both PI3-K and Akt increase in the early stages of reperfusion following ischemia in rabbit spinal cord [16]. Activation of Akt is also induced by a sublethal ischemic insult in gerbil and rat hippocampus [14,21]. Activation of Akt rescues neurons from cell death in gerbil hippocampus after ischemia, which is abolished by wortmannin, an inhibitor of PI3-K, suggesting that PI3-K activity is probably required for Akt-mediated neuroprotection [21]. Although phosphorylation of both residues Ser^{473} and Thr^{308} is generally known to be required for full activation of Akt [3,13], Akt seems to be phosphorylated only at residue Ser^{473} after ischemic condition in rat hippocampus [14] and during the course of hypoxia/reoxygenation in PC12 cells [19]. Akt phosphorylation at Thr^{308}, another phosphorylation site, has not been observed under both normal and ischemic/hypoxic conditions in neurons [14] and neuron-like cells [19]. The mechanisms for the lack of Akt phosphorylation at Thr^{308} [14,19] remain to be determined. Akt can be constitutively activated and increase basal phosphorylation levels of both Ser^{473} and Thr^{308} by an attachment of the myristoylation signal [13] at the N-terminus which targets Akt to the plasma membrane [13]. To investigate whether Thr^{308} on Akt with the myristoylation signal [13] at the N-terminus can be phosphorylated in PC12 cells in the course of hypoxia/reoxygenation, we compared PC12 cells over-expressing Akt with the src myristoylation signal at the N-terminus and control PC12 cells transfected with vector only.

Full-length murine Akt1 cDNA [5] was obtained from a murine cDNA library (TaKaRa Bio) by polymerase chain reaction (PCR) using *Pyrococcus furiosus* (Pfu) polymerase (Promega). The start methionine of Akt was removed. Myr-Akt cDNA (Akt cDNA attached with the src myristoylation signal (Myr) sequence [3,13] at the 5′ end) was created also by PCR with the Akt cDNA as template. The PCR products were separated electrophoretically, purified and treated with HindIII and XhoI, then inserted into the multiple cloning site of the cytomegalovirus promoter (pCMV)-Tag4 expression vector (C-terminal FLAG tagging vector) (Stratagene) using a Rapid DNA Ligation Kit® (Boehringer Mannheim).

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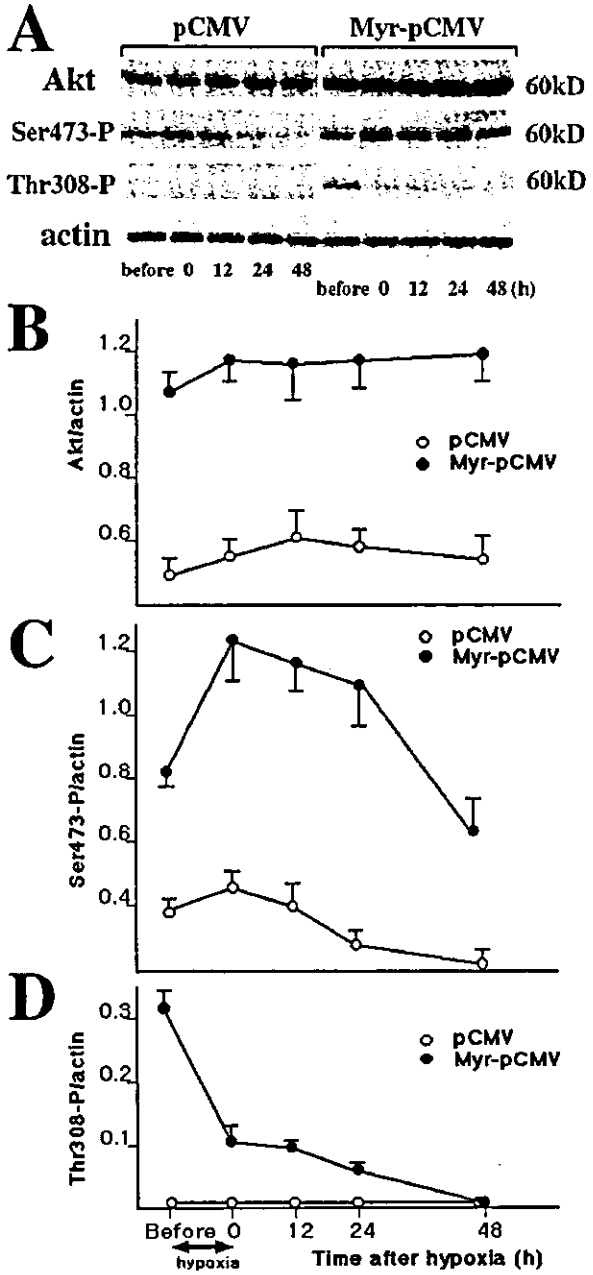

Fig. 1. (A) Representative immunoblots showing expression of total Akt, Akt phosphorylated at Ser^{473} (Ser^{473}-P), Akt phosphorylated at Thr^{308} (Thr^{308}-P) and actin, as a function of time. (B–D) Semi-quantitative determinations of total Akt (B), Ser^{473}-P (C) and Thr^{308}-P (D), relative to actin, as a function of time. Each data point represents the mean value ± SEM ($n = 3$).

The transformed competent cells (Epicurian coli® XL-2 blue: Stratagene) containing ligation constructs (Myr-Akt-pCMV plasmid) were streaked onto Luria-Bertani medium (LB)-kanamycin-agar plates. The accuracy of the Myr-Akt-pCMV plasmid was confirmed by restriction analysis and full sequencing for the inserted Myr-Akt cDNA using primer extension methods (TaKaRa Bio).

PC12 cells (JCRB 0733; Human Science Research Resource Bank, Osaka, Japan) were transfected with Myr-Akt-pCMV plasmid or pCMV vector only (without Myr-Akt cDNA) using Effectene™ Transfection Reagent (Qiagen). At the first subculture, 48 h after transfection, culture medium was supplemented with 0.75 mg/ml of G418 (Calbiochem) to positively select for stable integrants. PC12 cells transfected with Myr-Akt-pCMV plasmid (Myr-pCMV cells) and PC12 cells transfected with pCMV vector only (pCMV cells, for control) were exposed to hypoxia using a multigas incubator (Sanyo MCO-175M). Briefly, the cells were plated at a low density ($5 \times 10^4/cm^2$) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and without G418 for 24 h in culture dishes, then they were placed into the multigas incubator (2.0% O_2, 5% CO_2, balance N_2) at 37 °C for 12 h [19]. In a previous study, under almost identical experimental conditions, partial oxygen pressure in the medium reportedly decreased to approximately 20 mmHg at 60 min, and then remained at this level thereafter [10]. The cells were then returned to normoxic atmospheric conditions for subsequent analysis at 0, 12, 24 and 48 h after the hypoxia.

