Essential Role of Ascorbic Acid in Neural Differentiation and Development: High Levels of Ascorbic Acid 2-Glucoside Effectively Enhance Nerve Growth Factor-Induced Neurite Formation and Elongation in PC12 Cells

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The aim of this study was to investigate the role of ascorbic acid (AsA) in neural differentiation induced by nerve growth factor (NGF) in PC12 cells, a neural precursor cell line. Cells were cultivated in the presence of L-ascorbic acid-2-*O*-α-D-glucopyranoside (AA-2G) and evaluated for potentiation of neural differentiation induced by a small amount of NGF. AA-2G enhanced the proportion of neurite-bearing cells in a concentration-dependent manner (<1 mM). When cultivated with relatively high concentrations of AA-2G (1–5 mM), the cells tended to extend neurites further and to form a neuron-like network. The resultant number of neurite-bearing cells was apparently increased by the addition of AA-2G, with a concurrent decrease in the number of total cells in the same culture dish. These results suggest that AsA in the brain not only promotes NGF-induced differentiation of neural precursor cells but also participates in the development of neural network-forming cells.

Key words —— neurite formation, ascorbic acid 2-glucoside, differentiation, ascorbic acid, PC12 cell

INTRODUCTION

It is well known that ascorbic acid (AsA) is present at high concentrations in mammalian brains.[1–5] Therefore, AsA is predicted to play a critical role in the formation and maintenance of the neural microenvironment. Several studies have demonstrated that AsA acts as an antioxidant in the brain and has neuroprotective effects in models of cerebral ischemia.[6–8] In addition, AsA has been shown to be a neuromodulator of dopamine- and glutamate-mediated neurotransmission[9, 10] and to be an essential cofactor for catecholamine synthesis,[11] as well as an activator of the release of noradrenaline and acetylcholine from synaptic vesicles.[12] However, the physiological role of AsA in the brain has not yet been clarified.

The development of the central nervous system proceeds through several stages including generation and proliferation of precursor cells, differentiation of precursor cells into neurons and glial cells, selection of other precursor cells by apoptosis, and subsequent functional maturation of differentiated neurons to form synaptic circuits. In fetal rat brains, AsA levels rapidly double from the 15th to the 20th days of gestation.[4] Such a drastic change of AsA level at this time suggests that AsA plays a critical role in the development of the brain.

PC12 cells derived from a rat pheochromocytoma have been used as a model cell line for neural differentiation.[13] The cells respond to nerve growth factor (NGF), undergoing a morphological change that generates a phenotype similar to that of sympathetic neurons.[14] NGF binds to its specific receptor TrkA on plasma membranes, followed by tyrosine phosphorylation of TrkA. This process triggers the activation of a mitogen-activated protein kinase (MAPK) cascade that is associated with neuronal survival and neurite outgrowth.[15–19] The activation of extracellular signal-regulated kinase (ERK), the final member of MAPKs, is considered to be essen-

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tial for neuronal differentiation of PC12 cells.[20—23)]

In this study, we examined the potentiating effect of AsA on NGF-induced neurite outgrowth in PC12 cells in the presence of a stable ascorbate derivative, L-ascorbic acid-2-O-α-D-glucopyranoside (Ascorbic acid 2-glucoside; AA-2G), and discuss the physiological role of AsA in the development of the brain.

MATERIALS AND METHODS

Materials —— NGF, Dulbecco's modified Eagle's medium (DMEM), N1 medium supplement, and peroxidase-labeled secondary antibody were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). AA-2G was a product of Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan) and AsA was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Cell Culture Technologies (Tokyo, Japan) and horse serum (HS) was from GIBCO (Grand Island, NY, U.S.A.). Primary antibody against phospho-p44/42 MAPK for immunoblotting was obtained from Cell Signaling Technology, Inc. (Boston, MA, U.S.A.).

Cell Culture and Evaluation of Neurite-forming Cells —— PC12 cells were obtained from Health Science Research Resources Bank (Osaka, Japan) and maintained under the culture conditions described by Greene and Tischler.[13)] Cells were grown in DMEM medium supplemented with 10% FBS and 10% HS in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. All experiments were performed using cells within 10 passages of the initial passage.

PC12 cells were suspended in culture medium and seeded at a cell density of 2.0×10^4 cells/well in 24-well collagen-coated plates (Asahi Techno Glass, Chiba, Japan). After incubation for 6 hr, cells were washed once with DMEM and medium was exchanged to DMEM with 1% N1 medium supplement (N1 medium) containing the indicated concentration of AA-2G. After incubation for 24 hr, NGF was added at a final concentration of 0.2 ng/ml and cells were cultivated for a further 48 hr.

