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Aluminum-induced apoptosis in PC12D Cells

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Abstract

The addition of aluminum-maltol complex to PC12D cells induced a time-dependent and concentration-dependent growth inhibition as well as cell death, whereas aluminum chloride or maltol alone did not affect the viability of PC12D cells. Apoptosis of differentiated PC12D cells was assessed by using terminal deoxynucleotidyltransferase-mediated 2′-deoxyuridine-5′-triphosphate nick end labeling (TUNEL) technique to detect DNA strand breaks *in situ*. The number of TUNEL-positive cells treated with aluminum-maltol increased with time in the treatment cultures. The ability of aluminum ion to elevate intracellular reactive oxygen species was determined by fluorescence in PC12D cells loaded with the oxidant-sensitive dye 2′,7′-dichlorofluorescin diacetate. Aluminum ion incorporated to PC12D cells causes apoptotic cell death by enhancing the generation of reactive oxygen species.

Abbreviations: DMEM – Dulbecco's minimal essential medium; DCF-DA – 2′,7′-dichlorofluorescin diacetate; TUNEL – terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Introduction

Aluminum is abundant elements in the earth crust, and shows a weak toxic effect on the experimental animals and cell culture (Ganrot 1986; Sato *et al.* 1998). Low biological toxicity of aluminum is assumed to be due to the insolubility of aluminum hydroxide at neutral pH (Ganrot 1986) and further to lower incorporation into the cells (Sato *et al.* 1998). However, increased aluminum levels in the brain are related to some neurodegenerative diseases, and aluminum is recognized as a causative agent in human encephalopathy (Crapper *et al.* 1973; Perl & Brody 1980; Perl *et al.* 1982). Aluminum toxicity is accompanied with an increased lipid peroxidation in the brain (Fraga *et al.* 1990). Recently, we showed that aluminum can enhance the iron-mediated lipid peroxidation by stabilizing ferrous ion the prooxidant (Yoshino *et al.* 1999). In the present study, we tested the *in vivo* effect of aluminum incorporated as an aluminum-maltol complex (Bertholf *et al.* 1989). Aluminum induced apoptotic cell death of PC12D cells, which was assessed by detecting the DNA strand breaks *in situ*.

Materials and methods

Materials

Maltol(3-hydroxy-2-methyl-4H-pyran-4-one) and 2′, 7′-dichlorofluorescin diacetate (DCF-DA) were products of Sigma-Aldrich-Japan (Tokyo, Japan). Aluminum maltol complex was prepared according to the method of Bertholf *et al.* (1989).

Cell cultures and treatment conditions

PC12D cells were grown in Dulbecco's minimal essential medium (DMEM) (Gibco BRL) supplemented with 5% fetal bovine and 10% horse serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were seeded at a concentration of 1×10^5 cells/ml and were grown at 37 °C in 5% CO_2 in the absence and presence of different concentrations of aluminum

chloride, aluminum-maltol complex and maltol. Differentiation of PC12D cells was induced by supplementing nerve growth factor (NGF) of 50 ng ml to the medium. Cell viability was measured at appropriate times by trypan blue staining (Mills *et al.* 1996).

TUNEL staining in aluminum-treated cells

To determine *in situ* if aluminum incorporated caused DNA fragmentation, a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method (Gavrielli *et al.* 1992) was used (Apotosis *in situ* detection kit; Wako, Osaka, Japan).

Reactive oxygen species assay

For the measurement of intracellular reactive oxygen species, 2′,7′-dichlorofluorescin diacetate (DCF-DA) of 300 μM was administered to the PC12D cells in DMEM medium, and then washed twice with phosphate buffered saline followed by the addition of NGF of 50 ng ml. Aluminum maltol complex or aluminum chloride of 25, 50 and 100 μM was added to the medium 1 h after the addition of NGF. The DCF-loaded cells were cultured at 37 °C for 3 days. Fluorescence of the cells was monitored at excitation and emission wavelength of 475 and 525 nm, respectively with fluorescent plate reader (Fluoroscan ascent).