Immunoblotting and detection were performed as described elsewhere [19]. The chemiluminescence signals from the CCD camera were integrated for 30 s into the computer memory directly from the membranes using the Chemi Doc system (Bio Rad). The density of each pixel and the number of pixels were measured and calculated. A density equal to or lower than the background was eliminated. Primary antibodies used were anti-total PKB/Akt (Transduction Laboratories), anti-phospho-Akt (phosphorylated at Thr^{308}) (New England Biolabs, Inc.), anti-phospho-Akt (phosphorylated at Ser^{473}) (New England Biolabs, Inc.), and anti-actin (Progen).

In order to determine the populations of G1- and G2-phase cells, cells were immediately semi-fixed with 70% ethanol in phosphate-buffered saline (PBS), stained with propidium iodide (PI), and monitored for DNA content by fluorescent activated cell sorter (FACS) [19]. Analysis was performed for 5×10^4 cells.

Representative immunoblots for total Akt, Akt phosphorylated at Ser^{473}, Akt phosphorylated at Thr^{308} and actin (as inner control) are shown in Fig. 1A, and semi-quantitative determinations of expression levels of these proteins, relative to actin, are shown as a function of time in Fig. 1B–D, respectively. In pCMV cells, expression of total Akt relative to actin only modestly increased up to 12 h reoxygenation (Fig. 1B). In Myr-pCMV cells, the basal expression levels of total Akt were approximately 2.2-fold higher than in pCMV cells, which were also modestly upregulated during hypoxia and reoxygenation (Fig. 1B). Expression levels of Akt phosphorylated at Ser^{473} were approximately 2.1-fold higher in Myr-pCMV cells compared with pCMV cells before the onset of hypoxia, which further increased during hypoxia, and then gradually decreased to the basal levels (Fig. 1C). The upregulation of Akt phosphorylated at Ser^{473} after hypoxia was modest in pCMV cells (Fig. 1C). Akt phos-

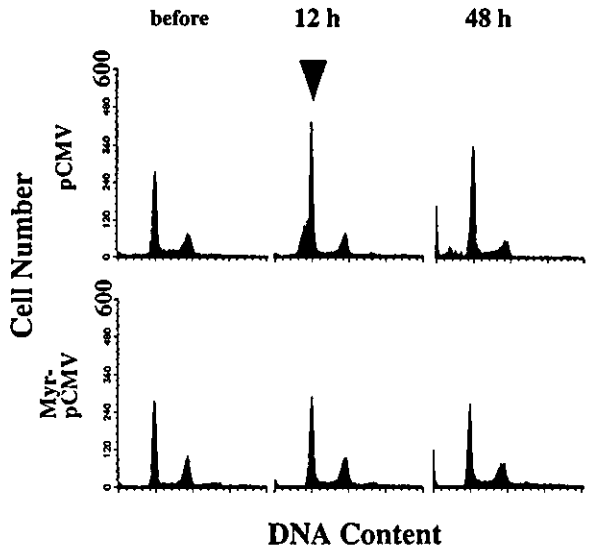

Fig. 2. Representative histograms of DNA content, indicating the proportion of cell population in each cell cycle phase, after hypoxia. The arrowhead in pCMV cells at 12 h after hypoxia (top) indicates a rise in the peak suggesting G1-arrest. Myr-pCMV cells do not show G1-arrest (bottom).

phorylated at Thr^{308} was not detected in pCMV cells at any period during the examination, but was expressed in Myr-pCMV cells before hypoxia, which, however, was immediately downregulated during hypoxia, and then diminished at 48 h reoxygenation (Fig. 1D).

Representative histograms of DNA content, indicating the proportion of each cell cycle phase, for both cell lines, after hypoxia, are shown in Fig. 2. pCMV cells showed a higher peak representing the G1 population compared with other peaks, suggesting G1-arrest, at 12 h after hypoxia (Fig. 2, arrowhead), and at 48 h the sub-G1 population (possibly corresponding to apoptotic cells) was increased (Fig. 2, top). However, in Myr-pCMV cells, the G1 population remained unaltered (Fig. 2, bottom).

The present study showed that expression of both Ser^{473}- and Thr^{308}-phosphorylated Akt were upregulated in Myr-pCMV cells than pCMV cells before hypoxia. However, while expression of Ser^{473}-phosphorylated Akt was transiently increased during hypoxia/reoxygenation in both pCMV and Myr-pCMV cells, expression of Thr^{308}-phosphorylated Akt steeply decreased in Myr-pCMV cells, and was absent in pCMV cells throughout the observation periods. These findings suggest that Akt can be phosphorylated at Thr^{308} also in PC12 cells, but this is altered with hypoxic conditions. The mechanisms of phosphorylation of Akt have been studied primarily in growth factor-mediated PI3-K activation [1,8]. Activation of PI3-K results in an increase in phosphatidylinositol-3,4,5-triphosphate [PI (3,4,5) P3] activity, which induces translocation of Akt to the plasma membrane by its binding to the pleckstrin homology (PH) domain. This event leads to a conformational change of Akt which alters the phosphorylation sites (Thr^{308}, Ser^{473}) accessible to 3-phosphoinositide-dependent protein kinases (PDKs) [2,6,9,17,18]. Phosphorylation of Thr^{308} is catalyzed by PDK1 [4,6,20] through interaction with a fragment of protein kinase C-related kinase-2, the PDK1-interacting fragment (PIF) [4]. The PDK1/PIF complex can phosphorylate Akt at both Thr^{308} and Ser^{473} residues, resulting in full activation of Akt [4]. However, in cells derived from a $PDK1^{-/-}$ mouse, Akt can be phosphorylated only at Ser^{473} but not Thr^{308} after insulin-like growth factor-1 treatment [20], suggesting that PDK1 is essential only to phosphorylation at Thr^{308} but not at Ser^{473}. Although the mechanisms of phosphorylation of Ser^{473} remain to be further studied, the two sites are likely to be independently regulated. Given that PDK1 is localized at the inner surface of the plasma membrane [6], and that translocation of Akt induced by the myristoylation signal to the plasma membrane is sufficient to lead to full activation of Akt for both wild-type Akt and mutant Akt without PH domain [13], the normality of membrane structure and functions may be important for phosphorylation of Akt at Thr^{308}. In the early phase of hypoxic neuronal damage, disruption of membrane potential results in depolarization and subsequent Ca^{2+} influx and intracellular Ca^{2+} overload, which in turn causes lipid peroxidation and membrane damage via phospholipase A_2 and cyclooxygenase [7]. These early features of hypoxic membrane damage may impair PDK1 functions and suppress phosphorylation of Akt at Thr^{308}, although the Ca^{2+}-triggered cascade can also influence neuroprotective signaling which includes the Akt pathway [22].

