2004

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NeuroToxicology 25 (2004) 859–867

NeuroToxicology

Aluminum Maltolate-Induced Toxicity in NT2 Cells Occurs Through Apoptosis and Includes Cytochrome *c* Release

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Received 7 October 2003; accepted 17 December 2003

Available online 4 February 2004

Abstract

Aluminum (Al) compounds are neurotoxic and have been shown to induce experimental neurodegeneration although the mechanism of this effect is unclear. In order to study this neurotoxic effect of Al, we have developed an in vitro model system using Al maltolate and human NT2 cells. Al maltolate at 500 μM caused significant cell death with a 24-h incubation and this toxicity was even more evident after 48 h. Lower doses of Al maltolate were also effective, but required a longer incubation for cell death. Nuclear fragmentation suggestive of apoptosis was observed as early as three hours and increased substantially through 24 h. Chromatin condensation and nuclear fragmentation were confirmed by electron microscopy. In addition, TUNEL positive nuclei were also observed. The release of cytochrome c was demonstrated with Western blot analysis. This in vitro model using human cells adds to our understanding of Al neurotoxicity and could provide insight into the neurodegenerative processes in human disease.

Keywords: Aluminum; Apoptosis; Cellular models; Mitochondria; Cytochrome *c*; Neurodegeneration

INTRODUCTION

Apoptosis or programmed cell death is a regulated process of death in cells that facilitates the removal of extra, aged or damaged cells. In contrast to necrosis, apoptosis is an ordered operation with characteristic apoptotic morphological changes that include nuclear condensation and fragmentation, DNA damage, cell shrinkage, membrane blebbing, and the formation of membrane-bound apoptotic bodies (Huppertz et al., 1999).

Mitochondria are key mediators of apoptosis. Early in apoptosis, the mitochondrial transmembrane potential ($\Delta\psi M$) collapses (Brown et al., 1999), indicating the opening of the permeability transition (PT) pore. When it opens, the PT pore facilitates the equilibration of ions in the intermembrane space, which disconnects the respiratory chain, possibly causing the outer membrane to burst and release cytochrome *c* into the cytosol. Cytochrome *c* release initiates an irreversible cascade that activates programmed cell death (Liu et al., 1996). Several proteins are critical in the regulation of apoptosis. Bax causes the release of cytochrome *c* from mitochondria, driving apoptosis forward (Jürgensmeier et al., 1998). In contrast, Bcl-2 interferes with caspase activity following Bax induction (Rosse et al., 1998), and can also prevent cytochrome *c* from being released at all (Kluck et al., 1997).

Several different triggers have been used to induce apoptosis experimentally including hydrogen peroxide and staurosporine. In addition, aluminum (Al) has been shown to induce death via apoptosis in astrocytes

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doi:10.1016/j.neuro.2003.12.004

(Suarez-Fernandez et al., 1999). $AlCl_3$ has been demonstrated to preferentially accumulate in cultured astrocytic cells (Levesque et al., 2000), resulting in apoptosis. In contrast, Al maltolate accumulates at high levels in neurons, possibly attributable to its lipophilic nature (Levesque et al., 2000).

At 8%, Al is the third most abundant element in the earth's crust. Although it has no known biological function, it is constantly introduced into living systems through soil, water, food, and pharmaceutical agents (Crapper McLachlan, 1986). Despite its apparent lack of positive biological function, it has been reported to be toxic. The first indication of its adverse effects in humans was seen in patients undergoing long term hemodialysis treatment for chronic renal failure. Many of these patients developed hyperaluminemia due to contamination of the dialysis solution with Al and also treatment with oral phosphate binding gels to control hyperphosphatemia. This Al overload resulted in a fatal neurological syndrome (dialysis dementia) and a devastating metabolic bone disease known as dialysis osteodystrophy (reviewed by Mach et al., 1988). Al has been linked to neurotoxicity (Levesque et al., 2000; Perl and Pendlebury, 1986; Roll et al., 1989; Suarez-Fernandez et al., 1999; Troncoso et al., 1985; Tsubouchi et al., 2001) and implicated as a possible causative or contributing factor in neurodegenerative disorders particularly Alzheimer's disease (AD), although the exact mechanism by which it acts is far from clear (Crapper McLachlan, 1986; Shin et al., 1995; Strong, 2001).