In an another experiment to examine the effect of pretreatment with AA-2G, medium was exchanged to N1 medium 6 hr after cell seeding and then AA-2G was added at a final concentration of 100 μM for various periods. Cells were treated with NGF (0.2 ng/ml) for 24 hr and their neurite forma-tion was measured.

The neurite formation of PC12 cells was examined under a phase-contrast microscope (CK40, OLYMPUS (Tokyo, Japan)) equipped with a phase-contrast condenser, 10 × objective lens and charge-coupled device (CCD) camera (moticam 2000, SHIMADZU (Tokyo, Japan)) to capture the images. Cells with processes whose length was greater than the cell diameter were scored as differentiated in each microscopic field of view (0.48 mm^2). The ratio of neurite-bearing cells to the total number of cells counted was determined for each culture well and expressed as a percentage.

Kinase Assay for MAPK —— PC12 cells were seeded at 1.5×10^6 cells/dish on collagen-coated 60 mm dishes. After incubation for 6 hr, medium was exchanged to N1 medium containing 100 μM AA-2G. After incubation for 24 hr, NGF was added at 0.2 ng/ml and cells were cultivated for a further 1 hr. Then, the cells were washed with cold phosphate-buffered saline (PBS) and harvested by scraping. After centrifugation for 3 min at 3000 rpm, cells were solubilized in 50 μl of lysis buffer (25 mM 3-[N-morpholino]propane sulfonic acid (MOPS), pH 7.2, 15 mM EGTA, 40 mM β-glycerophosphate, 15 mM MgCl$_2$, 15 mM p-nitrophenyl phosphate, 0.2 mM Na$_3$VO$_4$, 50 μM p-aminobenzoic acid, 1 mM dithiothreitol, 2 μg/ml leupeptin, 1 μg/ml pepstain A, 10 μg/ml aprotinin, 1 μM phenylmethylsulphonylfluoride, 1% Triton X-100) and then centrifuged for 10 min at 10000 rpm. Protein concentration of the supernatant was measured by the Bradford technique.

Cell lysate samples were mixed with the same volume of sample buffer (125 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 10% sucrose, 0.01% bromophenol blue, 10% 2-mercaptoethanol) and boiled for 5 min. Samples (10 μg protein/lane) were separated on 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 2 hr at room temperature and then blocked overnight at 4°C. Membranes were incubated with mouse polyclonal anti-phospho-p44/42 MAPK for 1 hr, washed three times with phosphate-buffered saline − 0.05% Tween 20 (PBST), and then treated with peroxidase-conjugated anti-mouse IgG antibody. The peroxidase-active bands were visualized according to the enhanced chemiluminescence procedures.
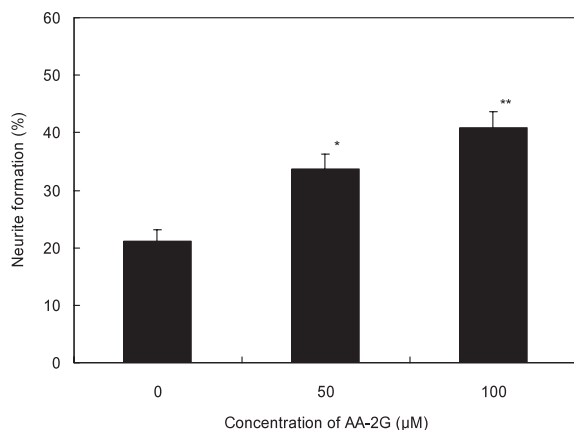
Statistical Analysis —— All values are expressed as the mean ± S.D. from triplicate wells for each treatment. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by least significant difference test (LSD). All percentage values were subjected to arcsine transformation before statistical analysis. A probability of $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of AA-2G on Neurite Formation in PC12 Cells

When PC12 cells were treated with NGF at concentrations of 0.1–10 ng/ml in the present study, cells dose-dependently formed neurites, with the maximum proportion of neurite-bearing cells reaching about 70% at the highest concentration. To evaluate the potentiating effect of AA-2G and AsA on NGF-induced neurite formation, we added NGF at the concentration of 0.2 ng/ml, which generated 20–30% neurite-bearing cells. When AA-2G was present at concentrations of 50 and 100 μM throughout the culture period, significant potentiation of NGF-induced neurite formation was observed when compared treatment without AA-2G (Fig. 1).

Figure 2 shows the effect of pretreatment of AA-2G at a concentration of 100 μM to potentiate NGF-induced neurite formation. The potentiating effect of AA-2G was not observed in cells pretreated within 3 hr prior to addition of NGF, but sig-

nificantly observed in cells pretreated for more than 6 hr.