The present study revealed that G1-arrest after hypoxia was prevented in Myr-pCMV cells, despite the fact that Akt phosphorylation at Thr^{308} was immediately down-regulated during hypoxia and then diminished. Apoptosis is associated with checkpoints and their related molecules in the cell cycle [23]. In response to hypoxia, G1-phase checkpoints of the cell cycle are activated and p53 accumulates [11]. In our previous study, PC12 cells exhibited an increase in G1 population, a hallmark of G1-arrest, at 12 h post-hypoxia, before DNA fragmentation [19]. In an ischemic animal model, DNA fragmentation in spinal motor neurons might be associated with molecules related to G1-checkpoints [15]. Akt can promote the cell cycle progression by activation of p70 S6 kinase, which is required for ribosomal protein synthesis [12,24]. These findings suggest that Akt is activated only at Ser^{473}, which still has a neuroprotective function against hypoxia/reoxygenation in PC12 cells. Although PC12 cells differ somewhat from mature neurons in the human brain, the present study provides an important implication to further investigate the role of Akt as a candidate target for treatment against hypoxia.

In conclusion, under hypoxia/reoxygenation conditions, Akt was phosphorylated at Ser^{473}, but not at Thr^{308}, and prevented G1-arrest of the cell cycle in PC12 cells, suggesting that partially activated Akt can exhibit a neuroprotective function against hypoxic neuronal damage at least in PC12 cells.

We wish to thank Dr Taiju Utsugisawa for his technical advice, and Ms. Sakuyo Nagai for her technical assistance. This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Eur Neurol 2002;48:115–117

The Effect of Combined Therapy with Immunoadsorption and High-Dose Intravenous Methylprednisolone on Myasthenia gravis

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Myasthenia gravis (MG) is an autoimmune disease mediated by antibodies to the acetylcholine receptor of skeletal muscle (anti-AChR Ab) [1, 2]. Although the efficacy of plasmapheresis on MG had been reported only in a relatively small-scale controlled study for severe cases of MG [3], a significant reduction in serum anti-AChR Ab titer following plasmapheresis has been considered to be related to the efficacy of treatment [4–9]. However, the effect of plasmapheresis on clinical symptoms of MG did not continue, and the serum anti-AChR Ab titer increased relatively soon (within 1 month) after treatment [4, 7]. In other reports, addition of high-dose intravenous methylprednisolone therapy (HMP) immediately after plasmapheresis might have prevented the early phase deterioration in case studies with MG [10, 11]. Recently, particularly in Japan, the immunoadsorption method (IA) has been the preferred treatment for MG because of a lower rate of serum albumin loss and side effects to the circulating system, and because the effect is almost identical to plasma exchange or the double-filtration method [12]. We compared short- (1 month after each treatment) and long-term (serial treatments during 52–1,240 days) effects of combination therapy with IA and HMP (IA + HMP) and treatment with IA alone on clinical severity, titer of anti-AChR Ab and dose of oral prednisolone (PSL) needed for normal life in 29 patients with MG. Informed consent was obtained from all the patients. Statistical analysis was performed using the unpaired t test.

Backgrounds of the MG patients studied are shown in table 1. Thirty-four patients were consecutively enrolled into the study. Initially, they were randomly allocated to receive IA + HMP or IA alone. However, as the patients receiving IA alone (12 cases) did not suffi-

Table 1. Patient profiles

	Type of therapy	
	IA + HMP (n = 22)	IA (n = 7)
Age, years	40.2 ± 18.6 (n.s.)	36.7 ± 25.1
Gender (M/F)	6/16 (n.s.)	2/5
Classification (I/IIa/IIb)	3/10/9 (n.s.)	1/3/3
Anti-AChR-Ab-positive cases, %	68 (15/22) (n.s.)	71 (5/7)
Thymectomy, %	77 (17/22)	86 (6/7)
Thymic histology (remnant/hyperplasia/thymoma)	6/8/3	3/3/0

n.s. = Not significant. Classification according to Osserman [13].

Table 2. Comparison of the effect of combination therapy with IA + HMP and treatment with IA alone

	Type of therapy	
	IA + HMP (n = 22)	IA (n = 7)
Before treatment		
Severity score	7.61 ± 5.53	7.02 ± 4.50
Dose of oral PSL, mg	20.8 ± 11.2	22.1 ± 10.6
One week after each treatment		
Severity ratio	0.58 ± 0.31* (mean of 103 times)	0.86 ± 0.28 (mean of 32 times)
Anti-AChR Ab ratio	0.73 ± 0.33 (mean of 62 times)	0.68 ± 0.18 (mean of 19 times)
PSL dose ratio	1.01 ± 0.29 (mean of 103 times)	1.05 ± 0.17 (mean of 19 times)
One month after each treatment		
Severity ratio	0.47 ± 0.30** (mean of 103 times)	0.90 ± 0.33 (mean of 28 times)
Anti-AChR Ab ratio	0.43 ± 0.34** (mean of 62 times)	0.99 ± 0.32 (mean of 17 times)
PSL dose ratio	0.92 ± 0.32 (mean of 103 times)	0.97 ± 0.21 (mean of 17 times)
After total observation period		
Number of therapy (times)/total observation period (days)	4.2 ± 2.3/634.3 ± 402.0	3.8 ± 3.1/109.8 ± 70.1
Severity ratio	0.29 ± 0.31**	0.72 ± 0.32
Anti-AChR Ab ratio	0.38 ± 0.27**	0.70 ± 0.23
PSL dose ratio	0.58 ± 0.29**	1.03 ± 0.61