Intracisternal administration of Al compounds to New Zealand white rabbits has long been known to induce neurofibrillary pathology (Klatzo et al., 1965). However, apoptosis-related changes and oxidative stress (Ghribi et al., 2001a; Savory et al., 1999) have recently been observed including opening of the permeability transition pore, cytochrome *c* release, Bax and Bcl-2 translocation involving both mitochondria and endoplasmic reticulum, caspase activation, and TUNEL positive nuclei (Ghribi et al., 2001a, 2001d). In these studies, apoptosis was prevented with the administration of cyclosporin A (Ghribi et al., 2001b) and also glial cell derived neurotrophic factor (GDNF) (Ghribi et al., 2001c). The soluble Al compound, Al maltolate, also induces stress to the endoplasmic reticulum when it is administered to rabbits (Ghribi et al., 2001a, 2001d).

Here, we have developed an in vitro model of Al toxicity using human NT2 neuronal precursor cells and show that Al maltolate induces apoptosis including the release of cytochrome *c*.

MATERIALS AND METHODS

Cell Culture

Human teratocarcinoma (NT2) precursor cells (Stratagene) were grown in DMEM/F-12 (Gibco) growth medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1% (v/v) penicillin–streptomycin and maintained in 5% CO_2 at 37 °C. For Al treatment, Al maltolate was prepared as a stock solution of 25 mM in sterile water (Berthol et al., 1989) and then passed through 0.2 μm filter. Al was added to growth medium just before use. Unless specified, cells were grown on coverslips in six well plates and allowed to adhere for ~24 h prior to replacement of media containing from 0 to 500 μM Al maltolate.

Cell Viability (LDH) Assay

Cell viability was assessed using a lactate dehydrogenase assay. NT2 cells were grown in 96 well, round bottom plates at a density of 10,000 cells in 100 μl of media. After Al incubation (5–500 μM), 50 μl of media was removed and analyzed for LDH activity using the cytotoxicity assay kit from Promega.

Nuclear and Mitochondrial Visualization

NT2 cells were grown on glass coverslips placed in six-well plates. 1.5×10^5 cells in 3 ml media were plated for 24 h prior to addition of Al maltolate. Cells were washed with PBS followed by fixation in 4% formaldehyde for 15 min. Permeabilization was accomplished with an incubation in ethanol:acetic acid (19:1) for 20 min or 0.1% Triton X 100. Nuclei were stained with Hoechst 33258 (Sigma) at 10 μg/ml for 20 min or VectaShield mounting media with DAPI (Vector Laboratories). Mitochondria were visualized with CMXRos Mitotracker Red (Molecular Probes, Eugene, OR, USA). The mitochondrial polarity-dependent dye was added to culture media at 200 nmol for 20 min before being washed with PBS and fixation in 4% formaldehyde. Coverslips were mounted with VectaShield (Vector Laboratories) and the cells were observed under fluorescence with an Olympus microscope. Pictures were obtained using a digital camera and ImagePro+ software.

Electron Microscopy

NT2 cells were grown in six-well plates, directly on well bottoms without coverslips. Cells were incubated

in 500 μM Al maltolate for 24 h, rinsed in HBSS and then fixed in gluteraldehyde. Cells were postfixed in 1% osmium tetroxide, embedded and viewed at 150 kV using a Zeiss 902 transmission electron microscope with an EELS spectrometer.