Results

Aluminum-maltol complex above 60 μM prohibited the growth of PC12D cells after 2 days of exposure (Figure 1A). However, aluminum chloride or maltol alone did not show any toxic effect on the cells under the same conditions (Figure 1B). Figure 2 shows the effect of varying concentrations of aluminum and aluminum-maltol complex on cell growth. Aluminum-maltol above 70 μM completely inhibited cell growth, whereas aluminum or maltol up to 100 μM showed no inhibitory effect. These data imply that aluminum incorporated into cells as a form of aluminum-maltol complex showed cytotoxic effects.

The addition of NGF induces differentiation of PC12D cells. We characterized the aluminum-induced cell death of differentiated PC12D. Specific labeling of nuclear DNA fragmentation was performed by terminal deoxynucleotidyltransferase-mediated 2′-deoxyuridine-5′-triphosphate nick end labelling (TUNEL) technique (Gavrielli *et al.* 1992) to

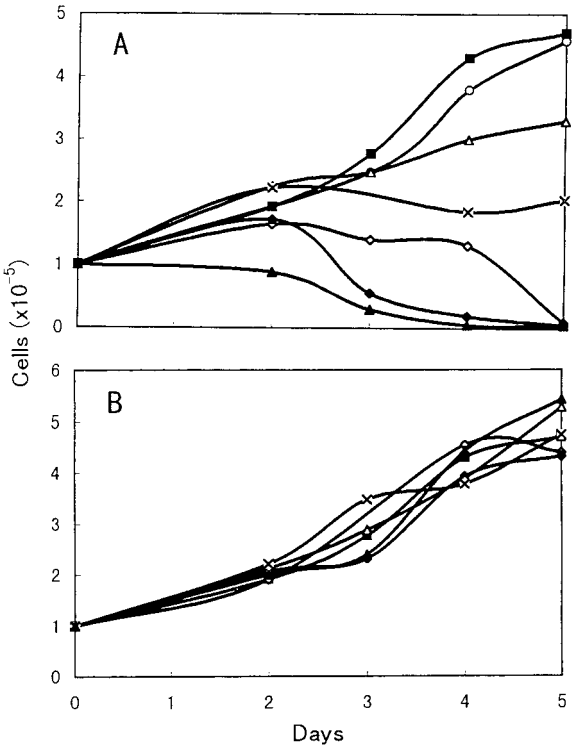

Fig. 1. The effect of aluminum on the growth of PC12D cells. PC12D cells (seeding density of 1×10^5 cells ml) were grown in DMEM medium supplemented with 5% fetal bovine and 10% horse serum in the absence and presence of different concentrations of aluminum chloride, aluminum-maltol complex and maltol. A. Aluminum-maltol complex added. ■, No addition; ◯, 10 μM; △, 30 μM; ×, 50 μM; ◇, 60 μM; ♦, 80 μM; ▲, 100 μM aluminum maltol. B. Aluminum chloride or maltol added. ▲, 20 μM $AlCl_3$; ◇, 60 μM $AlCl_3$; ♦, 80 μM $AlCl_3$. △, 60 μM maltol; ×, 120 μM maltol; ▲, 180 μM maltol.

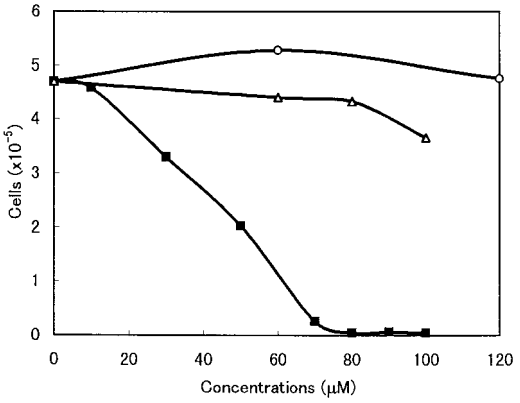

Fig. 2. The effect of increasing concentrations of aluminum on the growth of PC12D cells. Growth conditions were similar to those of Figure 1. Cell number 5 days after exposure to aluminum or aluminum maltol was plotted against aluminum or aluminum-maltol concentrations. ■, Aluminum-maltol added; △, Aluminum chloride added; ◯, Maltol added.

detect DNA fragmentation *in situ*, as a universal characteristic of apoptosis. TUNEL-positive cells were scarcely found in the control culture of PC12D cells (Figure 3A). Addition of aluminum-maltol caused clear manifestation of apoptosis of the cells, and TUNEL-positive cells increased with time in the treatment conditions (Figure 3B and 3C), and a late stage of aluminum induced apoptosis was characterized by chromatin aggregation and condensation (Figure 3C).