* $p < 0.05$, ** $p < 0.01$ (unpaired t test) compared with IA alone; the other IA + HMP data are not significant.

ciently respond to the treatment, they needed other therapies (intermittent HMP alone or IA + HMP) in the early phase of the study. Five of such cases in whom other therapies were required within 1 month after the first treatment with IA alone were excluded from the study. Thus, the final analysis was performed for 22 cases of IA + HMP (total observation period, 155–1,324 days) and 7 cases of IA alone (33–202 days). These patients had received medications of 10–40 mg/day of PSL together with 60 or 120 mg of pyridostigmine bromide/day before receiving IA. IA was performed using a plasma separator, OP-08® (Asahi Medical, Tokyo, Japan), and an adsorption column, TR-350® (Asahi Medical), through which 2,000 ml of plasma separated from the patient's blood passed [8, 12]. In the TR-350 col-

umn, a tryptophan-linked affinity immunoadsorbent semiselectively removes anti-AChR Ab. HMP was performed by serial intravenous injection of 1.0 g methylprednisolone/100 ml saline immediately after IA and on the mornings of the following 2 days. Clinical severity was strictly scored by the patients and the participating medical staff (not blinded to the treatment), according to the methods of Schumm et al. [14] and Besinger et al. [15] (excluding measurements of vital capacity) for ocular symptoms and generalized symptoms, respectively. The ratios (after/before the treatments) of the clinical severity score, the anti-AChR Ab titer among the patients showing elevated values and the dose of oral PSL per day required for normal life (together with 60 or 120 mg of pyridostigmine bromide/day) were calculated for individual patients (table 1).

One week and 1 month after each treatment, the mean severity ratio was significantly lower in the IA + HMP than the IA alone group ($p < 0.05$, $p < 0.01$; table 2). The mean anti-AChR Ab ratio was significantly lower in the IA + HMP than IA alone groups at 1 month ($p < 0.01$) after each treatment (table 2). Both the severity and anti-AChR Ab ratios increased within 1 month after IA alone, which was consistent with a previous report [4]. These findings suggest that IA + HMP produce more immediate effects on MG for up to 1 month compared with IA alone. The mean PSL dose ratio did not differ between the IA + HMP and IA alone groups, at least within 1 month (table 2).

After the total observation period, both the severity and anti-AChR Ab ratios were significantly lower in patients receiving IA + HMP than in patients receiving IA alone (0.29 ± 0.31 vs. 0.72 ± 0.32 and 0.38 ± 0.27 vs. 0.70 ± 0.23, respectively, $p < 0.01$; table 2). Although the total observation period was longer for the patients receiving IA + HMP than IA alone (table 2), no significant correlation was observed with severity ($r = 0.20$, $p = 0.48$), anti-AChR Ab ($r = -0.22$, $p = 0.42$) and PSL dose ratios ($r = -0.09$, $p = 0.77$). These results indicate that IA + HMP for a longer interval (mean, 151 days) is more effective than IA alone for a shorter interval (mean, 29 days). Although we did not compare the effects of IA + HMP and HMP alone in the present study, previous studies have reported that the effect of HMP continued for only 4–14 weeks [16], and that even frequent repetition of HMP (every fifth day intravenous administration of 2,000 mg of methylprednisolone) did not result in complete remission of MG symptoms [17]. In contrast, 4 of the present patients receiving IA + HMP demonstrated remission (no symptom with no medication for more than 1 year). Therefore, combined therapy with IA + HMP appears more effective than IA alone or, in all likelihood, HMP alone. Furthermore, the PSL dose ratio was significantly lower in patients receiving IA + HMP than in patients receiving IA alone (0.58 ± 0.29 vs. 1.03 ± 0.61, $p < 0.01$) after the total observation period (table 2), suggesting that IA + HMP treatment may allow for a reduced dose of oral PSL per day for normal life in MG patients. As the combined therapy with IA + HMP exhibits no serious complications, it can be used not only to overcome acute deterioration as plasma exchange but also to reduce the dose of oral PSL and aim at remission. The anti-AChR Ab ratio, together with clinical severity, can be an indicator for the therapeutic response to IA or IA + HMP for individual patients.

In conclusion, the combined therapy with IA and HMP appears to be more effective for the treatment of MG compared with IA alone, although further studies are needed to compare the effect of IA + HMP and HMP alone.

Acknowledgments

This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Neuroscience Letters 336 (2003) 105–108

Neuroscience Letters

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Alteration of 8-hydroxyguanosine concentrations in the cerebrospinal fluid and serum from patients with Parkinson's disease

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Received 26 September 2002; received in revised form 17 October 2002; accepted 19 October 2002

Abstract

In order to investigate the possible role of oxidative RNA damage in the pathogenesis of Parkinson's disease (PD), the concentrations of the oxidative stress marker 8-hydroxyguanosine (8-OHG) were measured in the cerebrospinal fluid (CSF) and the serum of patients with PD and control subjects. The concentration of 8-OHG in CSF in PD patients was approximately three-fold that in controls ($P < 0.001$). The concentration of 8-OHG in CSF decreased significantly with the duration of disease ($r_s = -0.46$, $P < 0.05$). However, the concentration of 8-OHG in serum was not significantly altered in PD patients compared to that in controls. In addition, the concentration of 8-OHG in CSF showed no correlation with that in serum in both the controls and PD patients suggesting that the 8-OHG concentrations in the CSF do not reflect those in serum and may be probably reflect those in brain tissue. These in vivo findings suggest a possible role of 8-OHG and increased oxidative RNA damage in the early stage of the development of PD.