TUNEL

NT2 cells were grown on glass coverslips in 6-well plates. Media containing 500 μM Al maltolate was added and allowed to incubate at 37 °C at incremental time intervals of 6, and 24 h. Cells were fixed in 4% formaldehyde for 15 min followed by a wash with PBS. DNA damage was assessed using the apoptosis detection kit, fluoroscein (Promega) following the manufacturer's instructions.

Counts

Apoptotic nuclei percentages were determined by obtaining images from 10 random fields on each slide. The total number of cells and the number with apoptotic morphology (strongly condensed or fragmented) were found for each image and then combined to yield a total for each slide. The number of TUNEL positive nuclei was obtained in a similar manner.

Western Blot

NT2 cells were grown to confluency in T25 flasks. The media was replaced with normal growth media or that containing 500 μM Al maltolate. After six hours in 5% CO_2 at 37 °C, cells were trypsinized and detached cells were pelleted at 300 g for 5 min. The cell pellet was resuspended in five volumes of homogenizing buffer (20 mM Hepes–KOH (pH 7.5), 10 mM sucrose, 10 mM KCL, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/ml pepstatin and 12.5 mg/ml of *N*-acetyl-Leu-Leu-Norleu-Al) (Liu et al., 1996) and homogenized in a Teflon homogenizer. The resulting suspension was centrifuged for 5 min in a microcentrifuge. The supernatant was then centrifuged at 100,000 × *g* using a Beckman Optima LE-80K ultracentrifuge for 30 min to obtain cytosolic extract. The protein concentration was determined using the Bradford method and BSA as a standard (BioRad). Cytosolic and mitochondrial extract was mixed with 2x Lamelli sample buffer and frozen at −80 °C until used for electrophoresis. 10 μg of protein was loaded onto precast 4–20% gradient SDS-PAGE gels (BioRad) and subsequently transferred to PVDF membrane. Membranes were blocked with 5% non-fat dry milk and incubated with monoclonal antibody to human cytochrome *c* (Pharmingin, Lexington, KY, USA). A monoclonal antibody to β-actin (Sigma) was used as a gel loading control. Protein bands were visualized with chemiluminescence (Vector Laboratories). An antibody to cytochrome oxidase IV (Molecular Probes) was used to determine the extent of mitochondrial contamination of the cytosol.

RESULTS

Aluminum is a Potent Neurotoxin for Cultured NT2 Cells

Cell viability was determined by measuring lactate dehydrogenase (LDH) release into tissue culture media. This assay allowed determination of the appropriate cell density and Al maltolate concentrations for further study. 500 μM and 250 μM Al maltolate-induced noticeable death of NT2 cells after a 24-h incubation (Fig. 1) and effectively depleted cells from the culture after 48 h. Lower doses of Al maltolate (50–100 μM) were also neurotoxic, but required longer incubation times to elicit similar neuronal death observed at 250 μM and 500 μM at 24 h.

Aluminum-Induced Neurotoxicity Occurs via Apoptosis

To assess the type of death evoked by Al maltolate, we used Hoechst 33258 or DAPI to examine nuclear morphology. Nuclear fragmentation consistent with apoptotic morphology was observed as early as three hours following incubation with Al maltolate. At 24 h, numerous fragmented and condensed nuclei could clearly be observed in the culture (Fig. 2B). The percentage of apoptotic nuclei had increased at 6 h and rose dramatically through 24 h (Fig. 2C). Under phase microscopy, the size of some cells with apoptotic nuclei was clearly reduced (data not shown).