Effect of AA-2G on ERK Phosphorylation in NGF-treated PC12 Cells

In the MAPK cascade, it has been reported that the third member of MAPK, ERK 1/2, is important and necessary for neuronal differentiation of PC12 cells.[20–23] Therefore, we examined the phosphorylation response of ERK 1/2 in NGF-treated PC12 cells that were pretreated with or without AA-2G (100 μM) for 24 hr before addition of NGF. These cells were treated with NGF (0.2 ng/ml) for 1 hr and cell lysates were analyzed for their ERK 1/2 phosphorylation by the Western blotting technique. As shown in Fig. 3, phosphorylation of ERK 1/2 was apparently induced by the treatment of NGF. However, the pretreatment with AA-2G hardly enhanced NGF-induced ERK 1/2 phosphorylation. Similarly, enhanced or prolonged expression of phosporylated ERK 1/2 was not observed in cells treated with NGF

Fig. 2. Effect of Pretreatment of AA-2G on NGF-induced Neurite Formation in PC12 Cells

PC12 cells were cultured in media containing 100 μM AA-2G. After incubation for various times, NGF (0.2 ng/ml) was added and cells were cultivated for a further 48 hr. Significantly different from the NGF-treated group without AA-2G; ** $p < 0.01$.
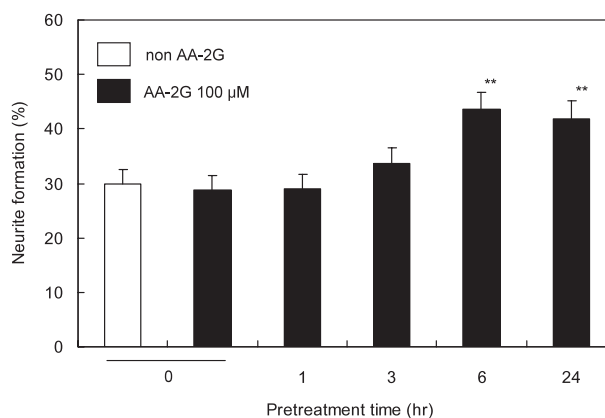
Fig. 1. Effect of AA-2G on NGF-induced Neurite Formation in PC12 Cells

PC12 cells were cultured in media containing the indicated concentrations of AA-2G. After incubation for 24 hr, NGF (0.2 ng/ml) was added and cells were cultivated for a further 48 hr. Significantly different from the NGF-treated group without AA-2G; * $p < 0.05$, ** $p < 0.01$.
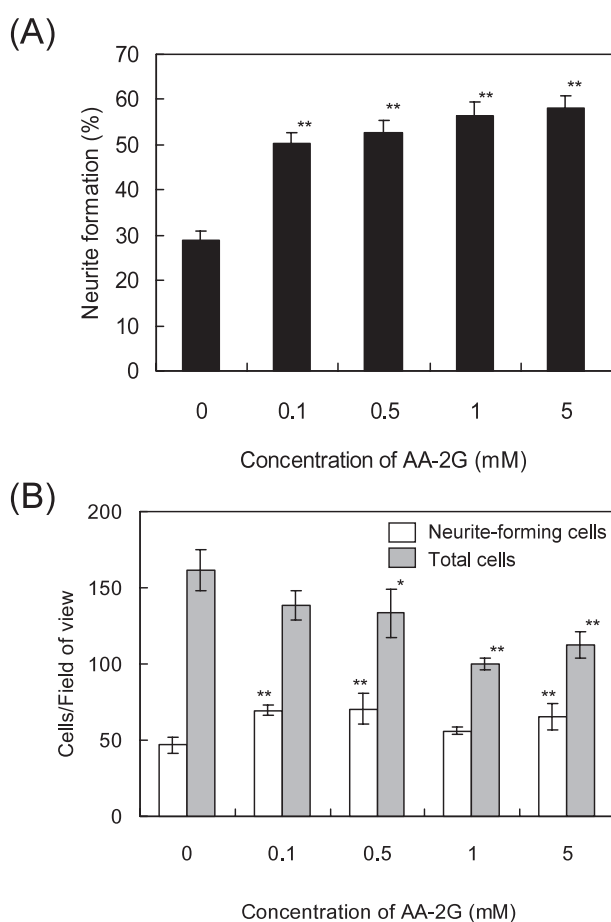
Fig. 3. Effect of AA-2G on the Phosphorylation of ERK 1/2 Induced by NGF

PC12 cells were treated with or without 100 μM AA-2G for 24 hr and then NGF (0.2 ng/ml) was added to the medium. After 1 hr, cells were collected and cell lysates were analyzed for their phosphorylation of ERK 1/2 by Western blotting and immunostaining.

for 24 hr (data not shown).