Keywords: Parkinson's disease; 8-hydroxyguanosine; RNA; Oxidative stress; Cerebrospinal fluid

Parkinson's disease (PD) is characterized pathologically by progressive degeneration of dopaminergic neurons in the sabstantia nigra. The cause of dopaminergic neurodegeneration in PD still remains unknown. Recently, several studies have established an association of oxidative stress with PD [2,5,17,27]. Oxidative damage results from impaired oxidative balance in which reactive oxygen production exceeds cellular antioxidant defenses, leading to damage to proteins, lipids, and nucleic acids. The free radical theory of aging [3,9] suggests a major role for oxidative stress in age-related cellular dysfunction. The central nervous system is particularly vulnerable to oxidative damage because of its higher energy requirements, higher oxygen consumption rate, and less active antioxidant defense systems compared with other organs [8]. In addition, the dopaminergic neurons are particularly exposed to oxidative stress because the metabolism of dopamine, whose concentration is high in the substantia nigra, can lead to formation of H_2O_2, which in the presence of ferrous iron can give rise to the highly toxic hydroxyl radical [10,13].

8-Hydroxyguanosine (8-OHG), a good marker of hydroxyl radical damage to RNA, has been found in a wide variety of disorders and after various environmental insults, and 8-OHG has been proposed as a useful systematic marker of oxidative stress [7]. Since RNA is turned over rapidly, the level of 8-OHG reflects the steady state oxidative balance at the time of its determination, rather than the history of oxidative damage [4,20]. Recent histochemical studies showed a marked accumulation of 8-OHG cytoplasmic RNA within the cerebral neurons of patients with PD. [17,27]. However, quantitative analysis of 8-OHG in the cerebrospinal fluid (CSF) or brain tissue of PD patients has not been reported. In order to investigate the potential role of oxidative stress and oxidation of RNA in the pathogenesis of PD, we measured the concentrations of 8-OHG in the CSF and the serum of patients with PD and investigated its relationships to the duration and severity of disease.

Subjects were 24 untreated patients with PD [12 males and 12 females, age 63.3 ± 10.5 years (mean ± SD)] and 15 controls [seven males and eight females, age 62.3 ± 9.4 years (mean ± SD)]. Controls were neurologically normal patients who underwent lumbar anesthesia for minor surgery. Diagnosis of PD was made according to Koller's criteria [11]. The severity of disease was assessed based on

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0304-3940/02/$ - see front matter © 2002 Elsevier Science Ireland Ltd. All rights reserved.
doi:10.1016/S0304-3940(02)01259-4

the Hohen and Yahr scale. In patients with PD, duration of the disease was 2.8 ± 2.0 years (mean ± SD), and the average mean Hohen and Yahr scale was 2.5 ± 1.0 (mean ± SD). Informed consent was obtained from all the patients or their families.

CSF and blood were obtained between 9:00 and 10:00 AM after overnight bed-rest and before breakfast. For serum preparation, venous blood samples were centrifuged at 1000 *g* for 5 min at 4 °C. CSF and serum samples taken from the patients were rapidly frozen and stored at −80 °C until assayed. The free 8-OHG concentration was determined according to the method of Shigenaga et al. [21] with modifications. Briefly, 1 ml of CSF or serum was absorbed in a solid-phase extraction cartridge (Bond Elut C18 3 ml/200 mg: VARIAN, Harbor City, CA) and eluted by 1.5ml of methanol. The eluate was concentrated with a centrifugal evaporator and dissolve in 100 μl of distilled water. After filtration with a 0.45 μm membrane filter, 40 μl of the solution was analyzed using high-performance liquid chromatography (MCM C18 reversed phase column 250 × 4.6 mm; MC Medical, Tokyo, Japan) with an electrochemical detector (Coulochem II Model 5200; ESA Inc., Bedford, MA). The mobile phase consisted of 10 mM NaH_2PO_4 and 6% (for CSF) or 4% (for serum) methanol. The electrode potentials were maintained at 0.3 V for the guard cell, 0.15 V for detector I, and 0.25 V for detector II. The flow rate was 1.0 ml/min and the column temperature was kept at 20 °C. The limit of detection for 8-OHG was 20 pM. The 8-OHG standard was obtained from Cayman Chemical Co. (Ann Arbor, MI).

Statistical analysis was performed using a nonparametric Mann–Whitney *U*-test or the Spearman rank correlation coefficient (r_s). The significance level was set at $P < 0.05$.

The concentration of 8-OHG in CSF of control subjects ranged from 40 pM to 140 pM. There was no significant correlation of 8-OHG concentration with age ($r_s = -0.25$, $P = 0.35$). The concentration of CSF 8-OHG was significantly and remarkably increased in patients with PD compared with the controls (288 ± 129 pM vs. 97 ± 32 pM; $P < 0.001$, Fig. 1a). The concentrations of 8-OHG in the CSF of PD patients showed a significant negative correlation with the duration of disease ($r_s = -0.46$, $P < 0.05$, Fig. 1b), but did not show a significant correlation with Hoehn and Yahr scale ($r_s = -0.25$, $P = 0.23$). The concentration of 8-OHG in serum in PD patients (1.49 ± 0.54 nM) was not significantly altered compared to that in controls (1.42 ± 0.59 nM). In addition, the concentration of 8-OHG in CSF was not correlated with that in serum in both the controls ($r_s = -0.10$, $P = 0.71$) and PD patients ($r_s = -0.06$, $P = 0.76$). Cell counts (0.5 ± 0.7 /mm^3) and protein concentrations (26.7 ± 6.2 mg/dl) in CSF in PD patients were within normal range. The concentration of CSF 8-OHG showed no significant correlation with cell counts ($r_s = 0.10$, $P = 0.63$) nor with protein concentration ($r_s = 0.13$, $P = 0.55$).