Chromatin condensation and nuclear fragmentation were further confirmed by electron microscopy. Nuclei of control cultures appeared healthy and round, fully enclosed within the nuclear membrane (Fig. 3A). Al-treated cultures contained nuclei that appeared irregular and separated into fragments (Fig. 3B). In addition, nuclei in experimental cultures contained patches of condensed DNA consistent with apoptotic

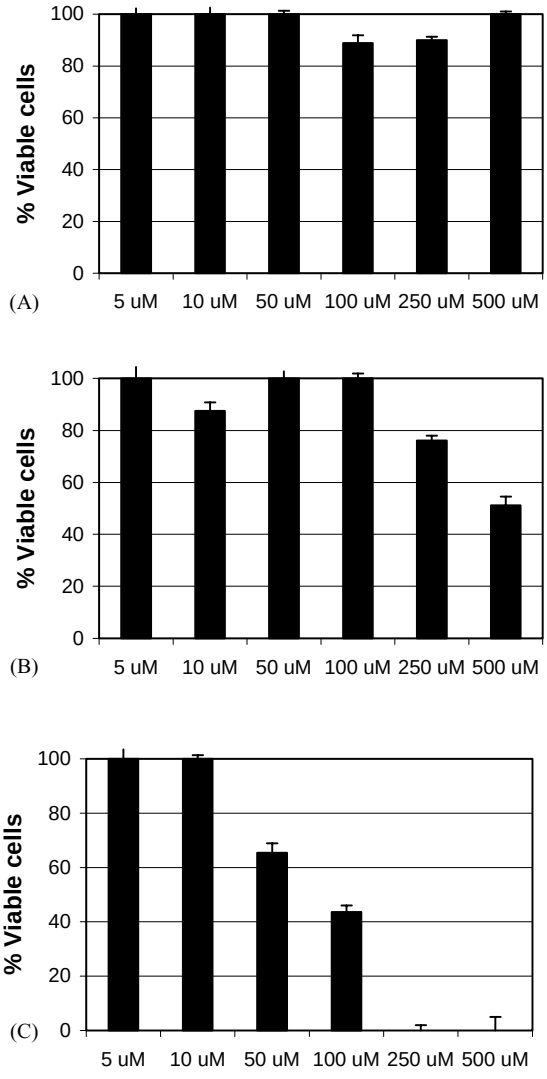

Fig. 1. Al maltolate induces cell death Cell viability was determined by measuring the amount of LDH released into the culture media at 2 h (A); 24 h (B) and 48 h (C). Incubation with 250–500 μM Al maltolate-induced substantial cell death at 24 and 48 h. Lower doses of Al maltolate also induced death of NT2 cells, however, longer incubation times were required. Data is representative of three independent experiments with similar trends. Values are ±S.D.