Potentiation of NGF-induced Neurite Formation by High Concentrations of AA-2G

Next, we examined whether high concentrations of AA-2G potentiate or inhibit the neurite formation induced by NGF in PC12 cells. High concentrations of AA-2G at 1–5 mM significantly enhanced NGF-induced neurite formation, with the highest proportion reaching approximately 60% (Fig. 4A). At concentrations of more than 0.1 mM, AA-2G treatment significantly increased the generation of neurite-forming cells, together with significant decrease in the number of total cells in a concentration-dependent manner (Fig. 4B). Consequently, neural differentiation was apparently enhanced in cells treated with higher concentrations of AA-2G.
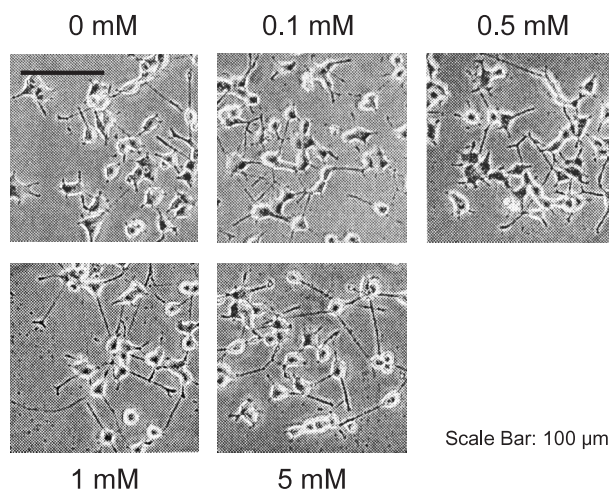
There were also typical changes in the morphology of neurite-bearing PC12 cells treated with high concentrations of AA-2G (Fig. 5). At concentrations of more than 1 mM, AA-2G treatment generated neural cells possessing remarkably long neuritis and resulted in neural network-like features.

Effect of AsA on Neurite Formation in PC12 Cells

AA-2G has been shown to be very stable under various oxidative conditions and release AsA moderately upon hydrolysis by cell-surface α-glucosidases in serum-free cell cultures.[24] Therefore, we hypothesized that AsA released from AA-2G could be responsible for potentiating neurite formation in PC12 cells.

When a single dose of AsA at 5–500 μM was added at the culture starting time and 24 hr after NGF (0.2 ng/ml) was added to induce differentiation, the number of neurite-forming cells did not increase at less than 100 μM, but the number of total cells in the culture decreased at more than 25 μM (data not shown). In addition, treatment with more than 250 μM AsA was severely cytotoxic to PC12 cells. These results demonstrate that a single addition of AsA at the culture starting time of culture exerts no effect on NGF-induced neurite formation in PC12 cells.

It has been shown that antioxidants such as NAC and dithiothreitol inhibit NGF-induced neurite formation in PC12 cells.[25] The intracellular H_2O_2 level has also been reported to increase in the process of NGF-induced neurite outgrowth. These

Fig. 4. Effect of High Concentrations of AA-2G on NGF-induced Neurite Formation in PC12 Cells

PC12 cells were cultured in media containing the indicated concentrations of AA-2G. After incubation for 24 hr, NGF (0.2 ng/ml) was added and cells were cultivated for a further 48 hr. (A) Changes in the proportion of neurite-bearing cells; (B) Changes in the numbers of neurite-bearing cells and total cells in dishes. Significantly different from the NGF-treated group without AA-2G; *$p < 0.05$, **$p < 0.01$.

Fig. 5. Microscopic Photographs of PC12 Cells Treated with NGF and AA-2G

findings suggest that antioxidative activity hardly contributes to NGF-induced neural differentiation. Taken together, we speculate that AsA supplied from AA-2G in this study may have effects other than antioxidative activity in the observed neural differentiation.