The present study showed the significant three-fold

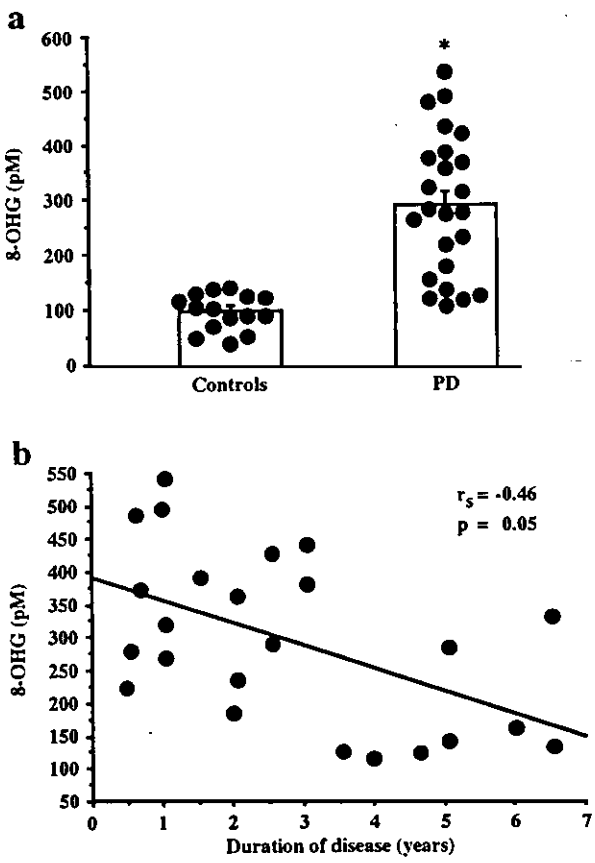

Fig. 1. (a) Concentrations of 8-OHG in the cerebrospinal fluid from patients with PD and controls (Scattergram and mean ± SEM). *$P < 0.001$ compared with controls using the Mann–Whitney *U*-test. (b) Concentrations of 8-OHG in PD patients as a function of duration of disease. rs, the Spearman rank correlation coefficient.

increase in 8-OHG concentrations in the CSF of PD patients compared with controls and the negative correlation between the concentration of 8-OHG and the duration of disease. The concentration of 8-OHG in the CSF may in most part reflect those in the brain rather than blood, because 8-OHG is hydrophilic. The concentrations of 8-OHG in serum did not significantly differ between controls and PD patients. Further, the concentrations of 8-OHG in serum showed no correlation with those in CSF in both the controls and PD patients.

In PD patients, previous studies on postmortem tissues have provided some evidence to support the occurrence of oxidative stress, including increased levels of iron [6], decreased levels of reduced glutathione [18], and increased oxidative products of lipid, protein, and DNA [1,2,26]. Our data suggest the possibilities that RNA oxidation is abnormally accelerated in the cerebral tissue of PD patients and that increased oxidation of RNA occurs in the early stages of PD. These findings are consistent with those of previous reports on semi-quantitative immunohistochemical studies using anti-8-OHG monoclonal antibody in autopsy brains

[17,27]. These previous studies reported that 8-OHG immunoreactivity was increased significantly in the substantia nigra neurons of PD brains compared with the controls. Although we cannot specify the brain location with increased 8-OHG production, the possibility that the present findings reflect the increase of RNA oxidative stress in the substantia nigra cannot be ruled out.

RNA is more vulnerable to oxidative stress than DNA both in vitro and vivo [7,24], probably because unlike nuclear DNA, RNA is single-stranded and is not covered with protective histones. The relative paucity of oxidative damage to DNA may be explained by DNA repair mechanism, while the only known compensation for increased oxidation of RNA is its higher turn over rate [25]. The fidelity of RNA, a molecule that can be viewed as the disposable soma of genetic information, has been much less studied than that of DNA, which is the heritable germ line. However, there may be unidentified RNA repair mechanism that are involved in important RNA modifications such as editing and splicing [22]. Recent studies have reported transcriptional mutation of RNA in vulnerable neurons of Alzheimer's disease (AD) and Down's syndrome [23] as well as aberrant RNA, possibly resulting from RNA processing errors, in affected neurons of amyotrophic lateral sclerosis [12]. Although the consequences of oxidization of RNA are not fully understood, oxidative damage of RNA may interfere with correct base pairing and could compromise the accuracy of translation [19].

Whether oxidative stress plays a role in the early phase in the pathogenesis of the disease or is secondary to the neuropathological changes in PD has not been determined. In AD and Down's syndrome, previous studies demonstrate that the oxidization of RNA precedes Aβ deposition [14,15]. Oxidization of RNA within nerve cells appears to be a common feature of neurodegenerative diseases such as AD, multi-system atrophy, and dementia with Lewy bodies [27], which are closely related to aging [14,16,17,27]. The alteration in the CSF of neurodegenerative diseases other than PD remains to be investigated. The present in vivo findings demonstrate the importance of RNA oxidation in the early phase of PD development.

We wish to thank Miss Yoko Iwa for her technical and secretarial assistance. This study was supported in part by the Ministry of Education, Science, and Culture and the Ministry of Health, Labour and Welfare, Japan.

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SHORT REPORT

ABSTRACT: We compared the early effects of FK506 on clinical severity, interleukin-2 (IL-2) production by phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMs), and serum levels of acetylcholine receptor antibodies between myasthenia gravis (MG) patients with elevated (>1250 pg/ml, n = 9) or normal (<1250 pg/mL, n = 10) levels of PBM IL-2 production. Reduction in clinical severity and PBM IL-2 production were significantly greater in the patients with elevated IL-2 production than those with normal PBM IL-2 production in the first month of treatment.

Muscle Nerve **27:** 245–248, 2003

EFFECTS OF FK506 ON MYASTHENIA GRAVIS PATIENTS WITH HIGH INTERLEUKIN-2 PRODUCTIVITY IN PERIPHERAL BLOOD MONONUCLEAR CELLS

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Accepted 16 September 2002

Myasthenia gravis (MG) is an autoimmune disease generally mediated by antibodies against the acetylcholine receptor (AChR) of skeletal muscle (AChR-Ab).[11,15] Production of these antibodies in B cells depends upon AChR-specific T cells.[7] Although both Th1 and Th2 cytokines generally play important roles in the pathogenesis of MG,[19] T-cell proliferation and activation are apparently induced by interleukin-2 (IL-2), particularly in severe cases of MG.[2,3] Treatment with the IL-2 fusion toxin DAB_{389} IL-2, which is toxic to cells with high-affinity IL-2 receptors, prevents experimental autoimmune MG (EAMG).[9] These findings suggest that the IL-2–mediated immunoresponses in MG arise from functional abnormalities of T cells.