To examine the role of reactive oxygen species in aluminum toxicity, intracellular production of reactive oxygen species was monitored by fluorescence in PC12D cells treated with aluminum-maltol (Figure 4). 2′,7′-Dichlorofluorescin diacetate can be oxidized intracellularly to 2′,7′-dichloro-fluorescein, which is fluorescent and oxidant generation was estimated by increasing fluorescence of DCF-loaded cells. Administration of aluminum-maltol to the cells increased fluorescence intensity with culture time. Significant increase in the reactive oxygen species was observed in the DCF-loaded cells 3 days after administration of aluminum-maltol of 75 μM (Figure 4), whereas $AlCl_3$ itself did not increase the fluorocence resulting from reactive oxygen species (data not shown).

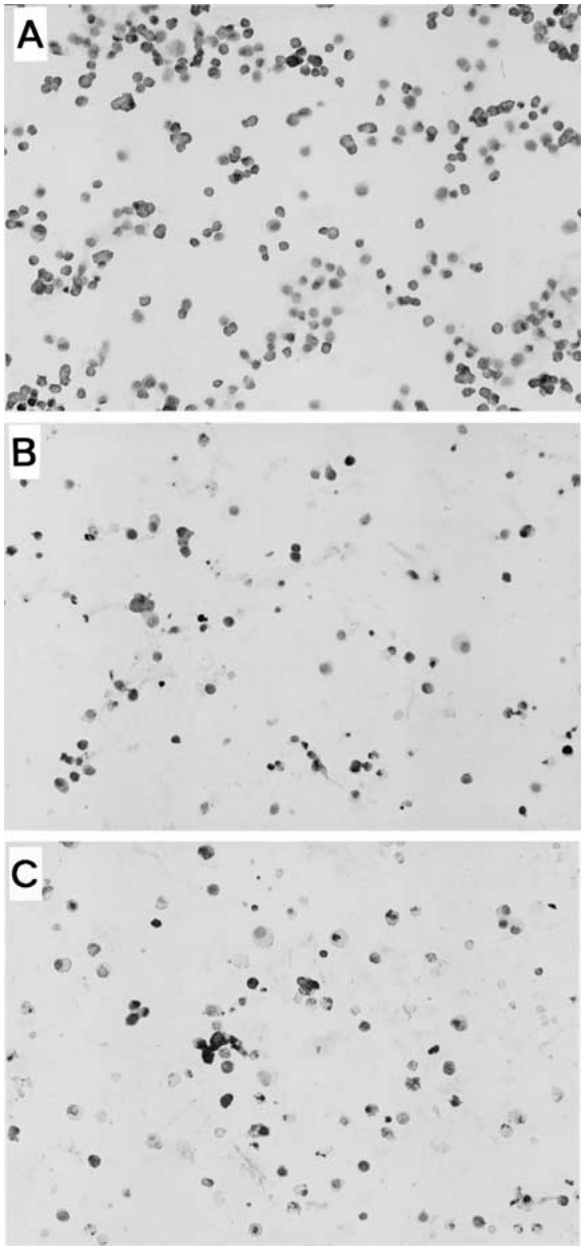

Fig. 3. TUNEL staining of differentiated PC12D cells. Cells were seeded at a concentration of 1×10^5 cells/ml, and NGF of 50 ng ml was supplemented to the medium. (A) Control culture without aluminum supplement. Magnification ×200. (B) Aluminum-maltol complex of 100 μM was added to the medium 2 h after the NGF supplement, and cultured for 3 days. Magnification ×200. (C) Aluminum-maltol was added to the medium, and cultured for 5 days. Magnification ×200.