DISCUSSION

It has been indicated that endogenous production of NGF gradually decreases with aging in humans and its reduction may participate in neurodegenerative diseases. Therefore, it is important to search for biological modulators that can ameliorate the age-dependent decrease in NGF in human brains. The concentration of NGF (0.2 ng/ml) used in this study induced PC12 cells to differentiate into neurite-bearing cells at a low proportion of about 20% of the total cells counted, although the length of neurites was still short. When PC12 cells were pretreated with AA-2G at 50–100 μM and then induced to differentiate by NGF, AA-2G significantly potentiated NGF activity (Fig. 1). However, no potentiating effect of AA-2G was observed in cells pretreated with AA-2G for less than 3 hr, suggesting that AA-2G is required for a longer culture time (Fig. 2). In addition, AA-2G alone exhibited no differentiation-inducing activity at the concentrations tested (data not shown). It is well known that cells cultured in ordinary culture media *in vitro* lack intracellular AsA, meaning that these cells are not in a physiologically normal condition. The addition of AA-2G to the culture media of cells such as lymphocytes and fibroblasts resulted in efficient accumulation of AsA in cells throughout the culture period. Consequently, these results indicate that the potentiating effect of AA-2G on NGF-induced neurite formation in PC12 cells is due to the accumulation of AsA into the cells.

The treatment of PC12 cells with relatively high concentrations of AA-2G significantly enhanced neural differentiation together with an increase in neurite-forming cells and a decrease in the total cell number in the culture dishes (Fig. 4). Furthermore, as the concentration of AA-2G increased, the neurites markedly expanded and formed neural network-like features (Fig. 5). Although a single addition of a high level of AsA (> 250 μM) at the culture starting time caused severe cytotoxicity, high levels of AA-2G showed a positive effect on neural development. It is likely that AA-2G is hydrolyzed by cellular enzymatic action and then the released AsA is continuously incorporated into the cells throughout the culture. This behavior would not cause cytotoxicity via a prooxidant action but rather maintain the physiological redox state or modulate signal transduction for differentiation. So far, it has been described that NGF induces differentiation to neural cells through the activation of its specific receptor, TrkA, followed by the phosphorylation of MAPK cascade. We therefore analyzed the phosphorylation levels of ERK1/2 in cells treated with NGF in the presence or absence of AA-2G. However, AA-2G treatment did not elevate the phosphorylation of ERK1/2 (Fig. 3). This suggests that the contribution of intracellular AsA to the activation of the MAPK cascade induced by NGF is small, and that there may be another mechanism in which NGF action is enhanced by AsA.

Differentiated PC12 cells cultured in the presence of high concentrations of AA-2G elongated their neurites markedly and formed a neural network-like assembly, whereas undifferentiated PC12 cells underwent cell death when treated with a single addition of AsA at more than 250 μM and with high concentrations of AA-2G (>1 mM). This phenomenon resembles neonatal neural development. In mammalian cerebral cortices, AsA is present at high concentrations and in the fetal rat brain these high levels of AsA are further doubled from the 15th to the 20th days of gestation.[26] It is generally known that the number of neural precursor cells is reduced by apoptosis during the process of neural development. These lines of evidence strongly suggest that AsA plays an essential role in the assembly of the central nervous system. It is therefore likely that the maintenance of high levels of AsA in the brain is necessary for the physiological differentiation and development of neural precursor cells and neural stem cells.

There are many studies describing the relationship between neuronal differentiation and the redox state. Recent studies have reported that reactive oxygen spices (ROS) participate in intracellular signaling pathways in a variety of cells by modulating gene expression, cellular growth, and differentiation.[27–29] ROS was specifically shown to be involved in the NGF-induced differentiation of PC12 cells.[30, 31] *N*-Acetyl-L-cysteine (NAC), a representative antioxidant, has been demonstrated to inhibit NGF-induced neurite outgrowth in PC12 cells[25, 32, 33] Among the many antioxidant flavonoids, only a limited number including flavone

and flavonol can potentiate differentiation.[34] These findings suggest that antioxidative activity hardly contributes to NGF-induced neural differentiation. Taken together, we speculate in this study that AsA supplied from AA-2G may exert cell modulating effects other than antioxidative activity in neural differentiation.

AsA has been recently reported to induce differentiation of embryonic stem cells to cardiac myocytes.[35] This finding supports that the potentiating effect of AsA on NGF-induced neurite outgrowth may be dependent on the action of AsA itself as a cellular redox state-modifying factor, but not on its antioxidant ability. It was also demonstrated that the redox state is involved in the differentiation of neural stem cells.[36–38] AsA has been reported to induce differentiation of embryonic cortical precursors and midbrain neuroblasts into neurons and to alter gene expression involved in neurogenesis, maturation, and neurotransmission.[39–42] Our present study indicates the possibility that AsA may regulate the redox state in the neural cells and play a crucial role in the differentiation and development of the brain. However, mechanism of neuronal differentiation regulated by AsA is not yet clear. Investigation of this mechanism may lead us to understand phenomena such as the maintenance of brain function, defects of memory, and aging of the brain.

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