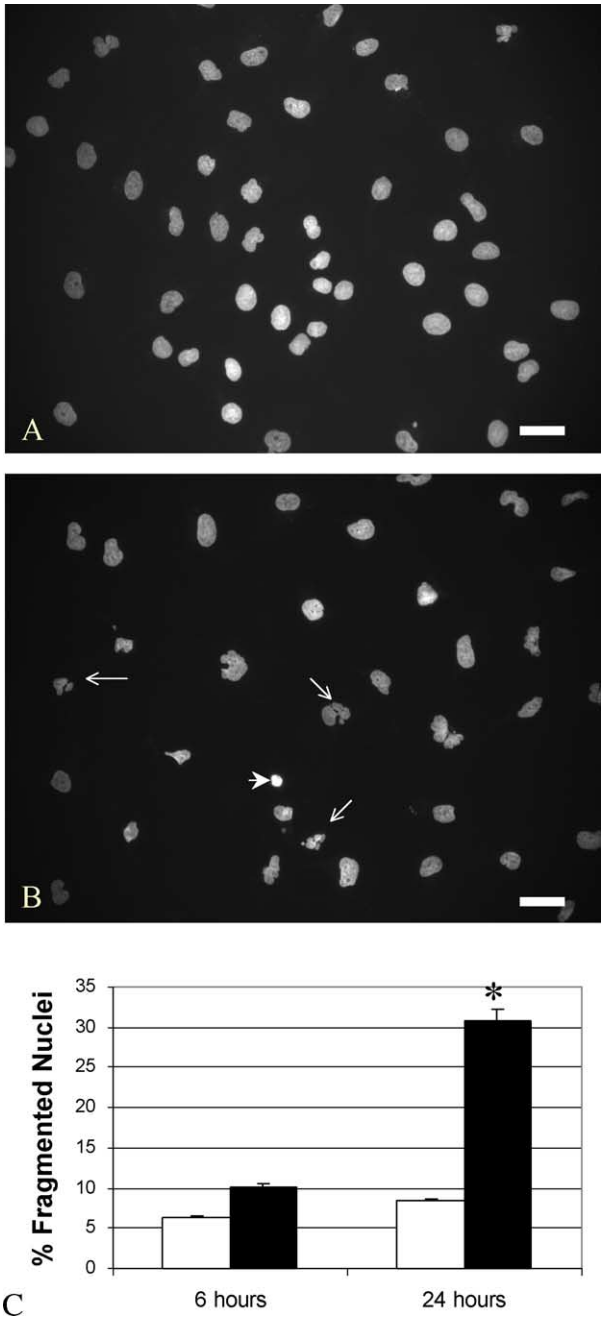

Fig. 2. DAPI staining of NT2 cells revealed large round or oval nuclei (A). Following 24 h incubation in Al maltolate, the nuclei take on a bright, condensed (arrowhead) or fragmented (arrows) morphology (B). The percentage of nuclei with a fragmented, apoptotic morphology had increased as early as 6 h and increased further at 24 h (C). Values are means ± S.E.M. and representative from three independent experiments. Scale bar = 40 μm (*$P < 0.001$).

morphology. Many of the separated nuclear fragments were surrounded by membrane although some of the nuclear vesicles appeared ruptured.

Al-treated cells exhibited DNA damage, a key marker of apoptosis, as indicated by TUNEL positive nuclei (Fig. 4D). A significant increase in the number of cells with DNA damage and nuclear fragmentation was observed at six hours and a 10-fold increase in damage was observed by 24 h (Fig. 4F). This occurred with a clear decrease in cell number in Al maltolate-treated cultures (Fig. 4E).

Al-treated NT2 Cells Undergo Apoptosis Including the Release of Cytochrome c

CMXRos is a mitochondrial specific dye that is taken up in a membrane polarity dependent manner.

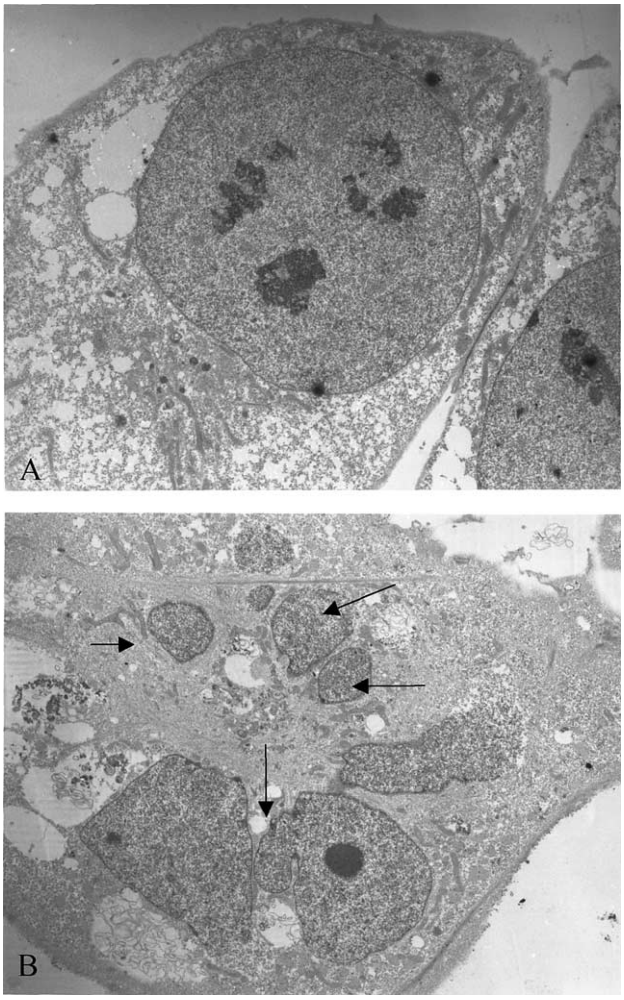

Fig. 3. Electron microscopy confirmed nuclear condensation in Al maltolate-treated cultures. Nuclei of cells in control cultures (A) appeared healthy with a round, even morphology. In contrast, nuclei of Al maltolate-treated cultures (B) were typically fragmented (arrows).