Whether the IL-2–mediated immunoresponses involve elevated IL-2 productivity in T cells remains to be determined. However, the immunosuppressant, FK506, which rapidly inhibits transcription of the IL-2 gene and inhibits early-phase T-cell activation,[13] is reportedly an effective treatment for MG[6,8] and prevents induction of EAMG.[18] Furthermore, serum concentrations of IL-2 as well as IL-2 production in phytohemagglutinin (PHA)- or IL-2–stimulated peripheral blood mononuclear cell (PBM) culture were elevated in MG patients.[10,14,19] Therefore, in patients with elevated PBM IL-2 production, IL-2 productivity may correlate with functional abnormalities of T cells in the pathogenesis of MG, and may be a viable target for treatment with FK506.

To determine whether symptoms of MG patients with elevated PBM IL-2 production respond more markedly to treatment with FK506 than those of patients with normal PBM IL-2 production, we compared the effects of FK506 on clinical severity, PBM IL-2 production, and serum AChR-Ab titer between these two groups.

PATIENTS AND METHODS

Subjects were 19 MG patients between the ages of 22 and 82 years (mean age 45.3 ± 17.5 years; 2 men and 17 women) and 48 non-MG controls for determination of normal PBM IL-2 production, who presented with mild neurological symptoms including a history of previous tension headaches or benign positional vertigo, but with no history of autoimmune diseases (mean age 44 ± 14.7 years; 18 men and 30 women). The diagnosis of MG was based on widely accepted criteria.[4] The serum AChR-Ab titer was elevated (>0.5 nmol/L) in 14 MG patients. Eleven MG patients underwent thymectomy (thymoma, 4;

Abbreviations: AChR, acetylcholine receptor; EAMG, experimental autoimmune myasthenia gravis; IL-2, interleukin-2; MG, myasthenia gravis; PBMs, peripheral blood mononuclear cells; PHA, phytohemagglutinin

Key words: FK506; interleukin-2; myasthenia gravis; peripheral blood mononuclear cells; severity

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Published online 7 November 2002 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mus.10285

Table 1. Profile of patients and comparison of the effects of FK506 in patients with elevated (>1250 pg/ml) and normal (<1250 pg/ml) levels of PBM IL-2 production.

	IL-2 levels (pg/ml)	
	>1250 (n = 9)	<1250 (n = 10)
Age (years)	44.1 ± 22.0	46.3 ± 15.1
Gender (M/F)	1/8	1/9
AChR-Ab–positive cases (%)	100*	50
Thymectomy (%)	56	60
Time since thymectomy (month)	60.3 ± 30.1 (n = 5)	37.5 ± 42.7 (n = 6)
Patients receiving prednisolone (%)	44	40
Time since beginning prednisolone (month)	61.7 ± 30.0 (n = 4)	43.2 ± 64.0 (n = 4)
Before treatment with FK506		
Severity score	9.9 ± 4.5	6.9 ± 4.0
IL-2 production (pg/ml)	1907.9 ± 815.3§	418.8 ± 211.8
One week after beginning FK506		
Severity score/Severity ratio	5.6 ± 4.6*/0.55 ± 0.32†	6.1 ± 3.5/0.88 ± 0.18
IL-2 production ratio	0.64 ± 0.36	0.98 ± 0.39
Anti-AChR antibody ratio	1.09 ± 0.40	0.92 ± 0.31 (n = 5)
Plasma FK506 (ng/ml)	6.3 ± 1.6	6.6 ± 1.9
One month after beginning FK506		
Severity score/severity ratio	4.0 ± 2.9*/0.44 ± 0.30‡	6.2 ± 3.2/0.94 ± 0.08
IL-2 production ratio	0.54 ± 0.28†	1.16 ± 0.75
Anti-AChR-Ab ratio	1.02 ± 0.40	0.99 ± 0.22 (n = 5)
Plasma FK506 (ng/ml)	6.4 ± 1.3	5.8 ± 1.9

*$P < 0.05$ (Wilcoxon signed rank test) compared with before treatment.
†$P < 0.05$, ‡$P < 0.01$, §$P < 0.001$ (Mann–Whitney U-test) compared to patients with normal (<1250 pg/ml) PBM IL-2 production.

hyperplasia, 3; remnant, 4). Eight patients were taking low-dose (<10 mg/day) oral prednisolone together with 60 or 120 mg of pyridostigmine bromide daily before receiving FK506, but doses were unaltered during the course of the present study. None of the patients received any treatment other than FK506 and low-dose oral prednisolone. Informed consent was obtained from all subjects prior to blood sampling. The protocol for this study was approved by the ethics committee of our institute.

FK506 (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was administrated orally at a daily dose of 3 mg, which is approximately 20% of that given initially to organ transplant patients.[5] Blood sampling and examination of PBM IL-2 production[10,14] were performed before treatment, and at 1 week and 1 month after the beginning of treatment. PBMs were isolated by density-gradient centrifugation, and 5×10^5 cells/well were then cultured in 1.0 ml of standard RPMI medium supplemented with 1% fetal bovine serum and 20 μg/ml of PHA in a humidified atmosphere containing 5% CO_2 at 37°C for 48 h. This cell suspension was then centrifuged for 5 min at 3000 × *g*, and the supernatant harvested for measurement of IL-2. IL-2 concentration (pg/ml) was measured by solid-phase enzyme-linked immunoassay (ELISA), using the Human Interleukin-2 Assay Kit (Otsuka Pharmaceuticals Co. Ltd., Tokyo, Japan).

Given that the mean PBM IL-2 production of the 48 control patients was 537 ± 295 pg/ml, we defined elevated PBM IL-2 production as >1250 pg/ml (mean + 2 SD of controls). Table 1 compares the clinical characteristics of the patients with elevated (>1250 pg/ml, $n = 9$) PBM IL-2 production and those with normal (<1250 pg/ml, $n = 10$) PBM IL-2 production. Clinical severity was determined by patients and participating medical staff (blinded to the level of IL-2 production), according to the methods of Schumm et al.[12] and Besinger et al.[1] (excluding measurements of vital capacity) for ocular and generalized symptoms, respectively. The ratios (post-treatment to pretreatment) of clinical severity score, PBM IL-2 production, and AChR-Ab titer for patients showing elevated AChR-Ab titer were calculated for individual patients at 1 week and 1 month after beginning FK506 administration (Table 1).

Changes in the clinical severity score at 1 week and 1 month into the treatment compared with before treatment were evaluated with the Wilcoxon signed rank test. Differences between the two groups were evaluated with the Mann–Whitney *U*-test. The significance level was $P = 0.05$.