Discussion

Aluminum ion is closely related to some neurodegenerative diseases, but the addition of a higher concentration of aluminum to cultured cells showed a weak toxic effect such as only a slight alteration of cell surface structure (Sato *et al.* 1998; Vorbrodt & Trowbridge 1993). The experimentally low toxicity of aluminum can be explained by its slight incorporation to cells (Sato *et al.* 1998) and insolubility at neutral pH (Ganrot 1986). The mechanism of incorporation of aluminum into cells remained unknown, but some evidence indicates that aluminum complexed with transferrin and citrate may be a preeminent carrier (Martin 1986; Martin *et al.* 1987). Aluminum-maltol complex was effectively absorbed by experimental rabbit, and aluminum was shown to accumulate in some tissues (Bertholf *et al.* 1989).

In this paper we showed that aluminum-maltol induces apoptosis in PC12D cells. No cytotoxic effects of aluminum itself suggest that aluminum-maltol complex can be incorporated to PC12D cells resulting in apoptotic cell death. Maltol alone did not show any toxic effect on PC12D cells, although maltol can induce apoptotic cell death of some neuroblastoma cell

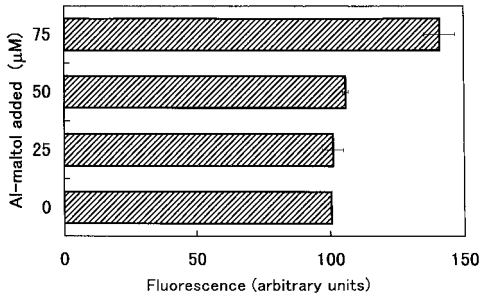

Fig. 4. Aluminum-dependent generation of reactive oxygen species in PC12D cells. DCF-DA was added to the PC12D cells supplemented with aluminum-maltol for the measurement of intracellular reactive oxygen species, and cultured for 3 days. Fluorescence was monitored as described in the Materials and methods. Values were obtained from three separate experiments. Data are expressed as the mean ± SD, and significant difference ($P < 0.001$) was obtained between the group administered with 75 μM aluminum-maltol and the control.

lines and primary murine fetal hippocampal neuronal cultures (Hironishi *et al.* 1996). Maltol toxicity may vary depending on cell species. Our present findings suggest that the apoptosis of PC12D cells can be mediated by aluminum incorporated into cells as aluminum-maltol complex, but not by maltol itself. Two types of apoptosis were defined: one type resulting in small DNA fragments associated with a typical ladder in agarose gel electrophoresis, and the other type that is the formation of large DNA fragments without DNA ladders (Fournel *et al.* 1995). Apoptosis of PC12D cells demonstrated by the TUNEL method was not associated with the observation of DNA ladders (data not shown), indicating that apoptotic processes of PC12D cells cannot activate some types of DNase, causing no production of small DNA fragments.

Apoptosis is closely related to the production of reactive oxygen species (Hansson *et al.* 1996). For example, inhibition of electron transport system by cyanide can induce apoptosis of PC12 cells (Mills *et al.* 1996). Aluminum ion can act as a prooxidant by stabilizing ferrous ion the initiating species for lipid peroxidation, and further by inhibiting the antioxidant action of flavonoids (Yoshino *et al.* 1999). Inhibition by aluminum ion of NADP-isocitrate dehydrogenase (Yoshino *et al.* 1992a, b), an antioxidant enzyme that produces NADPH necessary for the regeneration of reduced glutathione, is also responsible for the increased oxidative injury to cells. The prooxidant properties of aluminum including (a) the stabilization of reduced iron in the initiating species for lipid peroxidation, (b) attenuation of the antioxidant action of flavonoids and (c) the inhibition of NADPH-generating reactions may participate in the increase in the reactive oxygen species in the brain (Fraga *et al.* 1990; Bondy *et al.* 1998). Aluminum-induced apoptosis of PC12D cells may be, thus, due to the aluminum-mediated production of reactive oxygen species.

Growth inhibition and apoptosis of PC12D cells were observed in aluminum above 50 to 75 μM. Intracellular aluminum is largely localized within specific regions: overall aluminum concentration in the human cortex varies around 100 μM (Nixon *et al.* 1990), while the concentration within neurofibrillary tangles may be as much as 1 mM (Perl & Good 1991). Aluminum concentrations required for cellular toxicity of PC12D cells were within patho-physiological ranges. Aluminum ion may induce apoptosis by stimulating the production of reactive oxygen species under the *in vivo* conditions, and will be responsible for some neurological disorders.

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