Therefore, the dye can be used to demonstrate mitochondria with an intact membrane potential. Polarized mitochondria were observed in nearly all of the cells even following treatment with Al maltolate (Fig. 4C).

Release of cytochrome *c* to the cytoplasm was confirmed with Western blotting. Cytosolic extract was isolated from control and Al maltolate-treated cultures after a 6 h incubation. Cytochrome *c* was detected in the cytoplasm of Al maltolate-treated cells to a much greater extent than controls (Fig. 5). In order to verify that the cytochrome *c* in the cytosolic fraction was not due to mitochondrial contamination, an antibody to cytochrome oxidase IV was used. While present in the mitochondrial fraction, cytochrome oxidase IV was absent from the cytosolic fraction.

DISCUSSION

Although the neurotoxic effects of Al have been well established in conditions such as dialysis encephalopathy (Mach et al., 1988), the mechanism by which it elicits its effects remain to be elucidated. Here, we show that Al maltolate toxicity in human NT2 cells occurs via apoptosis and includes cytochrome *c* release. Our results indicate this neurotoxicity is time and concentration dependent, and occurs via apoptosis as shown by nuclear fragmentation and chromatin condensation, DNA damage, and cytochrome *c* release.

Al maltolate-induced substantial cell death of the NT2 precursor cells with a 24-h incubation. Concentrations of 250–500 μM Al maltolate were highly toxic, while lower concentrations required longer incubations to cause significant death. Similar concentrations of Al have been used in vitro and shown to be neurotoxic (Levesque et al., 2000; Suarez-Fernandez et al., 1999; Tsubouchi et al., 2001). In particular, Al has a narrow effective range with a large difference in outcome (Roll et al., 1989). Pulse or low concentration exposure appears to be less toxic than higher concentrations (Kashiwagi et al., 1998). We found that lower concentrations of Al maltolate (50–100 μM) did induce death but required longer incubations for a significant effect. It is possible that Al must first accumulate intracellularly to a critical level before triggering cell death. Such accumulation is likely to be concentration and time dependent as well as impacted by the Al species administered (Levesque et al., 2000). Accumulation below a certain threshold may have little or no effect. Alternatively, sub-lethal accumulated concentrations insufficient to induce apoptosis may still negatively impact the cytoskeleton or intracellular transport.

Cytochrome *c* release is a key event in the initiation of apoptosis (Liu et al., 1996). This release generally coincides with opening of the mitochondrial PT pore and loss of mitochondrial membrane polarity. An earlier study reported that cytochrome *c* could be released by staurosporine without concomitant depolarization of the mitochondrial membrane potential (Krohn et al., 1999). Al-induced apoptosis appears to occur through a similar mechanism. Previously, Al was shown to induce mitochondrial permeability transition (Toninello et al., 2000). However, when Al was bound to the PT pore, it held it in an intermediate, partially open position. It is possible that this is sufficient to trigger cytochrome *c* release.