RESULTS

At 1 week and 1 month into the treatment, the severity was decreased in MG patients with elevated PBM IL-2 production (9.9 ± 4.4 vs. 5.6 ± 4.6 and 4.0 ± 2.9, $P < 0.05$), but not in those with normal PBM IL-2 production (Table 1), and the severity ratio was significantly lower in MG patients with elevated PBM IL-2 production than in those with normal PBM IL-2 production (0.55 ± 0.32 vs. 0.88 ± 0.18, $P < 0.05$; and 0.44 ± 0.30 vs. 0.94 ± 0.08, $P < 0.01$, respectively) (Table 1). PBM IL-2 production was more markedly decreased following treatment with FK506 in the patients with elevated PBM IL-2 production than in those with normal PBM IL-2 production (1 month, $P < 0.05$) (Table 1). Given that FK506 inhibits IL-2 production, appears to act selectively on T cells,[13,16] and inhibits T-cell–dependent antibody production,[17] the marked decrease in IL-2 production may ameliorate functional abnormalities of T cells in the pathogenesis of MG. However, the mean AChR Ab ratio did not change, and was not different between the two groups (Table 1). The mean plasma concentration of FK506 did not differ between the two groups at either 1 week or 1 month after the beginning of treatment (Table 1). None of the patients exhibited significant side effects up to 1 month into the treatment, probably because of the low-dose administration (daily dose of 3 mg) of FK506.[5]

DISCUSSION

Our findings suggest that FK506 is more effective for MG patients with elevated PBM IL-2 production than those with normal PBM IL-2 production in the early stages of treatment. Among the 19 patients with MG, 17 had both generalized and ocular symptoms, and the other 2 patients showed either generalized or ocular symptoms. We compared the effects of FK506 on the severity ratio of generalized and ocular symptoms (generalized, $n = 18$; ocular, $n = 18$) in individual patients, and no significant difference was seen (0.64 ± 0.35 vs. 0.75 ± 0.34 at 1 month, respectively). The mean severity ratio at 1 month was lower ($P < 0.1$) in AChR-Ab–positive (>0.5 nmol/L) patients (0.63 ± 0.33, $n = 14$) compared with AChR-Ab–negative (<0.5 nmol/L) patients (1.05 ± 0.10, $n = 5$). This was probably due to a greater frequency of patients with high PBM IL-2 production in AChR-Ab–positive patients (9 of 14) than in AChR-Ab–negative patients (0 of 5). No significant difference in the severity ratio was observed between thymectomized ($n = 11$) and nonthymectomized ($n = 8$) patients (0.62 ± 0.38 vs. 0.76 ± 0.30 at 1 month, respectively) or between patients taking ($n = 8$) and those not taking ($n = 11$) oral prednisolone (0.63 ± 0.14 vs. 0.72 ± 0.24 at 1 month, respectively). However, these issues require longer-term observations.

In conclusion, FK506 is more effective in MG patients with elevated PBM IL-2 production than in those with normal PBM IL-2 production, at least in the early stages of treatment. Although the rapid amelioration of MG symptoms after FK506 has also been reported from other centers in Japan (unpublished results), its mechanisms remain to be studied. The present study has some limitations because of its unblinded and nonrandomized nature and the relatively small number of patients included. Further studies are needed to investigate long-term clinical outcome, and the influences of thymectomy and medication such as prednisolone, as well as the side effects of FK506 in larger numbers of patients. Longer term observation is necessary to determine whether FK506 has an effect on MG patients with normal PBM IL-2 production or whether the serum AChR-Ab titer is unaffected by the therapy. These longitudinal studies are currently in progress at our institution.

This study was supported in part by a grant from the Ministry of Education, Science and Culture, Japan.

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TIAの内科的治療　—抗血小板療法と抗凝固療法の適応—

Antiplatelet and anticoagulant therapy of TIA

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KEY WORDS
一過性脳虚血発作
抗血小板療法
抗凝固療法
アスピリン
ワルファリン

SUMMARY
TIA の概念は，単に病型としてではなく脳梗塞の前駆症状として考えられ，早期治療を必要とする疾患である．TIA の抗血小板療法の適応は，微小塞栓性及び血管不全性 TIA であり，アスピリン及びチクロピジンが選択され，ラクナ TIA ではシロスタゾールを選択するのが望ましいと考えられる．アスピリンの至適用量は75～325mgである．TIA の抗凝固療法の適応は，心原性 TIA であり，ワルファリンを選択し，至適用量は国際的には INR 2.0～3.0であるが，我が国では INR 1.9前後が望ましいのではないかと思われる．

はじめに

一過性脳虚血発作(transient ischemic attack：TIA)とは，脳虚血により一過性の局所神経症状を呈し，24時間以内に症状が消失する病態をいう[1]．典型的な発作は，2～5分で症状が完成し，2～15分間で消失する．TIA の概念は，単に病型としてではなく，脳梗塞の前駆症状として重視され，約1/3が数年以内に完成型脳梗塞に移行する[2][3]．TIA 患者の死因は，脳梗塞より心筋梗塞の方が多いとの報告があり[4]，TIA は脳梗塞だけでなく心筋梗塞の前駆症状として，予防医学的に早期治療を要する疾患である．

本稿では，TIA の抗血小板療法と抗凝固療法の適応と効果について，Evidence-Based Medicine (EBM) の立場から述べる．

抗血小板療法の適応

1．TIA 治療の基本方針

TIA 治療の基本方針は，TIA から脳梗塞への移行を防止することである．TIA の成因を確定し，適切な治療を行うために，原則として入院精査が望ましい．特に心原性 TIA，クレッシェンド TIA の場合は早期入院が必要である．クレッシェンド TIA とは，短期間に TIA 発作が頻発し，発作持続時間が長くなる病態で，脳梗塞への進展が切迫している TIA[5][6]であるため，即効性[7]-[11]のあるアスピリンが有効である．

薬物療法は，TIA の成因に対応した抗血栓薬が選択される．TIA の成因は，微小塞栓性 TIA[12]，脳血管不全性 TIA[13]，心原性 TIA，ラクナ TIA[14]の4つの機序が考えられるが，微小塞栓性，脳血管不全性及びラクナ TIA の治療では抗血小板療法が選択される．

微小塞栓性 TIA は，脳内主幹動脈