While a causal link between Al and Alzheimer's disease has not been demonstrated, several epidemiological studies have shown a statistically significant

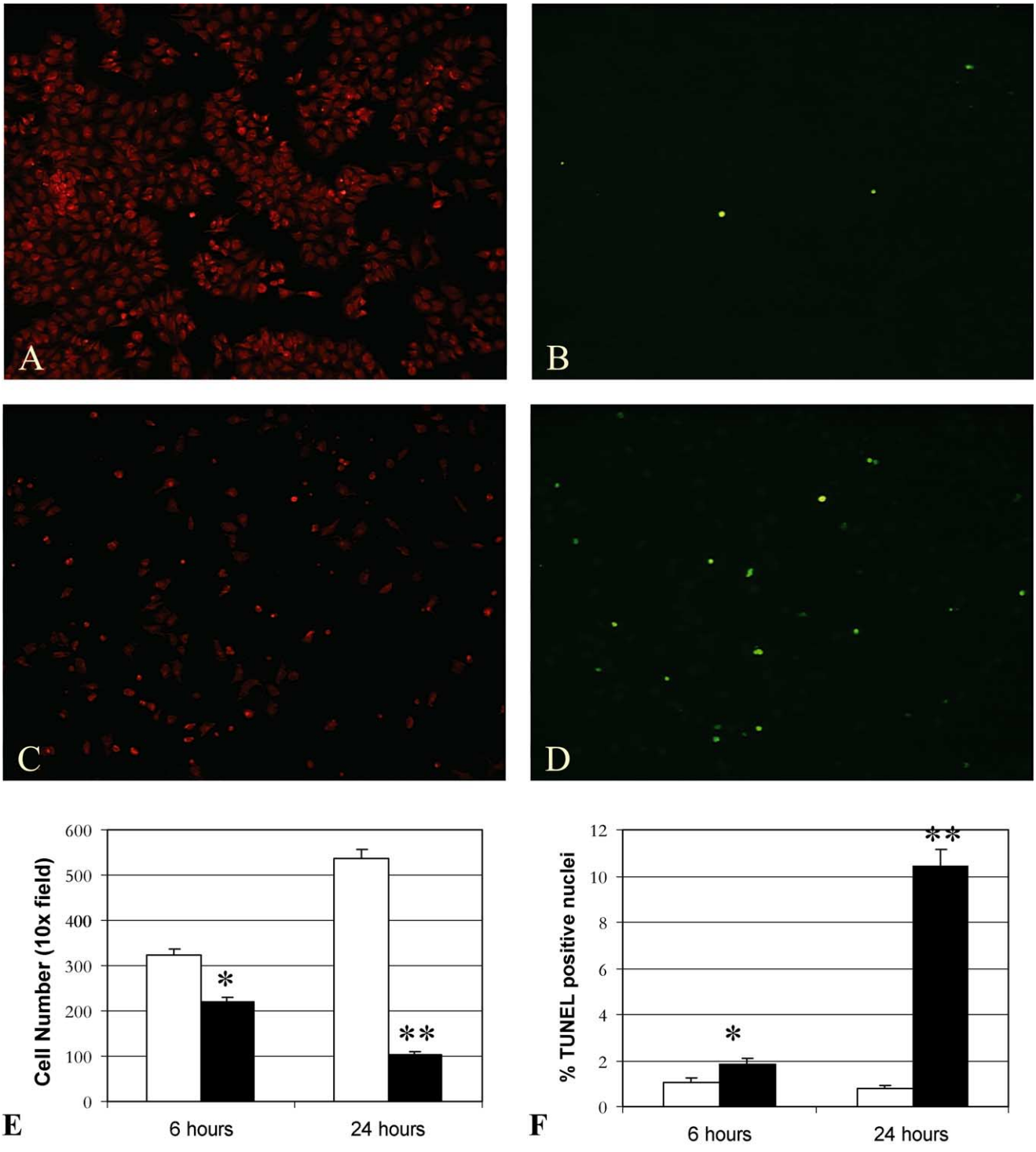

Fig. 4. Numerous NT2 cells can be observed following a 24 h incubation in control media (CMXRos, red A). Very few of these cells were TUNEL positive (B, green). However, after 24 h in Al maltolate (C) a clear reduction in cell number has occurred. Many of these cells were TUNEL positive (D, green). The number of cells in control cultures increases over 24 h but decreases in Al maltolate-treated cultures (E) which has more TUNEL positive nuclei as well (F). Values are means ± S.E.M. and representative from three independent experiments with similar trends (*$P < 0.5$, **$P < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

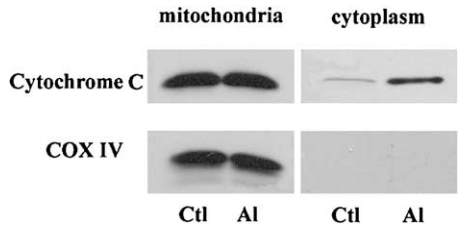

Fig. 5. Western blot analysis revealed a greater amount of cytochrome *c* in the cytoplasm of Al maltolate-treated cultures (Al) compared with controls (Ctl). The absence of cytochrome oxidase IV in the cytoplasm indicated that mitochondrial contamination of the cytoplasm was negligible.

positive link (reviewed by Flatten, 2001). Nonetheless, aluminum induces many of the same biochemical and pathological changes that occur in AD (reviewed by Strong). Regardless of any lack of evidence for a causative link, aluminum has potential as a trigger to cause cellular changes that mimic aspects of AD pathology (Perl and Pendlebury, 1986; Huang et al., 1997). If similar pathways are activated, Al-induced pathology may prove useful as a model to understand the sequence of events that occurs as neurons die in AD brain. New Zealand white rabbits have been used successfully in this regard (Ghribi et al., 2001a, 2001b, 2001b, 2001d; Kowall et al., 1989; Klatzo et al., 1965; Munoz-Garcia et al., 1986; Savory et al., 1999, 2001).

Aside from inducing apoptosis, Al negatively affects cytoskeletal proteins and axonal transport (Kashiwagi et al., 1998; Shea et al., 1997). Indeed, pulse exposure to Al maltolate blocks fast axonal transport leading to the accumulation of NF-L proteins in rat cortical neurons (Kashiwagi et al., 1998). Disrupted transport has been shown in AD (Cash et al., 2003; Praprotnik et al., 1996; Richard et al., 1989; Terry, 1996) and also amyotrophic lateral sclerosis (Williamson and Cleveland, 1999), however relatively few studies have examined this aspect of the disease (Kasa et al., 2000). Nonetheless, numerous factors implicated in AD including oxidative stress (de la Monte et al., 2000; Perry and Smith, 1997; Smith et al., 1995), APO ε (Tesseur et al., 2000), and APP-L (Torroja et al., 1999) have all been shown to affect axonal transport. The observation that AβPP can serve as a kinesin cargo receptor (Kamal et al., 2000, 2001) as well as the inhibition of kinesin-dependent transport by tau overexpression (Ebneth et al., 1998; Stamer et al., 2002) should not be overlooked. It would be interesting to see whether tau overexpression and Al affect intracellular transport via a similar mechanism.

Apoptosis has been suggested to play a role in AD pathology (Cotman and Anderson, 1995; Su et al., 1997) however, either the full cascade is not activated or appears to be aborted (Perry et al., 1998a, 1998b; Raina et al., 2001) An intriguing possibility is that apoptosis-related changes, oxidative stress, and axonal transport disruption are linked in a way that promotes neurodegeneration and involves cytoskeletal disruption (Srivastava et al., 1998). Since Al contributes to all of these factors (Ghribi et al., 2001b; Kashiwagi et al., 1998; Pratico et al., 2002), the model presented here may prove useful in understanding the mechanisms leading to neuronal death in neurodegenerative diseases.

ACKNOWLEDGEMENTS

This work was funded by the Jeffress Memorial Trust, the National Institutes of Health (AG-020996), an undergraduate research grant from the Virginia Academy of Sciences (K.J.S.G.) and an undergraduate research grant from the Liberty University Biology Alumni. The authors thank M.S. Forbes, Ph.D. for assistance with electron microscopy and P. Pramoonjago, Ph.D. for assistance with western blotting.

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